Stony Brook University

OFFICIAL COPY

The official electronic file of this thesis or dissertation is maintained by the University Libraries on behalf of The Graduate School at Stony Brook University.

© All Rights Reserved by Author.

Examining the Functional Role of the C-terminal Domain of the Insulin Like Growth Factor 1 Receptor Kinase Using Naturally Occurring Mutations Present in Human Cancers

A Thesis Presented

by

Christa Nicole Pappalardo

to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

December 2016

Stony Brook University
The Graduate School

Christa Nicole Pappalardo

We, the thesis committee for the above candidate for the
Master of Science degree, hereby recommend
acceptance of this thesis.

W. Todd Miller, PhD- Thesis Advisor
Professor and Chairman, Department of Physiology and Biophysics

M. Raafat El-Maghrabi, PhD- Second Reader
Research Professor, Department of Physiology and Biophysics

This thesis is accepted by the Graduate School

Charles Taber
Dean of the Graduate School

Abstract of the Thesis

Examining the Functional Role of the C-terminal Domain of the Insulin Like Growth Factor 1 Receptor Kinase Using Naturally Occurring Mutations Present in Human Cancers

by

Christa Nicole Pappalardo

Master of Science

in

Biochemistry and Cell Biology

Stony Brook University

2016

Insulin Like Growth Factor 1 Receptor (IGF1R) is a receptor tyrosine kinase that functions to control cell proliferation and apoptosis. Activation occurs upon the binding of the IGF1 ligand to the extracellular domain facilitating trans-autophosphorylation and thus activation. The receptor activates downstream signaling through both the MAPK and PI3K/Akt pathways that contribute to cellular proliferation and preventing cell death, respectively. IGF1R is overexpressed and hyperactive in many human cancers. We postulated that IGF1R could also be activated by mutation. We further hypothesized that particular C-terminal mutations could affect the ability of this domain to autoregulate the receptor. Mutations were chosen from the Catalog of Somatic Mutations in Cancer (COSMIC) database based on their location in C-terminal domain of the receptor and prevalence in various cancer patients. We conducted mutagenesis on the wild type IGF1R receptor to create four mutant constructs that were studied in vitro using R- (IGF1R-/-) mouse fibroblast cells. Following IGF1 stimulation the cells were harvested, the receptor immunoprecipitated, and kinase activity was studied using SDS-PAGE and western blot analysis. We found that the cancer associated mutations chosen did not affect the receptor's tyrosine kinase activity.

Table of Contents

List of Figures……………………………………………………………………………v

List of Abbreviations……………………………………………………………….....vi

Acknowledgements…………………………………………………………………..vii

I. Introduction………………………………………………………………………1

I.i Receptor Tyrosine Kinases and IGF1R……………………………………..1

I.ii IGF1R in cancer……………………………………………………………...4

II. Materials and Methods…………………………………………………………..6

II.i COSMIC……………………………………………………………………...6

II.ii Mutagenesis…………………………………………………………………7

II.iii Transformation……………………………………………………………..8

II.iv DNA Preparation and Sequencing………………………………………...8

II.v Cell Culture and Transfection……………………………………………...9

II.vi Immunoprecipitation……………………………………………………...10

II.vii Western Blotting…………………………………………………………10

III. Results……………………………………………………………………………...11

IV. Discussion…………………………………………………………………………13

References………………………………………………………………………………15

List of Figures

Figure 1. Extracellular Domain Variation in Tyrosine Kinase Families……………………......1

Figure 2. Basic Structure of IGF1R……………………………………………………………2

Figure 3. A Model For How IGF1 Binding Facilitates Receptor Trans-autophosphorylation……………………………………………………………….................3

Figure 4. Pathways Activated by IGF1R………………………………………………………4

Figure 5. Hallmarks of Cancer…………………………………………………………………5

Figure 6. Western blot analysis of IGF1R autophosphorylation in R- cells after transient transfection with 10ug *WT IGF1R*, *IGF1R A1206T*, and *IGF1R S1282I* with(+) and without(-) addition of IGF1 ligand………………………………………………………….....12

