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WHEN THE BEAR LOST ITS TAIL: TARGETING THE GRK/β-ARRESTINS DOWNSTREAM IGF1R IN NON-EPITHELIAL MALIGNANCIES

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Karolinska Institutet

Stockholm 2022

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Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, 2022
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ISBN 978-91-8016-859-5
Cover illustration: Based on the legend “How the bear lost its tail”, the image depicts a metaphor for the lost oncogenic power of the IGF1R following inhibition of the C-tail-dependent signaling. Illustration by Mireia Cruz De Los Santos.

When the bear lost its tail: targeting the GRK/β-arrestins downstream IGF1R in non-epithelial malignancies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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The thesis will be defended in public at Mangan, Bioclinicum J6:10, Akademiska stråket 1, 171 64 Solna Stockholm, Sweden.
December 16th, 2022, 9:00

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POPULAR SCIENCE SUMMARY OF THE THESIS

At the core of cell function and communication is a process known as cellular signaling. These signals are picked up by receptors on the membrane and converted to cellular functions through a domino effect of protein activation inside the cell. Cancer takes advantage cellular signaling to overcome natural limitations on cell multiplication and lifespan. One of the most studied plasma membrane receptors in cancer is IGF1R which uses two major groups of proteins known as signaling pathways to convert external signal to biological function. Past anti-IGF1R treatment strategies have failed, at least in part due to not having taken into account that each of these signaling pathways produce their own different outcomes and can be triggered together (balanced signaling) or individually (biased signaling). In this thesis we have focused on the later discovered signaling pathway which is controlled by the C-tail section of the receptor, and what role it plays in cancer. One of the major findings of this thesis is that the GRK/β-arrestin signaling pathway (C-tail dependent) is essential for cancer. Using this knowledge, we developed three strategies to target the GRK/β-arrestin biased signaling. More specifically our studies incapacitated the C-tail of the receptor (thereby losing the tail) in different ways and to different degrees. This thesis improves on the understanding of the role of IGF1R C-tail plays in cancer and unveils new possibilities for therapy.

ABSTRACT

Plasma membrane receptors are highly specialized cell surface structures that receive extracellular information and process them into biological responses. Among them, this thesis focuses on two major receptor families: the G protein-coupled receptors (GPCR) and the receptor tyrosine kinases (RTKs). One of the most studied RTKs, IGF1R has been demonstrated to be essential for cancer development in a wide range of tissues. Recognition of IGF1R signaling hijacking by twisted malignant processes was rapidly shadowed by anti-cancer therapeutics developments, yet with disappointing results in clinical setting. Failures in past anti-IGF1R strategies required bedside to bench shift and re-evaluation of the mechanism controlling IGF1R tumorigenesis. In particular its non-canonical, kinase-independent signaling capabilities, through use of the GRKs/β-arrestins system - prominent controller of GPCR-signaling-, is today acknowledged to orchestrate IGF1R oncogenic power.

This thesis aims to explore the GRK/β-arrestin system downstream IGF1R and uncover its targeting potential as a cancer therapeutic strategy in non-epithelial cancers.

Study I describes the functional roles of GRK isoform modulation in IGF1R downregulation to develop anti-IGF1R targeting strategies via inhibition of GRK2. Our results establish the potential for clinical applicability of cross-targeting the IGF1R through pharmacological inhibition of GRK2 in Ewing sarcoma by using paroxetine, a commonly prescribed antidepressant. In **Study II**, we investigate the therapeutic potential of p53 activation through targeting MDM2 in conjunctival melanoma. The use of Nutlin3 to reactivate p53 via inhibition of MDM2 proved more effective than siRNA inhibition of MDM2. This suggests that the additional effect of Nutlin3 on IGF1R degradation is highly beneficial in cancer targeting. This study reveals double-hit IGF1R/p53 targeting strategy as a potent therapy for recurring and metastatic conjunctival melanoma. In **study III,** we investigate the disruption of the p53/MDM2/IGF1R axis via unbalancing the β-arrestin system to improve treatment response to chemotherapy in malignant melanoma. This study demonstrates novel dual therapeutic strategy in which inhibition of β-arrestin1 signaling or β-arrestin2 hyperactivation can enhance response to chemotherapy. Considering the significance of IGF1R and downstream biased signaling in blood cancer, **study IV** investigates the individual effect of kinase signaling versus GRK/β-arrestin signaling downstream the receptor in leukemia. Our results uncover both arms of IGF1R signaling as targets for cell proliferation and survival (kinase) and cell differentiation (GRK/β-arrestin) in acute myeloid leukemia. This work establishes the potential of targeting IGF1R kinase and/or C-terminus to induce peripheral differentiation.

In summary, the findings described in the present thesis provide new insights for the therapeutic potential of non-canonical IGF1R signaling.

LIST OF SCIENTIFIC PAPERS

I. Inhibition of G Protein-Coupled Receptor Kinase 2 Promotes Unbiased Downregulation of IGF1 Receptor and Restrains Malignant Cell Growth
Crudden C, Takashi S, Song D, Dragomir MP, **CISMAS S**, Serly J, Nedelcu D, Fuentes-Mattei, Tica A, Calin GA, Girnita A and Girnita L
Cancer Research 2021 Jan 15;81(2):501-514.
PMID: 33158816

II. IGF-1R is a molecular determinant for response to p53 reactivation therapy in conjunctival melanoma
Dawei Song, **SONIA CISMAS**, Caitrin Crudden, Eric Trocme, Claire Worrall, Naida Suleymanova, Tingting Lin, Huiyuan Zheng, Stefan Seregard, Ada Girnita and Leonard Girnita
Oncogene. 2021 Nov 17. doi: 10.1038/s41388-021-02111-x.
PMID: 34785779

III. Competing engagement of β-arrestin isoforms balance IGF1R/p53 signaling and control response of melanoma cells to chemotherapy
SONIA CISMAS, Caitrin Crudden, Iara Trocoli, Naida Suleymanova, Sylvya Pasca, Benjamin Gebhard, Dawei Song, Shiyong Neo, Takashi Shibano, Terry J Smith, George A. Calin, Ada Girnita and Leonard Girnita
Submitted manuscript

IV. β-arrestin/GRK signalling downstream IGF1R prevents differentiation and promotes acute phenotype of myeloid leukemia
SONIA CISMAS, Sylvya Pasca, Catalina V. Gual, Takashi Shibano, Ada Girnita, George A. Calin and Leonard Girnita
Manuscript

CONTENTS

LIST OF ABBREVIATIONS

A-loop	Activation loop
ADP	Adenosine diphosphate
AML	Acute myelocityc leukemia
APL	Acute promyelocityc leukemia
ATP	Adenosine triphosphate
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
BRCA1	Breast cancer gene 1
CML	Chronic myelogenous leukemia
D1A	Dopamin 1A
DNA	Deoxyribonucleic acid
DTIC	Dacarbazine
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FGFR	Fibroblast growth factor receptor
GDP	Guanosine-5'-diphosphate
GEF	Guanine exchange factor
GPCR	G-protein-coupled receptor
Grb2	Growth factor receptor-binding protein 2
Grb10	Growth factor receptor-binding protein 10
GRK	G protein-coupled receptor kinase
GTP	Guanosine-5'-triphosphate
HEK293T	Human embryonic kidney cells 239T
IGF1/2	Insulin-like growth factor 1/2
IGF1R/IGF2R	Insulin-like growth factor 1/2 receptor
IGFBP	Insulin-like growth factor-binding protein
IP	Immunoprecipitation
IR	Insulin receptor
IRS	Insulin receptor substrate

JNK3	c-Jun N-terminal kinase 3
KO	Knockout
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
MEF	Mouse embryonic fibroblast
MEK	MAPK/ERK kinase
MDS	Myelodisplastic syndrom
mt	mutant
Nedd4	Neuronal precursor cell-expressed developmentally downregulated 4
pAkt	Phospho-Akt
PDGFR	Platelet-derived growth factor receptors
PDK1	3-phosphoinositide-dependent protein kinase 1
pERK	Phospho-ERK
PI3K	Phosphoinositide 3-kinase
PIP2	Phopsphatidyl-inositol bisphosphate
PIP3	Phopsphatidyl-inositol trisphosphate
pVHL	von Hippel-Lindau tumor suppressor
RTK	Receptor tyrosine kinase
SH2	Src homology region 2 domain
Shc	Src homology 2-containing protein
Sos	Son of sevenless
VEGFR	Vascular Endothelial Growth Factor Receptor
WB	Western blot
wt	wild-type
WT1	Wilms' tumour suppressor 1
β-arr	β-arrestin
β1AR	β1 adrenergic receptor
β2AR	β2 adrenergic receptor

1 INTRODUCTION

1.1 HALLMARKS OF CANCER

One of the most important causes of morbidity worldwide is cancer, which is responsible for 13% of all deaths each year (1). Cancer development is a complex and multi-step process in which normal cells acquire enhanced growth, survival, and dissemination properties. Originally six hallmarks of cancer development outlining the intricate design of carcinogenesis were described, later expanding to include two additional hallmarks and two enabling characteristics (Figure 1) (2):

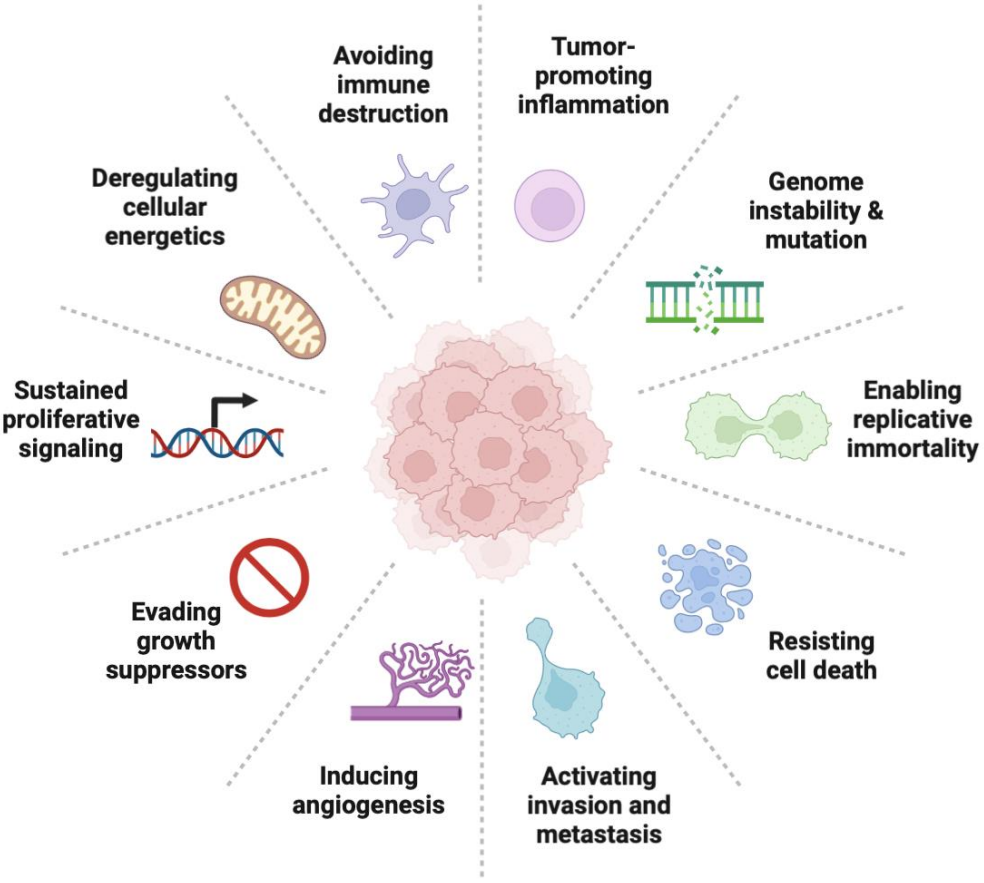


Figure 1. The hallmarks of cancer. Illustration is modified from Hanahan and Weinberg (1, 2) and created with BioRender.com.