Figure 7. Western blot analysis of IGF1R autophosphorylation in R- cells after transient transfection with 10ug *WT IGF1R*, *IGF1R S1882I, IGF1R E1289K,* and *IGF1R R1337C* with(+) and without(-) addition of IGF1 ligand………………………………………………13

Table 1. Anti IGF1R Therapeutic Strategies…………………………………………………..6

List of Abbreviations

IGF1R	Insulin Like Growth Factor Receptor
R-	IGF1R -/- Mouse Fibroblast Cells
COSMIC	Catalog of Somatic Mutations In Cancer
MAPK	Mitogen-Activated Protein Kinases
PI3K/Akt	Phosphoinositol 3 Kinase/ v-**Akt** Murine Thymoma Viral Oncogene pathway
PCR	Polymerase Chain Reaction
BME	Beta mercaptoethanol
DMEM	Dulbecco's Modification of Eagle's Medium
TBST	Tris-Buffered Saline and Tween 20
BSA	Bovine Serum Albumin
L1	Leucine rich domain 1
CR	Cysteine Rich domain
L2	Leucine rich domain 2
Fn1	Fibronectin type 1 domain
Fn2	Fibronectin type 2 domain
Fn3	Fibronectin type 3 domain
TM	Transmembrane domain
JM	Juxtamembrane domain
mAbs	Monoclonal Antibodies
TKIs	Tyrosine Kinase Inhibitors
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Acknowledgements

I would like to thank Dr. Todd Miller for giving me the opportunity to be a member of his laboratory. He welcomed me from the start and guided me throughout my experiments and classwork. His patience, kindness, and biochemical expertise have created a wonderful laboratory that fosters collaboration, curiosity, and success. I am very thankful for the opportunity to participate in graduate level research as a member of the Miller Laboratory. I am also thankful for his support of and contributions to my career goal of becoming a veterinarian.

Special thanks to Dr. Raafat El-Maghrabi for being a second reader of my thesis and for offering invaluable support and advice during this process.

Further thanks go to the members of the Miller Laboratory including Barbara Craddock who continuously taught me new protocols and offered help whenever I found myself in need, Richard Delle Bovi and Harini Krishnan for IGF1R specific experimental advice, and Stephen Collins and Yagmur Kan who offered additional support and advice along the way.

I would also like to thank Dr. Barnabas Gikonyo for giving me the opportunity to develop a foundation at Geneseo upon which I have begun to build my career. His unwavering support of and sacrifice to our research group will forever be admired. He continues to be endlessly supportive of my decisions and his mentorship continues to guide me to success.

Finally, I would like to thank my family who embraces my independence, ambitions, and wholeheartedly supports me throughout my education.

I. Introduction

I.i Receptor Tyrosine Kinases and IGF1R

In humans, there are 518 protein kinases, about 1.7% of all genes[1]. Approximately 90 of them are tyrosine kinases; the majority are receptor tyrosine kinases[2]. These kinases function as mediators of signaling in pathways including cell apoptosis, proliferation, and transcriptional activation/regulation, among many others[3]. Pathway mediation is achieved by protein phosphorylation where the activated kinase transfers a phosphate group from ATP to its substrate, a particular target protein. This modification yields changes in the activity of the target protein ultimately affecting one or more of the aforementioned pathways.