Of these hallmarks cellular signaling is critical to the regulation of all other (3, 4). Among various families of plasma membrane receptors conveying out-in information, two classes distinguish themselves as the main focus of this thesis; the GPCR and RTK receptor families.

1.2 G PROTEIN-COUPLED RECEPTORS

The G Protein-Coupled receptors (GPCRs), also known as seven transmembrane receptors (7TMR) due to their structure spanning the plasma membrane seven times, are the largest plasma membrane receptors family. Encompassing about 800 receptors in the human genome (5), they are responsible for controlling numerous critical physiological and pathological functions (6, 7). Many drugs currently in use target GPCR or GPCR related signaling across various pathologies, however, targeting of GPCRs is underutilized in cancer, where it makes up less than 10% of the market (8). All GPCRs share a similar structure consisting of a N-terminal extracellular domain, transmembrane domains, and a C-terminal domain. GPCRs can activate signaling following binding of different types of molecules including small peptides, proteins, amino acids, hormones, and lipids. For the entire class of receptors, G proteins and the β-arrestins are recognized as master regulators for all functional aspects (9-12).

1.3 G PROTEINS

The heterotrimeric G protein family convey information from the ligand bound GPCRs into biological responses. G-proteins complexes are composed of α, β and γ subunits and are classified by their distinct α subunit into four different G proteins: $G\alpha_s\beta\gamma$, $G\alpha_{i/o}\beta\gamma$, $G\alpha_{q/11}\beta\gamma$ and $G\alpha_{12/13}\beta\gamma$ (13, 14). $G\alpha_s$ act as stimulatory, and $G\alpha_i$ as inhibitory for adenylyl cyclase (15) and subsequently cAMP production. $G\alpha_q$ and $G\alpha_{12}$ are activators of phospholipase Cβ (PLCβ) while $G\alpha_{12}$ and $G\alpha_{13}$activate the Rho pathway. Each of these Gα subunits have their own various sub-group members (16). Gα protein isoform expression is tissue specific - some even exclusive to cell types (e.g., $G\alpha_{olf}$ (olfaction), a $G\alpha_s$ member present exclusively in olfactory neurons), while other Gα proteins are found ubiquitously (e.g., Gαq members Gαq, Gα11, Gα14 and Gα16) (17). The Gβ and Gγ subunits can also be found in various isoforms, numbering 5 Gβ and 12 Gγ encoded in the human genome. Gβ subunits share major sequence homology (80-90 %), as opposed to the Gγ whose expression widely varies (20-80 %), and again show a mixed pattern of cell specific or ubiquitous expression (13, 14, 17, 18).

The classical model describes the heterotrimeric G protein functionality as a binary switch. In the inactive state, the heterotrimeric G-protein complex (19-21) is made up of the α, β, and γ subunits, with the Gα bound to GDP through the nucleotide pocket. Conformational changes to the ligand-bound GPCR allows for interaction with their specific G protein and activates the GDP/GTP exchange through the dissociation of the heterotrimeric G protein complex. After dissociation, both Gα and Gβγ are enabled to act as free signaling activators. In most cases, these subunits interact with secondary messengers and modulate downstream signals (13, 17, 22-26) including activation of the mitogen-activated pathway (MAPK), cAMP, phosphoinositide 3-kinase (PI3K)-Akt and protein kinase A (PKA).

In a negative feedback system, G protein-coupled receptor kinases (GRK)/ arrestins (10, 20, 22, 27, 28) system is activated to prevent or "arrest" the G protein signaling.

1.4 GRK/β-ARRESTIN SYSTEM

The central dogma of GPCR signal transduction (29) describes receptor conformational change which triggers dissociation of Gα and Gβγ and subsequent activation of signaling pathways through various secondary messenger molecules. An integral part of this dogma, commonly described as desensitization, involves GRK phosphorylation of the receptor followed by β-arrestin binding to the phosphorylated receptor (9, 12). This results in a physical uncoupling of the receptor from the heterotrimeric G-protein and signal termination (30).

1.5 G PROTEIN RECEPTOR KINASES (GRKs)

GRKs are receptor associated kinases that phosphorylate GPCRs initiating β-arrestin activation and recruitment resulting in signal desensitization (31, 32). The first recognized GRK isoforms were described for rhodopsin and β-adrenergic receptors phosphorylation and named as GRK1 and GRK2 (31) respectively. To date, there are seven GRK isoforms (GRK1–GRK7) (33) encoded in the human genome, that are divided into three groups based on their structural similarities: GRK1/7, GRK2/3, and GRK4/5/6. GRK1 (rhodopsin receptor kinase) and GRK 7 are visual GRKs expressed only in retinal tissue. GRK4 is expressed in the testis, while GRK 2, 3, 5 and 6 are found to be ubiquitously expressed (22, 33).

GRKs are serine/threonine kinases that phosphorylate the C- terminus or the third intracellular loop of activated GPCRs (34). Multiple studies have shown that GRK isoforms have functional diversity but also significant functional overlap (23, 25). Moreover, different GRK isoforms can compete for binding of the same receptor. Though the mechanism behind GRK phosphorylation is highly conserved within the family of hundreds of GPCRs, phosphorylation by different GRK isoforms produced similar and/or opposing outcomes (34) as evidenced by the difference in growth phenotypes in knockout (KO) mice models. GRK2 KO mice (-/-) produced stunted growth phenotype and did not survive past embryonic day 12-15 due to heart failure and severe hypoplasia [58]. Conversely, GRK3 and GRK5 KO (-/-) did not affect viability [69], suggesting that specific functions in development can be carried out by more than isoforms.

Studies indicate that the biological outcome of GPCR activation is critically dependent on the pattern in which GRKs phosphorylate the receptor (9). GRK-dependent phosphorylation of the GPCR controls β-arrestin recruitment results in a modelling of distinct patterns (35-37) as related to subsequent effects and is acknowledged as a "barcode hypothesis (38).

1.6 B-ARRESTIN

First described as involved in the process of desensitization (arrest) of β-adrenergic receptor by the β-adrenergic receptor kinase, arrestins (39-41) come in four different flavors. Visual arrestin (1 and 4) are exclusively found in the retina (42) while arrestin 2 and 3 (β-arrestin 1 and 2) are ubiquitously found in all tissues. β-arrestins share structural similarities (11) and are involved in the regulation of all non-visual GPCRs (43, 44), with similar or differing outcomes in terms of GPCR expression and signaling (9, 44, 45). These outcomes depend primarily on the stability of the β-arrestin/receptor interaction, separating GPCRs into two major classes (46). Class A receptors (e.g., dopamine D1A receptor, μ-opioid receptor, β_2-adrenergic receptor), transiently interact with β-arrestin to form a weakly bound GPCR/ β-arrestin complex. The receptor is then recycled and made available for new signal activation in a process known as resensitization (31, 47-49). Class A receptors preferentially bind β-arrestin 2 over β-arrestin 1. In contrast, β-arrestins strongly bind to class B receptors (e.g., angiotensin II type 1A and vasopressin V2 receptors) (50) and internalize together with the receptor via endosomes, and are eventually degraded. These receptors can form stable GPCR/ β-arrestin signaling complexes with both β-arrestin 1 and 2 equally (50). β-arrestin isoform recruitment to the GPCR and, the stability of this interaction is controlled by the GRK phosphorylation *barcode.*

β-arrestin isoform specific KO mice models as evidenced by single isoform KO (βarr1-/- or βarr2-/-) in which survival (51, 52) was not affected while double KO mice (βarr1-/- and βarr2-/-) proved embryonic lethal (50). This suggests the two isoforms can replace each other's function under specific conditions. The GRK/β-arrestin system is essential to all aspects of receptor biology, controlling receptor internalization and trafficking as well as signaling and desensitization.

Today, understanding of β-arrestin function is not limited to signal arrest, but has expanded to include activation of G-protein independent signaling, thus marking β-arrestins master regulators of both signaling pathways (22, 53, 54).

The discovery of β-arrestin signaling as an alternative pathway to G protein signaling opens the field of functional selectivity for GPCR activity aka biased signaling (11, 22), this concept defines the ligand/receptor conformation able to selectively initiate only one of the two signaling pathways available for a given receptor.

Traditionally, receptors were thought to be in an inactive (*off)* conformation on the plasma membrane and following ligand binding, changes to the receptor conformation result in a so called active or *on* position. Studies have shown that some agonists and antagonists are able to selectively activate or inhibit different pathways downstream the receptor. Thus, the on/off model has expanded to include the possibility of multiple active conformations in which some agonists/antagonists interact with the receptor in a "biased" manner, preferentially

activating G protein signaling while simultaneously inhibiting β-arrestin signaling or vice versa (Figure 2). This *multi-state* model is a more accurate representation of these receptor conformation possibilities and describes how a single GPCR cand produce variations between G protein and β-arrestin signals (30, 55).

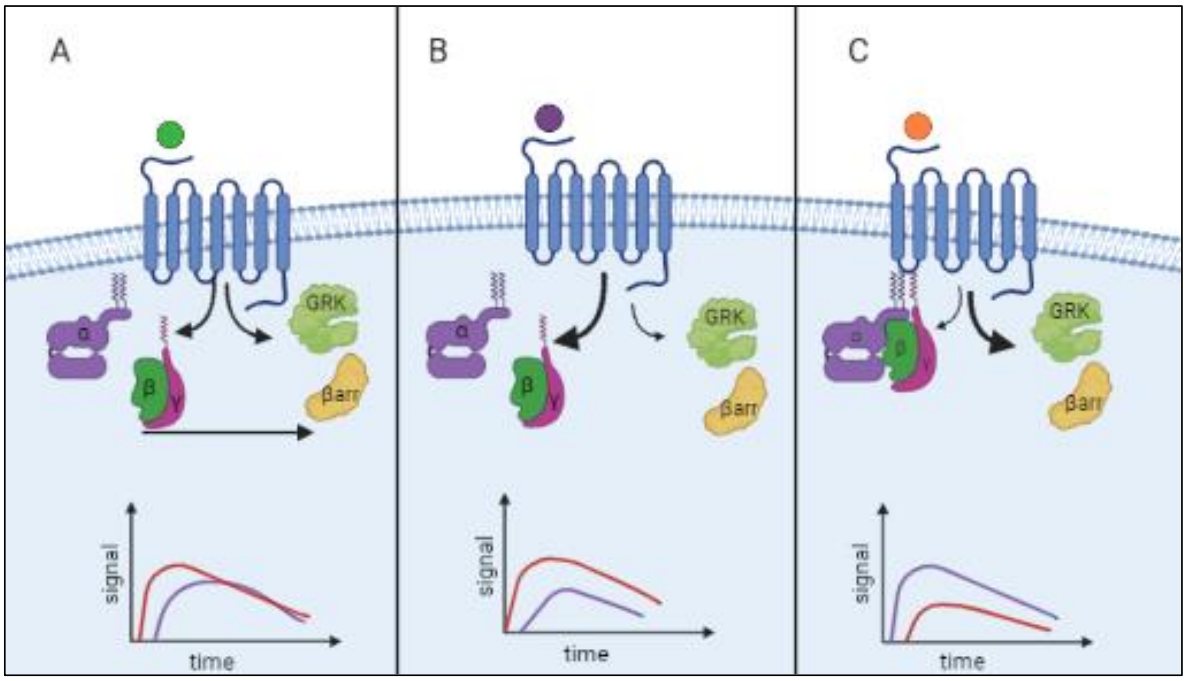


Figure 2. GPCR Biased signaling. (A) Balanced signaling in which the receptor equally activates G protein and β-arrestin signaling pathways. Biased signaling: preferential activation of either G protein signaling (B) or β-arrestin signaling (C).

With new understanding of GPCR activation and interaction with small molecules (10), biased signaling has also provided novel clinical drug targets and therapeutic implications of selective targeting for specific signaling pathways with a reduction in side effects (22, 27).

1.7 RECEPTOR TYROSINE KINASES (RTKs)

RTKs are cell surface receptors containing a kinase domain that relay extracellular signals into biological effects such as cell survival, growth, proliferation, differentiation, migration, and invasion. In contrast to the limited number of cancer-targeting agents against GPCRs, the much smaller RTK family has been a major target for drug discovery (56) for the last two decades. The RTK family is made up of about 60 receptors divided into 20 sub-families based on their common sequence homology. They share a similar structure consisting of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase. Each RTK is activated by (and named by) a specific ligand produced locally (autocrine or paracrine loop) or secreted into the blood stream from distant sites (endocrine).

With two notable exceptions, all RTKs are monomers, with dimer formation following ligand binding of two RTK monomers. Dimerization results in the cross phosphorylation (trans) of the two kinase domains and amplifiers intrinsic receptor kinase activity (22, 57, 58). The two prominent exceptions of RTK expressed as preformed dimers are insulin receptor (IR) and insulin-like growth factor 1 receptor (IGF1R) (59).

1.7.1 The Insulin-like Growth Factor 1 Receptor (IGF1R)

The IGF system is made up of receptors - the Insulin receptor (IR), Insulin-like growth factor receptor (IGF1R), the insulin-like growth factor 2 receptor (IGF2R), and IR/IGF1R hybrid receptor, as well as seven binding proteins (IGFBP 1-7) and three ligands: Insulin, IGF1, and IGF2 (25, 30, 55, 58). IGF1R and IR share around 70% structural homology, with 100% identical organization within the tyrosine kinase domain (60). As preformed dimers, IGF1R/IR are made up of two monomer units, each consisting of one α extracellular subunit and one β subunits, connected by disulfide bonds. The β subunit consists of an extracellular part, a transmembrane region, and an intracellular domain which in turn is divided into three regions: the juxtamembrane domain, the tyrosine kinase domain, and the C-terminal tail (22). The two αβ monomers are also interconnected by disulfide bonds (60).

Due to its kinase activity, IGF1R has historically been classified as an RTK. The inactive receptor keeps a low basal kinase activation through its inhibitory conformation. A specialized section within the kinase domain also known as the activation loop (A-loop) prevents ATP and substrate access to the active site of the kinase. The A-loop contains three tyrosine residues located at 1131, 1135 and 1136 (30, 61) positions and are responsible for initiation of receptor autophosphorylation. After ligand binding, the three tyrosine residues of the A-loop are phosphorylated by their counterpart on the opposite dimer subunit (in *trans),* starting with Tyr 1135 which is no longer locked in the cis-position (30, 62). Phosphorylation of Tyr 1131 and Tyr 1136 follows in quick succession (30, 61, 62). Phosphorylation of the A-loop greatly increases the IGF1R kinase activity and induces autophosphorylation of multiple tyrosine residues within β-subunit of the receptor, including Tyr950. This process generates docking sites for downstream signal molecules such as Shc and IRS (63, 64) to bind to the receptor and activate downstream tyrosine kinase signaling pathways (22, 24, 25, 27, 30, 57, 62). Among them, MAPK/ERK and PI3K/Akt signaling pathways orchestrate essential processes involved in the initiation and maintenance of the malignant phenotype (22, 27, 55, 57).

1.8 IGF1R SIGNALING

MAPK/ERK is one of the most important signaling cascades and is activated once tyrosine residues on the IGF1R are phosphorylated by the adaptor proteins IRS or Shc. The phosphorylated tyrosine residues on Shc are recognized by Grb2 through the src homology

domain (SH2) and after interaction with the Ras guanine nucleotide exchange factor (GEF) (21), son of sevenless (SOS), through its SH3 domain, trades GTP for GDP on Ras. Raf, a serine/threonine kinase, activated by interaction with RasGTP, activate dual-specificity protein kinase kinases (MEKs) which in turn activate extracellular signal-regulated kinases (ERK1 and 2) by way of tyrosine and threonine phosphorylation. Once activated, ERK1/2 translocate to the nucleus and phosphorylate several kinds of transcription factors (Elk, c-Fos, c-My, etc.) regulating cell proliferation and apoptosis (65).

PI3K, the other main signaling pathway activated by IGF1R is the result of the phosphorylated receptor and IRS interaction with class 1 PI3K, p85/p110 complex. PI3K activation initiates phosphatidylinositol 4,5-biphosphate (PIP_2) phosphorylation and subsequent phosphatidylinositol 3,4,5-biphosphate (PIP_3) synthesis where it binds phosphoinositide-dependent kinase-1 (PDK1) and Akt. After binding to the inner membrane, Akt is phosphorylated at the Thr308 and Ser473 residues (66, 67). Activated Akt phosphorylates and inhibits many substrates including Bcl-2, the Bcl-2-associated death promoter (BAD) (68), caspase 9, the pro-apoptotic effector protein glycogen synthase kinase-3β (GSK-3β) Forkhead box O-class protein (FOXO) and mTOR (69)regulating key cellular processes such as glucose metabolism, protein synthesis and cell survival.

IGF1R can activate several other signaling cascades. The ligated receptor can activate Jun N terminal kinase (JNK) and p38. IGF1R interacts with other signaling pathways through downstream signaling molecules such as Grb10, an adaptor protein which binds to IGF1R tyrosine residues located between amino acids 1229 and 1145 as well as Tyr 1316 (30, 70). Grb10 interacts with neuronal precursor cell-expresses developmentally regulated 4 (Nedd4) to ubiquitinate IGF1R (57, 71). Other IGF1R-substrate interactions include CrtII, CrL, RACK1, Focal adhesion kinase (FAK), Syp, GTPase-activating protein, C-terminal Src kinase and suppressor of cytokine signaling 2 (SOCS2) (57).

1.8.1 IGF1R as an RTK/GPCR hybrid

RTKs and GPCRs have been known to share signaling pathways in a paradigm known as receptor cross-talk and involves the GPCR dependent modulation of RTK activity or *vice versa*. RTK transactivation and the ability of RTKs to utilize GPCR components for signaling are the two mechanisms at the core of RTK/GPCR cross-talk (72, 73). Examples of RTK involved in cross-talk with various GPCRs include of epidermal growth factor receptor (EGFR) (74), vascular endothelial growth factor receptor (VEGFR), Platelet-derived growth factor receptor (PDGF) and nerve growth factor receptor (NGF) (73).

Similar to their interaction with GPCRs, β-arrestins were found to control IGF1R (25, 75, 76) trafficking and signaling. β-arrestins isoforms 1 and 2 were shown to enhance ubiquitination of IGF1R by mouse double minute 2 homolog (MDM2) (77). Moreover, IGF1R internalization and trafficking dependency on β-arrestins was demonstrated by the increase of

receptor internalization in β-arrestin1 overexpression. In contrast, inhibition of β-arrestin1 by way of dominant negative mutant blocked internalization of the receptor (78). GRK involvement in IGF1R function has also been demonstrated with isoforms 2/3 and 5/6 identified as mediators of IGF1R/β-arrestins interaction. GRK2 and 6 control β-arrestin isoform recruitment through phosphorylation of distinct serine residues at the C-terminus with divergent outcomes. GRK2 phosphorylation preferentially supports transient β-arrestin binding, while GRK 6 isoform results in a stronger, stable IGF1R/β-arrestin binding and subsequently a shift towards higher receptor degradation (12) and MAPK activation (57) (Figure 4).

Thus, IGF1R receptor directly utilized at least two GPCR components: GRKs and β-arrestin (Table 1). Taken together, the extent in which IGF1R utilizes GPCR components is not unique among RTKs suggesting there is a need to reclassify IGF1R as an RTK/GPCR functional hybrid.