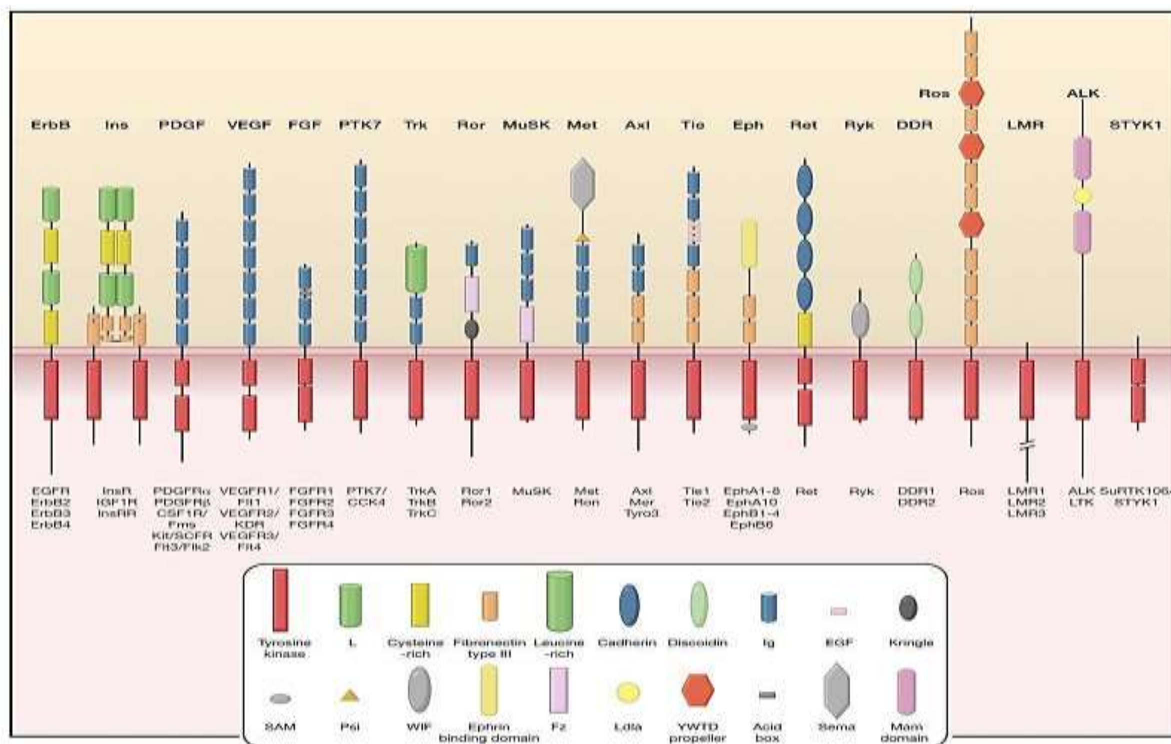

Figure 1. Extracellular Domain Variation in Tyrosine Kinase Families. 20 subfamilies of receptor tyrosine kinases share similar overall structure but show differences in domain composition[3].

Basic structural characteristics of receptor tyrosine kinases (RTKs) consist of a variable extracellular domain responsible for binding ligand, a single-pass transmembrane helix that unites the extra and intracellular portions of the receptor, a juxtamembrane domain that can

serve to regulate activity, a catalytically active kinase domain that phosphorylates proteins, and finally a C-terminal domain that can also participate in regulation. Though the general architecture of RTKs is conserved, each receptor is functionally different depending on cellular localization, ligand specificity, and domain structure which ultimately determine the downstream signaling pathways that are regulated. Differences in domain structure are particularly evident, as depicted in Figure 1 which shows the extracellular domain variation across different families of RTKs[3]. Intracellular domains similarly vary allowing receptors to interact with different downstream proteins thus affecting different pathways.