GPCR characteristics	**IGF1R /GPCR hybrid**
Binding to the ligand activates signaling through heterotrimeric G proteins	indirectly
GRKs phosphorylate serine residues on the activated receptor	yes
Leading to the creation of binding sites for β-arrestins	yes
β-arrestins recruitments cause desensitization of the signal	?
Initiation of a second β-arrestin-dependent signal	yes
Receptor endocytosis through either recycling or degradation pathways	yes

Table 1. IGF1R utilizes GPCR components.

IGF1R has long been an attractive target for cancer therapies, and due to the classification as an RTK, all strategies have so far been aimed at inhibiting its intrinsic kinase activity accounting for the failure of past clinical trials (79). However, in light of recent updates, it is obvious that IGF1R kinase signaling is only part of the story, as evidenced by the use of blocking antibodies which act as biased agonists in addition to downregulating the receptor (25, 57). Thus, a paradigm shift was necessary in approaching anti-IGF1R targeting strategies in cancer (22, 25, 30, 80).

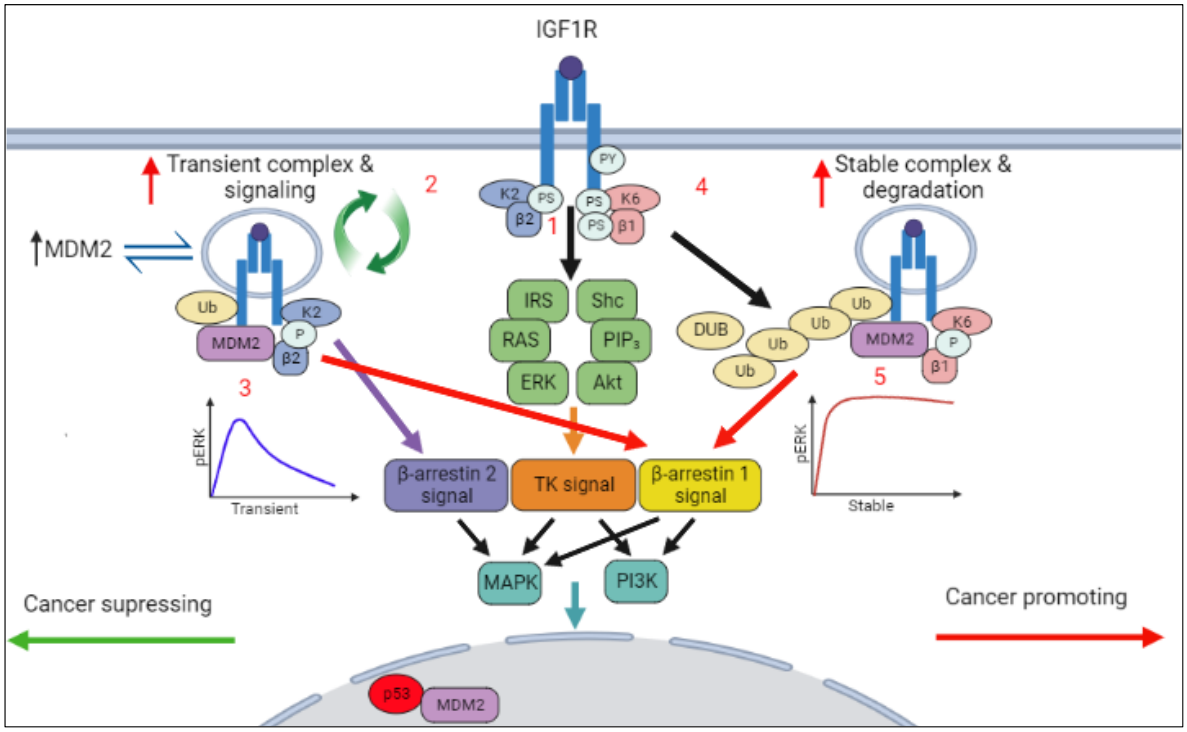


Figure 3. Updated model for IGF1R signaling: IGF1R canonical kinase signaling (1) activates PI3K and MAPK signaling pathways downstream the receptor and subsequent biological effects. Non-canonical, kinase-dependent IGF1R signaling is mediated through β-arrestins/receptor interaction. In conditions with low-ligand concentrations, IGF1R is phosphorylated by GRK2, preferential interaction with β-arrestin 2 (2). This interaction is transient, the receptor is internalized and rapidly recycled back to the surface with minimal activation of the β-arrestin 2-dependent MAPK signaling (3). In the presence of ligand, (4) β-arrestin 1 binds and forms stable complexes with GRK6-phosphorylated receptor (5). This interaction leads to increased receptor degradation, and sustained cancer-promoting MAPK signaling.

1.9 IGF1R AND THE TUMOR SUPPRESSOR P53 PATHWAY

Inactivation of the p53 tumor suppressor pathway is a major hallmark of cancer. Wild type p53 stimulates transcription of its own repressor, MDM2. In normal conditions, p53 levels are tightly controlled by the MDM2 ubiquitin ligase. MDM2 binds, inactivates and ubiquitinates p53. As a result, p53 loses its transcriptional power and is translocated to the cytoplasm where it is degraded via the proteasome maintaining p53 at a low basal level. Under conditions of DNA damage or oncogenic stress, the p53/MDM2 interaction is restricted leading to p53 nuclear accumulation. As such, several downstream genes are transactivated including cyclin-dependent kinase inhibitor (CDKN1A), BAX, PUMA, NOXA and MDM2 resulting in cell cycle arrest (81), senescence, and cell death (82-85). In

cancer, p53 is inactivated either by mutation or increased MDM2 expression (81). Mutations of p53 are found in 50% of cancers and most are missense mutations largely affecting its transcriptional activity. However, the majority of the mutant p53 protein variants can still interact with MDM2. Moreover, overexpression of MDM2 inactivates p53wt ensuring a balanced p53/MDM2 system.

MDM2 was also identified to act as a ubiquitin ligase for IGF1R in what has become a complex IGF1R/p53/MDM2 axis (86) often exploited by cancer cells. As IGF1R and p53 compete for the same E3 ubiquitin ligase, increased expression level of p53 (due to mutational status) lowered IGF1R ubiquitination and degradation by MDM2. With lower IGF1R degradation rates due to MDM2 sequestration by p53, the receptor is recycled at higher rates. This results in enhanced activation of PI3K/MAPK signaling pathways downstream the IGF1R (77, 86, 87) ensuing the proliferation and survival of cancer cells. At a transcriptional level, IGF1R is suppressed by wtp53 both directly and through interaction with several IGF1R transcriptional inhibitors (88-91), including the p53 homolog proteins p63 and p73 (92). Conversely, mutant p53 has been shown to enhance IGF1R transcription (93-96). p53 also protects the receptor from ligand activation through increased transcription of IGFBP2 and IGFBP3 as well as inhibition of IGF-2 expression (96-98).

β-arrestin 1 has been found to mediate the MDM2-dependent degradation of IGF1R. The subsequent β-arrestin 1-induced activation of PI3K/MAPK signaling pathways downstream the receptor promotes MDM2 binding and enables p53 degradation (99), through the exact mechanism is still unknown.

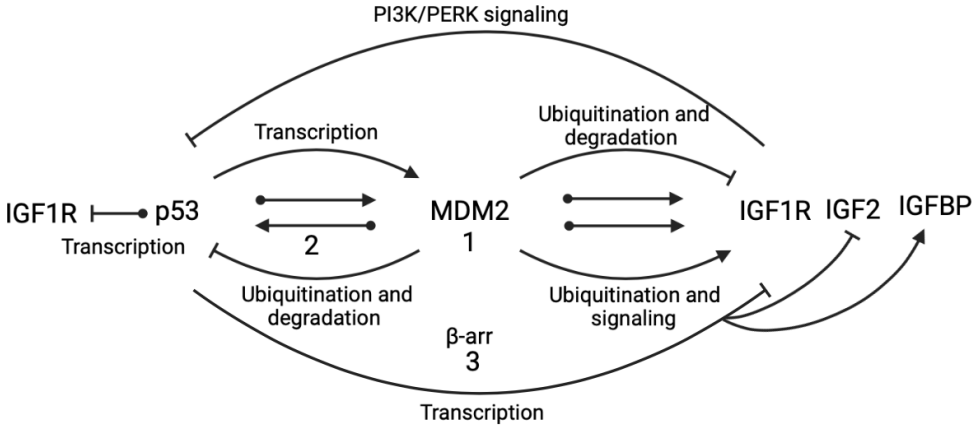


Figure 4. IGF1R/p53/MDM2 axis: p53 and IGF1R proteins compete for the same ubiquitin ligase MDM2. Strong negative feedback mechanisms are in place to maintain the status quo between the tumor suppressor p53 and tumor promoting IGF1R

The IGF1R and p53 pathways are therefore linked in a complex relationship on multiple levels (Figure 3). Understanding this onco-suppressor/onco-promoter interplay is essential to

comprehending the basic process of IGF1Rs role in cancer promotion and a key aspect in the improvement of anti-cancer therapeutic strategies (86, 100).

1.10 EWING'S SARCOMA

Ewing's sarcoma (ES) is an aggressive form of childhood cancer. ES are tumors of the bone and soft tissue, usually originating in the lower extremities and pelvic area (osseous sites) or the trunk and extremities (extraosseous sites). Up to a quarter of patients develop metastasis commonly located in the bone, long or bone marrow. The standard therapy involves chemotherapy induction followed by local treatment consisting of surgery and/or radiotherapy resulting in a 5-year overall survival rate of 60-80% depending on staging at the time of diagnosis (101). Characterized by the t (11; 22) (q24; q21) chromosomal translocation and the resultant gene fusion between EWS (chromosome 22) and FLI1(chromosome 11) (101) has been identified as an oncogene. The EWS-FLI1 protein, a main driver of ES transformation activates several transcription factor inhibitors such as NROB1, NKX2.2 as well as inhibits IGFFBP3 and TGFBR2 (102). EWS-FLI1 also enhances expression of several downstream targets known to be involved in growth and survival of cancer cells (103) including *IGF1* (104), *GLI1 (105), Myc* (106) *and ID2* (107). The fusion protein also leads to upregulation of *EZH2* and *SOX* and induction of cell differentiation (108, 109).

EWS-FLI1 is unique to ES and tumorigenesis appears to be dependent on the fusion protein making this an ideal target for therapy (110, 111). Despite the specificity, increased toxicity in clinical trials has rendered EWS-FLI1 targeting unsuccessful so far.

Signaling in ES involves, among other RTKs, IGF1R-mediated constitutive activation of the ERK1/2 signaling pathway suggesting the receptor plays an important role in ES tumorigenesis (103, 110-114). Multiple IGF1R inhibitors (115) have been investigated both in *vitro* and in *in vivo* studies, including robatumumab (116), R1507 (117), ganitumab (118), cixutumab (119) and figitumumab (120). Moreover, IGF1R inhibitors in combination with mTOR inhibitors, which induce IGF1R dependent phosphorylation and signaling activation of Akt, have shown promise in preclinical models.

1.11 MELANOMA

Skin melanoma makes up around 3% of all skin cancers however due to its increased propensity towards metastasis, melanomas are responsible for about 65% of all skin cancer deaths (121, 122). Treatment can be curative in the event of diagnosis in the localized stages of disease, as opposed to metastatic melanoma where the prognosis is much less favorable.

Conjunctival melanoma (CM) is the most frequent mucosal melanoma and distinguishes itself within this category as being the only case with ultraviolet radiation (UVR) exposure as an etiologic factor. While CM incidence parallel the that of skin melanoma, the 10-year cumulative

local recurrence and mortality of CM remains high, and there are no consensus guidelines on the postoperative adjuvant therapies to decrease the risk of recurrence and metastasis.

Due to its demonstrated involvement in malignant proliferation, and survival, IGF1R has long been an attractive target of therapy in melanoma (123, 124). Considering IGF1R involvement in all aspects of cancer metastasis, melanoma represents a very relevant experimental model to explore possible therapeutic strategies to prevent, delay or cure metastatic disease. Moreover, the IGF1 system has been shown to be involved in drug resistance targeted therapy (57, 80, 125-127), standard chemotherapy (80, 128, 129) or radiation (130, 131) in melanoma.

1.12 ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is a heterogenous group of hematological malignancies characterized by impaired differentiation of myeloid cells and clonal proliferation of leukemic blasts. This translates into various clinical outcomes as a result of myeloid dysfunction with over 20% immature or undifferentiated blasts in the bone marrow and hindered growth of normal blood cells. AML is the most common and the deadliest type of leukemia diagnosed in adults, with a 5-year overall survival of just 33,5% (132). Etiological classification of AML can be de novo, secondary (patient has previous hematological malignancies or has undergone chemo-radiotherapy) or relapsed/refractory in cases of disease recurrence after treatment (133). Current treatment varies from the classical cytarabine-based chemotherapy to targeted therapies such as fms-like tyrosine kinase 3 (FLT3), isocitrate dehydrogenase 1 and 2 (IDH1 and IDH2) (134-136) and/or hematopoietic stem cell transplantation (HSCT). AML prognosis is dependent on staging at time of diagnosis and the cytogenetic and molecular characteristics. However only a few patients who relapse after complete remission (CR) survive for more than 5 years (133, 137). One of the most successful therapeutic strategies in preventing relapse is the induction of blast differentiation in acute promyelocytic leukemia (APL) with all-trans retinoic acid (ATRA) in connection to standard chemotherapy (138).

Among the most extensively investigated signaling pathways in cancer cells is the PI3K-Akt-mTOR pathway. This signaling pathway is integral to normal hematopoietic cells and is responsible for regulating proliferation, differentiation, and survival. Constitutive activation of the PI3K-Akt-mTOR pathway is found in over 60% of AML patients (139). This upregulation is thought to be caused by mutation in RTKs, increased GTPases activity and other membrane-bound proteins as well as FLT3 signaling (140). Found in about 55% of AML patients constitutive activation of PI3K-Akt-mTOR pathway has been linked to worse overall survival (138, 140-144). Akt is in turn constitutively activated by the enhanced PI3K-Akt-mTOR pathway, but also through autocrine IGF1/IGF1R signaling (145).

The upregulation of IGF1/IGF1R (146) is commonly described in multiple leukemia disease subtypes, including 70% of AML patients (145, 147). Moreover, IGF1R upregulation has been shown to protect leukemia cells from apoptosis and has been linked to disease progression, drug resistance (145, 147, 148), and even AML transformation of myelodysplastic syndrome (MDS) (149).

Several studies involving several IGF1R inhibitors have demonstrated their therapeutic potential in AML. NVP-ADW742, a small molecule inhibitor against IGFIR was shown to dephosphorylate Akt and downregulate the antiapoptotic protein BCL-2 in AML cells (150). Treatment with another small molecule IGF1R inhibitor, NVP-AEW541 resulted in enhanced response to etoposide chemotherapy as well as additional anti-apoptotic effects in primary AML (148). The dual IGFIR/IR inhibitors, BMS-536924 or BMS-554417 mitigated constitutive IGF1R activation and subsequent MAPK/PI3K downstream signaling (151, 152). Though preclinical and even some early clinical data for anti-IGF1R therapy in AML were promising, the low tolerance and high drug resistance seen in patients proved less than ideal. However, several clinical trials suggest IGF1R targeting in combination with standard chemotherapy and/or other targeted therapies could be a more viable strategy.

2 RESEARCH AIMS

The overall aim of this thesis is to explore different signaling arms downstream IGF1R in relation to their corresponding biological effects in non-epithelial cancers. The generated knowledge was further used to develop novel anti-cancer strategies.

Paper I: Investigate the potential of GRK modulation to control IGF1R downregulation without cancer protective β-arrestin biased-signaling activation.

Paper II: Investigate the effects of destabilizing the p53/MDM2/IGF1R axis on conjunctival melanoma growth and metastatic phenotype.

Paper III: Evaluate the contrasting properties of the β-arrestin system to control different signaling pathways as therapeutic target for cancer

Paper IV: Explore therapeutic potential of the GRK/β-arrestin biased signaling to induce cell differentiation in acute myeloid leukemia.

3 MATERIALS AND METHODS

Detailed specific materials and methods are described within individual papers. This thesis is aimed at investigating the mechanisms involved in biased IGF1R signaling and whether the components involved could be exploited for therapeutic gain. To achieve this, the materials and methods are designed to: investigate expression, analyze receptor function and biological effects as well as *in vivo* experiments.

3.1 ETHICAL CONSIDERATIONS

All animal models used in this study followed guidelines set by the local ethical authority. For the mice model in **Study 1**, xenograft studies were approved by the MD Anderson Cancer Center (MDACC) Institutional Animal Care and Use Committee. **Studies 2 and 3** explore in vivo models consisting of zebrafish embryos of up to 5 days post-fertilization. All animal care and handling were in compliance with local regulations at Karolinska Institute Zebrafish Core Facility.

3.2 CELL LINE MODELS

	Cell line
Study 1	Human embryonic kidney: HEK293T Ewing sarcoma: A673, CADO, RDES, SKES, and SKNMC Osteosarcoma: U2OS and Saos-2 Mouse embryonic fibroblasts (MEF) wild-type (WT) and knockout for β-arrestin1 (KO β1), MEFΔCT*
Study 2	Conjunctival melanoma: CM (CM2005.1), CM-1 (CRMM1), CM-2 (CRMM2)
Study 3	Malignant melanmona: BE, DFB, SK-Mel-28, MelJuSo SK-Mel-5, SK-Mel-2
Study 4	Acute myeloid leukemia: THP1, HL-60 Chronic myeloid leukemia: K562 HEK293T, HEK293T IGF1R KO (I5) **

Table. cell lines used/study

* MEF with an IGF1R–null background cells (R-) stably transfected with IGF1R with C-terminal tail truncation at residue 1245.

** HEK293T IGF1R KO (I5), CRISPR IGF1R knockout HEK293T clone permits the confirmation of transfected IGF1R mutant expression in the absence of endogenous IGF1R.

All cell lines underwent mycoplasma testing and STR profiling every 12 months.

3.3 EXPRESSION ANAYSIS

Western blot (WB)

Protein expression was analyzed by western throughout the thesis to characterize cell line panels and evaluate the effect of various treatments on expression of IGF1R, β-arrestins, Akt, ERK1/2, p53, MDM2 and various other proteins. For degradation experiments, serum starvation was used to bring signaling pathways to basal levels before stimulation with IGF1 for 12 and 24h. Treatment with various pharmacological inhibitors and transfection with plasmids or siRNAs were performed as detailed in the materials and methods of each individual paper. Cell fractionation was performed according to manufacturer's protocol detailed in paper 3. After treatment, samples were dissolved using lithium dodecyl sulfate (LDS) sample buffer. Separated proteins were run using SDS-PAGE with 4-12% BisTris gels and transferred on nitrocellulose membranes and analyzed for target proteins. Primary and secondary antibodies used are listed in the supplementary material of each individual paper included in the thesis.

FACS

Fluorescence activated cell sorting (FACS) was used to analyze expression of differentiation markers on live cells. Samples were treated for the indicated time points, collected, and washed with PBS. Following blocking, samples were stained with fluorescent-conjugated antibody in FACS buffer (2%BSA) according to protocol detailed in paper 4. After 20 min incubation at room temperature, cells were washes and analyzed. All samples were acquired on a Novocyte (ACEA Biosciences).

Immunohistochemistry (IHC)

Another method to evaluate protein expression used in papers 1, 2 and 3 was IHC in both 2D, 3D and animal models. Adherent cells, spheroids or mice tumor samples were processed, and fixed with 4% PFA with paraffin inclusion for spheroid and mice tissue samples. Immunostaining was performed in the appropriate manner for each sample type followed by 1% BSA serum blocking before primary and secondary antibody incubation. Immunostaining protocol details, primary and secondary antibodies and analysis methods used can be found in the individual papers.

Immunofluorescence (IF)

Following treatment, cells were seeded on collagen coated plates. Samples were fixed in 4% PFA, blocked in BSA and stained with primary antibody against MDM2 overnight in 4^0C and later secondary antibody incubation. Antibodies and staining protocol are detailed in paper 3. Cells were imaged by confocal microscopy and analyzed for nuclear signal to evaluate shift in target protein localization after treatment.