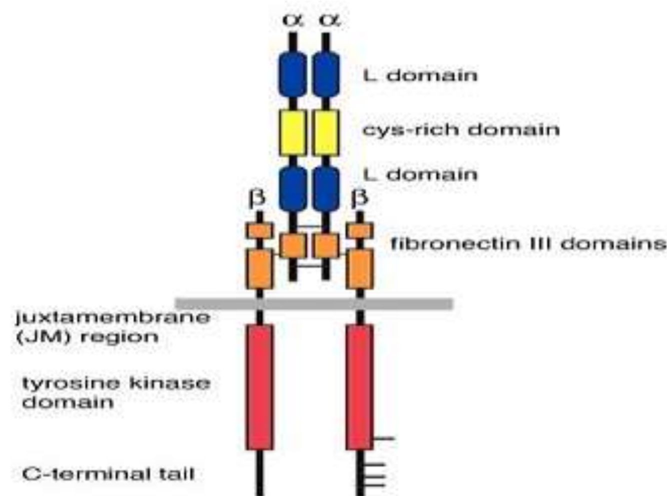

Figure 2. Basic Structure of IGF1R. IGF1R exists as transmembrane homodimer consisting of two αβ chains.

IGF1R, as shown in Figure 2 is composed of an extracellular alpha chain that includes two L domains (L1 and L2) linked by a cysteine rich (CR) domain, followed by two fibronectin domains that form disulfide bonds with the fibronectin domain (Fn3) of the beta chain. IGF1R exists as a homodimer of two monomers linked together via disulfide bonds between each alpha beta monomer’s fibronectin domains. The beta chain includes the transmembrane domain connected to the intracellular part of the receptor where the juxtamembrane, kinase, and C-terminal domains reside. The juxtamembrane domain has been shown to function as a

regulator of receptor activity[4]. The kinase domain is catalytically active and is responsible for phosphorylating tyrosine residues on the receptor's target proteins. Finally, the C-terminal domain has been shown to function as a means to propagate signaling as well as a requirement for receptor degradation[5-6]. Our hypothesis that the C-terminal of IGF1R functions in regulation is based on the presence of other RTKs, like Platelet Derived Growth Factor Receptor, that are regulated by the C-terminus[7].

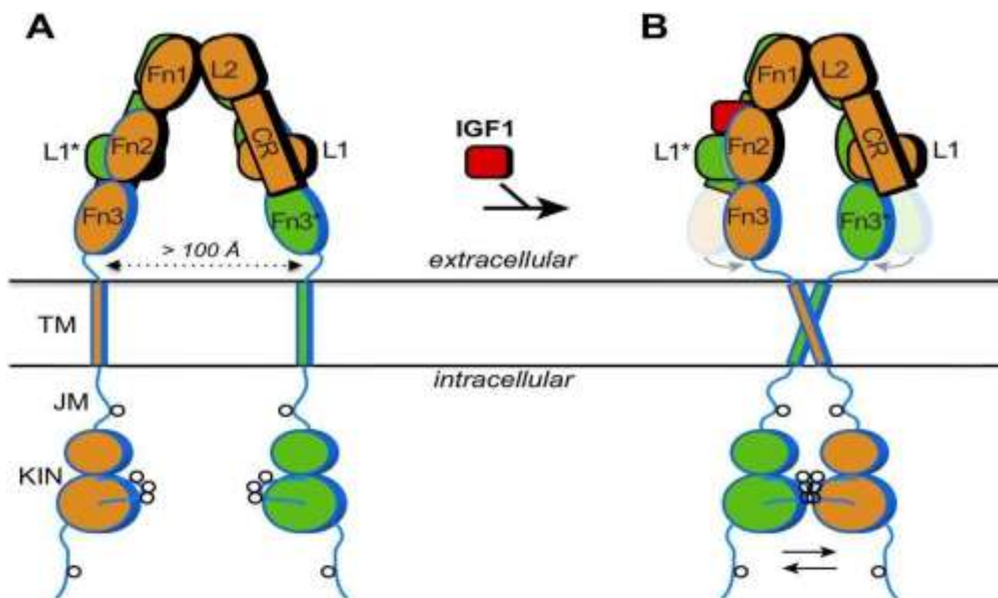

Figure 3. A Model For How IGF1 Binding Facilitates Receptor Trans-autophosphorylation. Upon IGF1 binding the two receptor monomers are brought closer together in the dimerized conformation that facilitates autophosphorylation and receptor activation[8].