3.4 FUNCTIONAL ANALYSIS

Western blot

Analysis of the kinase and GRK/βarrestin signaling branches activated by IGF1R can be partially distinguished kinetically. For this reason, through the thesis **western blot** analysis was consistently used to evaluate time-course and dose dependent phosphorylation by kinase and GRK/βarrestin signaling activation. Serum starvation was used to achieve basal level of the signaling cascades before IGF1 stimulation for the indicated times followed by cell collection and lysis. The wild-type form of the receptor activation is responsible for balanced downstream signaling of both MAPK and PI3K (pERK and pAKT) pathways. Peak levels of phosphorylated (p) IGF1R, pERK and pAKT protein levels ca be observed at 5/10 minutes after ligand stimulation followed by gradual decreased over the course of 60 minutes. As a scaffold protein keeping the proteins together, βarrestin1 biased signaling is a later occurrence as evidence by the sustained MAPK activation. Hence, across the papers included in this thesis, late pERK activity (60 minutes as % of peak) along with the relative phosphorylated (p) IGF1R, pERK and pAKT protein levels were analyzed to draw inferences regarding the balanced and imbalanced signal. **Immunoprecipitation** analysis of protein interactions was used to investigate the molecular interactions that initiate biased signaling. To validate results, quantification and statistical analysis was performed for multiple independent western blot experiments.

3.5 BIOLOGICAL EFFECTS

Cell viability assay

Cell viability was measured via PrestoBlue assay according to the manufacturer's instructions (Invitrogen, CA, USA). Fluorescence was measured using 560 nm excitation and 590 nm emission using a Tecan Infinite 1000 or a Varioskan Lux plate reader. Standard curve fluorescence measurement of known cell number was used to interpolate cell viability. In study 3, cell viability in combination treatment was measured using Incucyte and analysis was performed using the confluency mask software.

Spheroid formation assay

Spheroid formation was used in Studies 2 and 3 as a model for combination drug testing. This 3D model acts as a bridge between our 2D experiments and in vivo animal experiments. Advantages of this model are the shorter (5 days) experimental time compared to classical animal models, ideal model to investigate tumor growth, apoptosis, migration, invasion and response to single or combination treatment in a controlled system, before moving forward to in vivo experiments. However, not all cell lines can form spheroids, even in Matrigel. Other disadvantages are that cellular signaling may not be in line with the 2D model and the spheroid is not a complete reproduction of tumor growth in the human body.

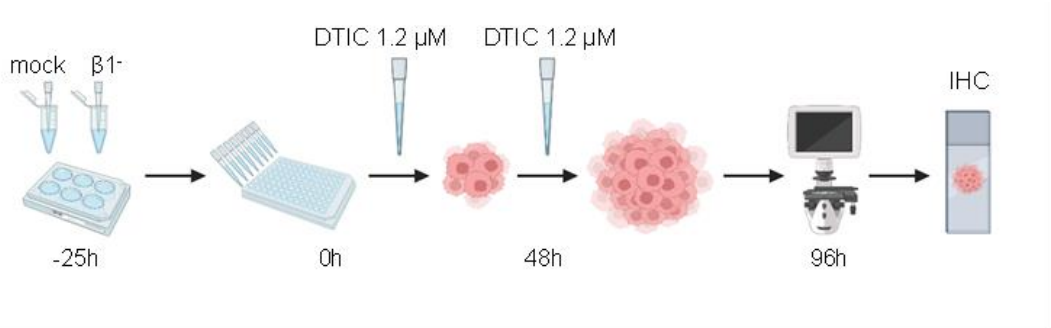


Figure. Spheroid formation assay work-flow.

Differentiation assay

Two different methods were used to evaluate and verify induction of differentiation in AML cell lines: FACS analysis of fluorescent-conjugated differentiation marker and live cell microscopy of leukemia cell phenotype by measurement of adherent cell population.

Cell cycle distribution analysis

Cell cycle distribution was used to investigate the effect of IGF1R activation on cell proliferation and survival. Experimentally treated AML cells were stained with DAPI and FACS analysis was used to evaluate specific changes to the cell cycle.

3.6 OPEN-SOURCE GENE EXPRESSION ANALYSIS: TCGA DATABASE

The Cancer Genome Atlas (TCGA) is an open-source database containing over 30 cancer types with clinical characterization, proteomics profiles and genomics data. TCGA is an easily accessible tool for both clinicians and researchers to better stratify prognosis identify new possible prognostic biomarkers for improved patient survival. The TCGA database is mostly made up of treatment naive primary tumors. This makes analysis of potential molecular gene signatures and patient outcome more accurate as considering the difficulty in obtaining tumor samples available for proteomics without previous exposure to standard or neoadjuvant treatment.

3.7 *IN VIVO* EXPERIMENTS

3.7.1 Zebrafish experiments

Zebrafish model

Animal models are one of the most frequently used approaches in the investigation of targeted therapy and combination therapy in cancer research. Over the last decades,

implantation of cancer cells from melanoma, sarcoma, leukemia, and various other cancers into zebrafish has been proven as one of the simplest, most robust model platforms in cancer studies. Due to high fecundity, transparency of embryos and 82% gene conservation to humans, the zebrafish model is high-throughput, and relatively low-cost. This model has several applications in the study of disease development, including cell invasion, metastasis, and tumor angiogenesis, in addition to the targeted drug response (153). Furthermore, the multitude of strains available to researchers from immune-deficient zebrafish strains lacking T, B and natural killer (NK) cells to vascular specific, fluorescent (green) strains allows for cancer cells labeled with a different color (e.g., red) florescent maker (CM-Dil) to be implanted in 2-day post fertilization (2 dpf) embryos to be tracked for up 7 dpf (154). Transplanted tumor cells can be assessed by fluorescence microscopy, confocal microscopy, flow cytometry, immunohistochemistry, or protein assays, to evaluate treatment response and cancer metastasis.

Figure. Zebrafish model work-flow.

Cancer cell preparation

Preparation of cancer cells pre-implantation is one of the first challenges in the use of a zebrafish model. CM-Dil stained single cell suspension is resuspended in polyvinylpyrrolidone (PVP) to reduce cell-to cell adherence and provide a matrix for the newly implanted tumor.

To achieve true single cell suspension, personal experience suggests reseeding 24 hours before injection to be necessary for easy, gentler dissociation of cell clumps and aggregates. Cells are then passed through 40µm cell strainer up to five times before forming the pallet by centrifugation and resuspension in 1ml PVP. Cell number is another key aspect of pallet preparation as a too high cell number leads to clump and aggregate formation both before and after pallet formation. On the other hand, if the cell number is too low pallet size could be insufficient to load the injection needle or ensure stable cell number/injection. Cell lines with high cell-to-cell adherence can form aggregates after preparation. Thus, if the experiment plan requires a higher number of embryos or for the cells to be injected at more than 30 min after preparation, needle reloading is often necessary. For this reason, a careful optimization should be done for each cell line considering cell number/pallet size balance to guarantee both sufficient cell suspension for the number of injections as well as maintaining a clump-free cell suspension.

Injection

Injection should be performed as accurately as possible to maintain consistent cell number and injection site throughout embryos. To verify accuracy, florescence microscopy is used on all injected embryos immediately after injection. Embryos with injection errors are excluded from the experiment, including burst perivitelline space, leakage into the yolk sac or blood stream or variations of fluorescent signal suggesting inequal number of cells between replicates. Embryos are then randomly distributed in treatment groups.

Analysis

Depending on the output data required, zebrafish analysis can be performed in several ways. Florescent microscopy at experimental end-point to evaluate tumor size and metastasis is the most common and was used in two of the four studies in this thesis. At day 3 post-injection, zebrafish imaging was analyzed for tumor size and metastasis foci in the tail using Photoshop or ImageJ software. In a separate study not included in the thesis, Zebrafish were dissociated and analyzed by FACS, cell sorting and western blot. Experimental target cells were erythrocytes and upon testing of different protocols, mechanical dissociation via 5ml syringe plunger of no more than 100 embryos/sample proved to produce the lowest debris while maintaining a high enough concentration of red blood cells.

Optimization of zebrafish experiments should be tailored to cell line characteristics, injection site and replicate requirements as well as analysis of target data.

3.7.2 Mouse model

Mouse xenograft models are an established in vivo animal experimental model used to mimic most conditions in human and was used in paper 1. Experimental cells were inoculated in male nude mice 5- to 8-week of age using a subcutaneous (s.c.) injection of 5*10^6 cells suspended in 0.1 mL sterile saline solution. Measurement of tumor volume was performed every 4 days and at a tumor growth of 65 mm^3, mice were treated via intraperitoneal (i.p.) injections. Mice were observed and sacrificed 32 days after of treatment, at a tumor volume measurement of 1,000 mm3 and sacrificed according to local ethical guidelines. Tumors were collected, measured, and processed for further analysis (IHC or frozen/RNA extraction). All animal care and handling were in accordance with and approved by the MD Anderson Cancer Center (MDACC) Institutional Animal Care and Use Committee.

4 RESULTS

4.1 STUDY 1: INHIBITION OF G PROTEIN–COUPLED RECEPTOR KINASE 2 PROMOTES UNBIASED DOWNREGULATION OF IGF1 RECEPTOR AND RESTRAINS MALIGNANT CELL GROWTH

Background and rationale:

Receptor downregulation without β-arrestin1 signaling activation is a requirement for effective targeting of IGF1R in cancer. Considering that IGF1R activation of β-arr1 is controlled by GRKs, we investigated the biological effects of GRK2 and GRK6 isoform modulation on IGF1R downregulation and subsequent biological effects.

Results and Discussion:

To evaluate the effect of GRK2 and 6 isoform modulation on IGF1R expression and signaling, we utilized transfection with small-interfering RNAs (siRNA) and plasmid overexpression. Resultant expression and function of the receptor were analyzed by western blot to investigate IGF1R degradation and signaling. Transgenic modulation demonstrated opposing effects of the two GRK isoforms, with GRK2 siRNA (-) and GRK6 overexpression (+) resulting in enhanced receptor degradation and GRK2 overexpression (+) and GRK6 inhibition (-) proving protective for the receptor. Additionally, GRK2 (-) and GRK6 (+) increased sustained ERK activation in response to IGF1R ligand stimulation, demonstrating β-arrestin biased signaling and these two conditions were taken forward for further experiments. Assessment of biological effects of GRK2 (-) and GRK6 (+) showed reduced proliferation in adherent conditions as well as anchorage-independent experiments suggesting the potential of a therapeutic shift towards a GRK6 biased system. Pharmacological inhibition of GRK2 via paroxetine (PX) – a serotonin reuptake inhibitor - widely used as an antidepressant for the treatment of mood and anxiety disorders - controls β-arrestin/IGF-1R association, initiating receptor degradation without the cancer-protective biased signaling. While PX treatment induced IGF1R downregulation in a time and dose dependent manner, PX showed no β-arrestin biased signaling activation of MAPK/ERK pathway. There results indicate the PX induced degradation of the receptor dependency on β-arrestin1 and its ability to interact with the IGF1R C-terminal. In U2OS, a cell line overexpressing MDM2, PX could induce activation of MAPK/ERK signaling in a transient manner. Using co-immunoprecipitation we demonstrated that, in ligand conditions, PX prevents β-arrestin2/IGF1R binding, promoting β-arrestin1 recruitment and enhanced MDM2-mediated ubiquitination and degradation of the receptor in a manner usually found particular to IGF1 stimulation. The manner in which PX alters the recruitment of β-arrestins isoforms and inhibits both IGF1R expression and β-arrestin signaling, was tested in a malignant model. In ES, where IGF1R/ β-arrestin1 biased agonism was previously demonstrated ineffective, PX treatment downregulated IGF1R and reduced receptor sensitivity to its natural ligand. Comparing the biological effects of biased agonist CP (anti-IGF1R antibody) and PX showed

PX inhibited colony formation in a dose dependent manner while CP proved ineffective. Our study identifies and validates that system bias (via GRK2 inhibition) can uncouple functional arms; downregulating the IGF1R without protective signaling, an approach with solid clinical feasibility as it employs a widely used drug with known toxicity.

4.2 STUDY 2: IGF1R IS A MOLECULAR DETERMINANT FOR RESPONSE TO P53 REACTIVATION THERAPY IN CONJUNCTIVAL MELANOMA

Background and rationale:

Reactivation of the tumor suppressor p53 by targeting the IGF1R/β-arrestin/MDM2 axis has previously been shown to as a promising approach in malignant melanoma. Our aim was to evaluate p53 reactivation via disruption of p53/MDM2 interaction as potential therapeutic strategy in CM.

Results and Discussion:

Using a panel of aggressive CM cell lines, we investigated the effect of p53 reactivation on cell viability. To increase p53 activation, we used two approaches: siRNA inhibition of the MDM2, or Nutlin3 an inhibitor of the p53/MDM2 interaction. Nutlin3 proved more efficient compared to siRNA inhibition of MDM2 suggesting a p53 independent mechanism at play. As MDM2 is a ubiquitin ligase for not just p53 but also IGF1R, we investigated the effect of Nutlin3 on IGF1R expression and function. Our entire panel was responsive to IGF1 stimulation and ligand-binding activated PI3K/AKT and MAPK/ERK signaling pathways downstream the receptor. Nutlin3 treatment induced IGF1R degradation and inhibition of cell viability in ligand conditions while the absence of the ligand did not produce the same effects. To further explore the mechanism, we investigated the role of β-arrestin1 in the Nutlin3 induced receptor downregulation. CM cells were transfected with siRNA for β-arrestin1 and analyzed by western blot. Results revealed inhibition of β-arrestin1 protects both IGF1R expression and cell viability from Nutlin3 induced effects. Moreover, β-arrestin1 overexpression increased IGF1R degradation in response to Nutlin3 and decreased expression of p53. This suggests Nutlin3 effect on IGF1R degradation and p53 reactivation is dependent on β-arrestin1. We compared mitomycin (MMC), an adjuvant commonly used in local therapy for CM known to enhance p53 activation, to Nutlin3 and analyzed IGF1R expression and function and biological effects. In both 2D and 3D experiments, all three conditions, MMC, Nutlin3 and combination treatment, induced an inhibition in tumor growth of about 30%. Through more sensitive to MMC treatment, Ki67 staining of CM spheroids show complete cell proliferation inhibition after Nutlin3 treatment. Zebrafish experiments validated our 3D model results, demonstrating Nutlin3 is a superior inhibitor of tumor growth and metastatic potential. Taken together, these results establish the potential of Nutlin3 and impairment of the p53/MDM2/IGF1R system as a novel treatment strategy for CM patients.

4.3 STUDY 3: COMPETING ENGAGEMENT OF B-ARRESTIN ISOFORMS BALANCE IGF1R/P53 SIGNALING AND CONTROL RESPONSE OF MELANOMA CELLS TO CHEMOTHERAPY

Background and rationale:

β-arrestins are essential in controlling p53/MDM2/IGF1R axis in cancer. In this study, our aim was to investigate the role of β-arrestin modulation as a possible avenue to enhance therapeutic response in melanoma.

Results and Discussion:

Using siRNA and plasmid overexpression to modulate β-arrestin isoforms in a panel of six malignant melanoma cells displaying wt (DFB, MelJuSo and SK-Mel-2) and mutant p53 (BE, SK-Mel-28 and SK-Mel-2), and we evaluated their effect on IGF1R expression and function. As such, in a β-arr2-biased (β-arr2^{+} and/or β-arr1^{-}) system, IGF1R receptor was degraded and the cancer-protective β-arrestin1 biased signaling was inhibited resulting in reduced cell viability in all cell lines. β-arrestins have been shown to play a role in transport of MDM2 from the nucleus to the cytoplasm. To evaluate the effect of β-arrestin isoform modulation on MDM2/p53 system, transfected cells were analyzed for MDM2 localization using immunofluorescence confocal microscopy. In β-arrestin2 shifted conditions, MDM2 translocation to the cytoplasm was enhanced in contrast to β-arrestin1 biased system. Cell fractionation validated our results displaying increased cytoplasmic expression in β-arrestin2 biased system and increased nuclear expression in a β-arrestin1 predominant conditions. Considering MDM2 is a ubiquitin ligase for p53, we investigated the effect of β-arrestin-induced MDM2 translocation on p53 activation. Experiments demonstrate that as opposed to β-arrestin1 biased conditions, the β-arrestin2 predominate state increased p53 stabilization while decreasing cell viability and the β-arrestin1 (-) condition was taken forward for further experiments. β-arrestin1 (-), induced downregulation of IGF1R and transient β-arrestin2 signaling while increasing p53 expression. Comparing DTIC treatment, shown to activate p53 in all but one mutant p53 cell line to β-arrestin1 (-) conditions resulted in a similar increase of p53 expression levels. However, β-arrestin1 (-) resulted in lower cell viability and combination treatment proved to be the most effective in both p53 reactivation and inhibition of cell proliferation. These results suggest downregulation of IGF1R in combination to p53 reactivation is beneficial as a dual targeting strategy in cancer. To verify the effect of combination therapy, we used a 3D spheroid model showing reduced tumor growth and increased p53 expression. Our zebrafish model confirmed the inhibitory effect of combination therapy on tumor growth and metastatic potential. These studies indicate that a β-arrestin1 high/p53wt phenotype produces a more aggressive melanoma. Analysis of TCGA data confirm this concept as the β-arrestin1 high/p53wt subgroup presented the worst overall survival. Our study demonstrates that disrupting the β-arrestin isoform in favor of β-arrestin2 could impair the cancer protective mechanism. Though direct β-arrestin targeting is not yet

possible, indirect targeting strategies could be employed to promote β-arrestin2 nuclear localization or inhibit the IGF1R/β-arrestin1 biased signaling.

4.4 STUDY 4: β-ARRESTIN/GRK SIGNALLING DOWNSTREAM IGF1R PREVENTS DIFFERENTIATION AND PROMOTES ACUTE PHENOTYPE OF MYELOID LEUKEMIA

Background and rationale:

PI3K-Akt-mTOR upregulation in acute myeloid leukemia (AML) has been shown to control proliferation, differentiation, and survival. One of the mediators for constitutive activation of PI3K/Akt is IGF1R, frequently upregulated in AML. In our study we aimed to investigate biased targeting of the two branches of IGF1R signaling pathways and their impact on the AML malignant phenotype as a possible strategy for AML.

Results and Discussion:

We evaluated the expression and function of IGF1R in a panel of acute and chronic myeloid leukemia. Stimulation with IGF1 was shown to induce proliferation in all cell lines and activate MAPK/ERK and PI3K/Akt signaling pathways. To further investigate the biological effects of IGF1R on AML, cell cycle analysis showed an increase in proliferative cells after IGF1 stimulation. Moreover, in acute myeloid leukemia cell lines, microscopy imaging shows an increase in adherent phenotype, a characteristic indicative of differentiation in absent/low-ligand conditions. This suggests that IGF1R is protective for the leukemia phenotype, and FACS analysis of differentiation marker CD14 confirmed the IGF1R-dependent maintenance of the undifferentiated phenotype. To investigate the biological effects of ERK signaling pathway (GRK/β-arrestin signaling) independent from Akt signaling pathway (kinase signaling) downstream IGF1R, we transfected plasmids containing various receptor mutants. Western blot analysis of pIGF1R, pERK1/2 and pAkt confirmed the dissociation of the two signaling pathways in AML and evaluation of biological effects showed the two branches produce different outcomes. As such, kinase activation controls cell proliferation and survival while GRK/β-arrestin signaling maintains the undifferentiated phenotype. TCGA data analysis show better overall survival in subgroups with low β-arrestin levels confirming our concept and the potential of targeting GRK/β-arrestin signaling in AML. This study demonstrated that the IGF1R C-terminus supports the undifferentiated phenotype in AML and provides a new targeting strategy in AML.

5 CONCLUSIONS

The present thesis provides insights into therapeutic strategies targeting the IGF1R by unbalancing the GRK/β-arrestin signaling in non-epithelial cancers.

Main findings of the thesis:

1. Oncogenic IGF1R signaling is essentially dependent on the C-tail of the receptor.
2. Various strategies targeting IGF1R-dependent signaling reverse malignant phenotype.

The key findings of each paper are summarized below:

Study 1:

The study describes the molecular mechanism and biological function of β-arrestin biased signaling downstream of IGF1R and explores its targeting as viable therapeutic strategy in anti–IGF1R cancer treatment.

Main finding: We identify the “system bias” as a novel strategy to target IGF1R though the GRK/β-arrestin system

Study 2:

Balancing IGF1R and p53 signals can be a compelling cancer strategy to inhibit metastasis and decrease the risk of relapse in CM.

Main finding: This study demonstrates β-arrestin targeting as a way to destabilize the p53/MDM2/IGF1R interaction.

Study 3:

Shifting the β-arrestin isoform balance towards a β-arrestin2 predominant system, can both mitigate IGF1R-induced cancer cell proliferation and survival as well as enhance the tumor suppressor activity of p53 thus improving therapeutic response.

Main finding: This study identifies modulation of β-arrestin isoforms as a strategy for targeting the p53/MDM2/IGF1R system.