As shown in figure 3 in its inactive state, IGF1R exists as a homodimer where the L1 domain of one monomer interacts with the FN2 and FN3 domains of the other monomer, linking the two together[8]. In this state the transmembrane domains exist at a considerable distance from each other. When IGF binds to the L1 domain of one of the monomers within the dimer, the interaction between that L1 domain and the other monomer's Fn2 domain is destabilized and results in the Fn2 and Fn3 domains of each monomer to come closer together. This closing-in movement allows the TM and kinase domains of each to interact with each other to facilitate

trans-autophosphorylation where the kinase domain of one monomer phosphorylates three tyrosine residues in the other monomer's kinase domain, and vice versa[8-9]. It should be noted, however, that an alternative model for the transmembrane regions has been proposed in which the two helices separate upon activation[10]. Despite having slightly different TM conformational changes, both models of the trans-autophosphorylation event yield an activated receptor involved in cell proliferation and survival via the MAPK and PI3K/Akt pathways, shown in Figure 4[11].

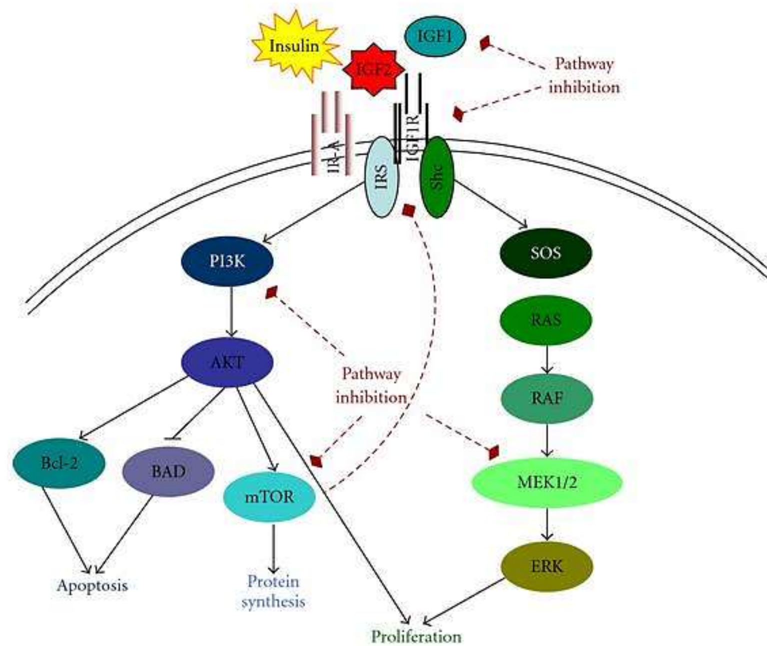

Figure 4. Pathways Activated by IGF1R. IGF1 binds IGF1R and facilitates the recruitment of adaptor proteins that mediate downstream signaling through PI3K and RAF/MEK1/2/ERK (MAPK) cascades[11].

I.ii IGF1R in Cancer

Cancer is a disease defined by the transformation of healthy cells into abnormal malignant cells. Extensive studies have yielded what we believe to be the many causes of, or contributors to cancer, collectively considered the hallmarks of cancer. Figure 5 shows the most recent interpretation of these factors as well as therapeutic strategies[12]. Though not explicitly included in the diagram, IGF1R plays a definite role in at least three of the hallmarks: cell death

resistance, proliferative signaling sustenance, and angiogenesis[13-15]. IGF1R related cell death resistance directly relates to the cellular pathways mentioned above where both result in proliferative signals and the inhibition of apoptosis. Proliferative signaling sustenance can be the consequence of mutant receptors that yield constitutively activated kinases, thereby maintaining constant growth signals throughout the cell. Angiogenesis is affected through the induction of other factors like vascular endothelial growth factor, VEGF, that cause the growth of new vasculature destined to feed tumor cells.