Study 4:

In the fourth and final paper, we investigated the effect of biased IGF1R signaling by isolating kinase signaling and GRK/β-arrestin signaling. Our result demonstrates the potential of both branches of IGF1R signaling as targets for proliferation and differentiation and provides the basis for a new strategy for acute myeloid leukemia.

Main finding: IGF1R signaling maintains cell stemness and undifferentiated leukemic phenotype.

6 POINTS OF PERSPECTIVE

This thesis explores non-canonical IGF1R signaling, essentially dependent on C-terminus of the receptor. Based on these findings we have developed novel targeted anti-IGF1R strategies in cancer (Figure 5).

Promoting the β-arrestin 2 binding to the C-tail of the IGF1R through pharmacological inhibition of GRK2 prevents receptor recycling with depletion of the IGF1R from the cell surface. This is a novel paradigm for the RTKs family and serves as a starting point for the redesign of specific RTK-targeting therapy for cancer or other pathological conditions.

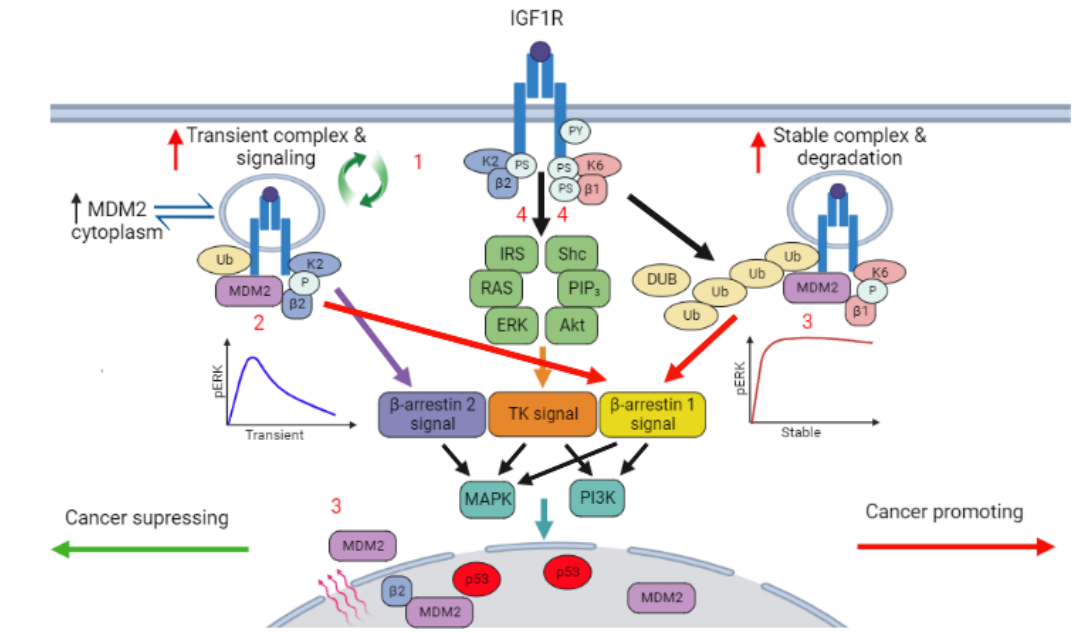
Pharmacological inhibition of p53/MDM2 system binding stimulates reactivation of p53 while the increased levels of free MDM2 binds to and degrades the IGFR at higher rates. This increased downregulation of IGF1R mitigates the activation of the cancer promoting β-arrestin 1 signaling.

Direct modulation of β-arrestin isoforms binding to the IGF1R C-tail with promotion of β-arrestin 2 signaling promotes p53 reactivation by β-arrestin 2-dependent translocation of MDM2 from the nucleus to the cytoplasm where it degrades IGF1R. At the same time β-arrestin 1 signaling is limited.

These novel insights into the destabilization of the p53/MDM2/IGF1R axis provide the basis a novel, dual-hit approach of simultaneous targeting two onco-crucial pillars; p53 and IGF1R pathways. From this perspective, targeting of IGF1R C-tail improves response to chemotherapy and p53 reactivating drugs.

Through further exploration of the C-terminal domain, the two signaling branches downstream IGF1R were demonstrated to control different aspects of the AML phenotype. Thus, targeting the C-tail through impairment GRK/β-arrestin signaling can induce differentiation of the leukemia clone. Inhibition of GRK/β-arrestin signaling has multiple potential clinical application in myeloid leukemias including enhanced response to current therapeutic strategies, inhibition of acute transformation from the chronic myeloid phenotype or progression from myelodysplastic syndrome to AML

In conclusion, our studies demonstrate that the IGF1R C-tail is critical to the cancer-promoting effect of IGF1R signaling. The use of several targeting strategies capable of mitigating the IGF1R signaling-dependent malignant phenotype described in this thesis offers a window into the possibilities of future applications of treatment strategies targeting IGF1R C-tail in most cancers.



Differentiation 4
Growth
Senescence 4
Apoptosis **2,3**

Novel therapeutic strategies:
A. Inhibition of GRK2 (paper 1)
B. Destabilization of the IGF1R/p53/MDM2 axis (papers 2 and 3)
C. Inhibition of IGF1R C-tail od kinase biased signaling (paper 4)

Proliferation
Oncogene transformation
Migration **2,3**
Invasion **2,3**
Metastais **2,3**

Figure 5. Therapeutic strategies targeting non-canonical IGF1R signaling. (A) Inhibition of GRK2 increased IGF1R degradation without activation of the cancer promoting β-arrestin 1 signaling (paper 1); (2) Destabilization of p53/MDM2/IGF1R axis using two approaches in which we favored MDM2/IGF1R interaction and reactivation of p53. Using Nutlin3 treatment, we suppressed p53/MDM2 interaction and induced p53 reactivation and concomitant IGF1R downregulation by MDM2, resulting in reduced tumor growth and metastatic potential (paper 2). Shifting the/β-arrestin signaling towards β-arrestin 2 transient signaling leads to translocation of MDM2 from the nucleus permitting p53 reactivation, to the cytoplasm where it binds to IGF1R and results in receptor downregulation (paper 3). (3) In paper 4, isolation of kinase signaling from GRK/β-arrestin signaling downstream the receptor impaired the AML malignant phenotype in two ways: kinase signaling inhibition decreased cell proliferation and survival while impaired GRK/β-arrestin signaling resulted in AML differentiation. In all four papers, impaired C-tail function to different degrees was demonstrated to be critical to the malignant phenotype.

7 ACKNOWLEDGEMENTS

This is where I thank everyone who supported me these last 4 years and helped me reach this point.

First of all, I would like to thank my main supervisor, Leonard Girnita. When I first came to your lab 4 years ago, you gave me a chance to work with you and restart my doctoral study. No part of this thesis could have been possible without your ideas and designs. You taught me how to plan, how to sort through my data and search for the diamond in the rough, and how to always find better and simpler ways to achieve my goals. You have taught me how to write, how present my data and how to "sell" the findings. Words fail to encompass how profoundly you have impacted the way I think about science. Your guidance has shaped me as a researcher. And I will never forget to plan to succeed lest I plan to fail.

Thank you to my co-supervisor George Calin for allowing me to collaborate with you on your exciting projects. Thank you for taking the time and having all those meetings and conversations, particularly the ones about leukemia and hematopoiesis. I deeply appreciate all your help and guidance with my projects. Your encouragement has been invaluable.

Takashi Shibano, my co-supervisor. I could not have asked for better technical guidance. I don't think I could have completed my projects without your input with troublesome optimization, sharing your protocols and late-night troubleshooting. Most importantly, you taught me how to use chopsticks, and that might be one the best skills I've acquired during my time here.

None of the papers included in this thesis could have been possible with my co-authors: Mihnea Dragomir, Julianna Serly, Daniela Nedelcu, Enrique Fuentes-Mattei, Andrei Tica, Ada Girnita, Naida Suleymanova, Eric Trocme, Iara Trocoli, Tingting Lin, Huyuan Zheng, Claire Worrall, Stefan Seregard, Benjamin Gebhard and Catalina Gual.

Thank you to my lab members, it has been a pleasure to work with you every day. David Song, you have been here throughout my PhD journey, and I am thankful for our time together from working on spheroids to shopping (my partner in crime) to cell culture room dancing. To Sylvya Pasca, the baby of our little family. Thank you for your help, for sharing your cells and for your help with the dreaded admin stuff. Not to mention all the laughs and the playlists. To Simin Zhang for your support, chats in the lab and going to the cinema with me. To Xiaohao Wang, for all fun times and optimizing protocols in the lab. This has been the best team to do my PhD with.

To Mireia Cruz De Los Santos, you were the first master student I supervised, and I am certain I learned just as much from you as you learned from me. Also, the bear turned out perfect, thank you for illustrating the cover.

A special thank you to Caitrín Crudden, my mentor for all the help and guidance.

To my collaborator, Terry J. Smith for inviting me to take part in your project and the inspiring conversations on your visit to Stockholm.

Thank you to my collaborators in the department, Andreas Lindquist, and Neo Shiyoung for not only allowing me to be a part of their project but also for being there for me whenever I had questions and for always having a smile or a joke ready.

Thank you to my friends in the department, who were always ready and willing to give a hand, lend a consumable, give advice whenever I was trying a new method or just a quick chat in between incubation and detection. You have all created such a pleasant, supportive, and creative work atmosphere, Le, Vicky, Pedro, Matko, Mireia, Andreas, Hao, Huaitao, Ninni, Jiwei, Konstantina, Ilias, Smaranda and Aarren (thank you proofing this thesis and making sure I stay on point). To my colleagues who were always ready with recommendation for a movie, a documentary or podcast, Muyi, Stefan, and Dominika. You guys have kept me sane.

To my friends back home, Diana, Adela, Irina, and Dana. You have believed in me, encouraged me and have been true cheerleaders for the better part of a decade. I am coming back home, and I could not be happier.

Last but not least, this thesis is dedicated to Danusia and Marius, my wonderful parents. You have been everything I could have asked for. You have always known when to encourage me, when to push me and when I needed a hug. I am the person I am today because of who you are and who you have raised me to be. Mom, thank you for fostering curiosity in me. For never allowing me to become bored and for making me ask questions and seek answers (sometimes by force). Thank you for teaching me to laugh, to be kind, to be organized and work hard. I love you. Dad, thank you for the unquestioning faith you have in me, for teaching me fairness, when to be silent and when to speak, for always supporting me and for passing on your awesome taste in music. I love you. You have been unwavering and unconditional in your love, you have always seen the best in me and have made me see the same, so this is just as much yours as it is mine.

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