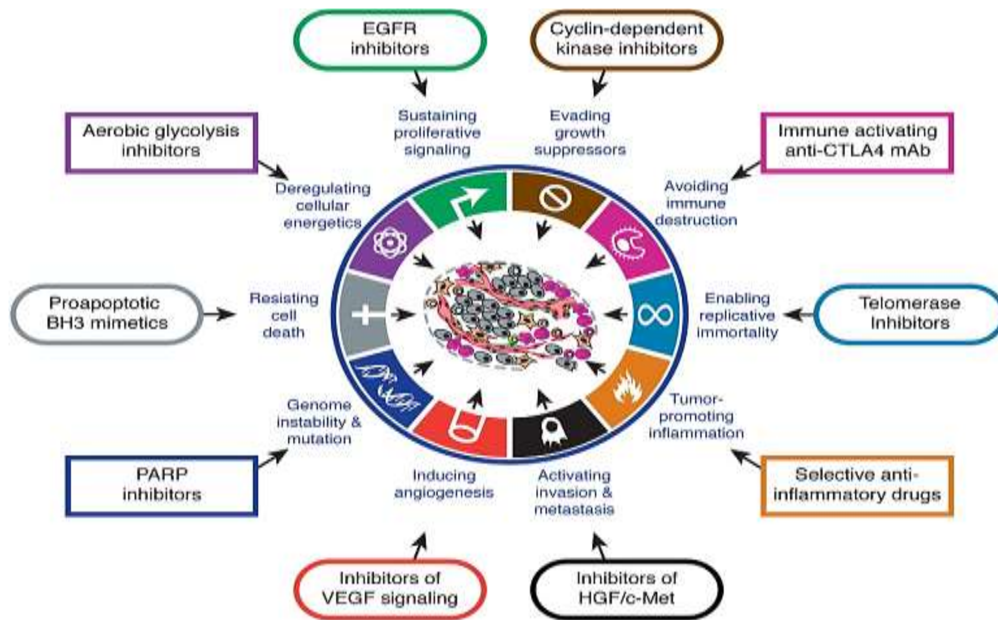

Figure 5. Hallmarks of Cancer. Abnormal cellular characteristics that contribute to cancer are shown, as are potential therapeutic strategies [12]

The basis of research on cancer related kinases like IGF1R is simple: the better we understand the kinase's activation, regulation, downstream signaling and how these are altered as a result of mutations, the more prepared we are to develop therapies that target dysfunctional receptors present in various cancer types. Current therapies being tested are monoclonal antibodies (mAbs) against IGF-1R, mAbs against IGF-1R ligand (IGF-1 and IGF-2), and IGF-1R tyrosine kinase inhibitors (TKIs), as described in Table 1[16]. These therapies target either

IGF1R or the IGF ligand and could potentially mitigate the aforementioned issues associated with hyperactive receptors. Although theoretically promising, clinical trials with various types of anti IGF1R drugs have not been successful. Data suggests that these clinical approaches can be feasible and effective but may only be beneficial to a limited subset of patients. Further studies are focusing on combinatorial strategies where anti IGF1R related therapies are being combined with targeting other pathways related to cancer like mTOR. These also could be promising but still warrant further studies on both IGF1R and its downstream targets[16].

Features of interest	mAb against IGF-1R	mAb against IGF-1 and -2	Small molecule TKI
Mechanism of action	• Block IGF-1R from ligand binding • Receptor degradation of IGF-1R homodimer and IGF-1R/IR hybrids • Possible ADCC (if IgG1)	• Neutralizing ligand from binding to IGF-1Rand IR-A	• Kinase inhibition intracellular ▲ (also inhibit ligand-independent activation, if relevant)
Signaling affected	• Specific • Inhibit signaling of: ▲IGF-1R ▲IGF-1R/IR-A hybrid • No effect on IR-A or IR-B	• Specific • Inhibit IGF-1 or IGF-2 signaling through: ▲ IGF-1R ▲ IGF-1R/IR-A ▲IR-A • No effect on insulin signaling	• Less specific • Inhibit signaling of RTKs (by any ligand): ▲IGF-1R ▲IGF-1R/IR ▲IR (to a lesser degree than for IGF-1R) • May inhibit targets beyond IGF-1R and IR (XL228; INSM-18)
Pharmacokinetics	• Long $t_{1/2}$ (days to weeks) • PK interaction less likely in combination regimens • Poor CNS uptake	• Long $t_{1/2}$ (days to weeks) • PK interaction less likely in combination regimens • Poor CNS uptake	• Short $t_{1/2}$ (hours)

Table 1. Anti IGF1R Signaling Therapeutic Strategies. Prospective therapeutic strategies include targeting either the IGF ligand or the receptor itself via antibodies or small molecule inhibitors[16].

II. Materials and Methods

II.i COSMIC

Cancer associated mutations were chosen by referencing the IGF1R gene variants documented in the COSMIC database. We chose mutants based strictly on their location in the C-terminal tail of the receptor which begins at the end of the kinase domain at amino acid 1266.

To further narrow our search we chose mutants with the highest prevalence in various tissue samples. These mutants included S1282I present in lymphoid neoplasm, E1289K present in endometrial and stomach cancers, and R1337C present in stomach and skin cancers. In addition to these C-terminal mutations we also included a mutation, A1206T near the end of the kinase domain present in lymphoid neoplasm and large intestinal cancers. We chose to include this non C-terminal mutation because of its prevalence as a mutated residue listed in the database.

II.ii Mutagenesis

Forward and reverse DNA oligos were designed to encode each mutation and its flanking sequences in the wild type receptor. We designed oligos greater than 30 nucleotides in length with maximal GC content where we ensured the ends of the oligos consisted of either G or C bases for efficiency. The forward primers utilized were as follows with the point mutation indicated in bold:

A1206T: 5'- GTC CTC TGG GAG ATC **ACC** ACA CTG GCC GAG CAG -3'

S1282I: 5'- GTC TCC TTC TAC TAC **ATC** GAG AAC AAG CTG -3'

E1289K: 5'- GAG AAC AAG CTG CCC **AAG** CCG GAG GAG CTG GAC -3'

R1337C: 5'- GGG GTG CTG GTC CTC **TGC** GCC AGC TTC GAC GAG -3'

Reverse primers were generated by using the reverse complement of the forward primer sequences. A previously cloned pBPV IGF1R wild type plasmid was utilized as template in the reaction. Site directed mutagenesis was carried out using a QuikChange II site directed mutagenesis kit (Agilent Technologies) as follows. For each reaction we combined 31µL deionized water, 5µL10X reaction buffer, 3µL pBPV IGF1R WT at 0.1µg/ µL , 5µL forward primer and 5µL reverse primer, both at concentrations of 25ng/ µL. This mixture was boiled for 4 minutes at 94∘C then iced for 2 minutes. We then added 1µL of the PFU polymerase and 1µL of the dNTP mixture.

The reaction was subjected to a thermocycler program as follows:

30 seconds at 95 °C followed by 18 rounds of 30 seconds at 95°C, 1 minute at 55°C, 32 minutes at 68°C. After the 18 cycles a 10 minute period at 68°C was programmed, followed by an infinite

4°C period post reaction completion. After PCR the reactions were digested for 1 hour with 1μL Dpn1 (QuikChange kit) at 37°C, and a second digestion with 1μL of Dpn1 (New England BioLabs) at 37°C for an additional hour to ensure complete digestion.

II.iii Transformation

The digested PCR reaction products were transformed into XL-10 Gold Ultracompetent E. coli cells (Agilent Technologies) using a protocol adapted from the manufacturer's transformation directions. 50μL of XL-10 Gold cells per reaction were prepared by thawing, the addition of 2μL BME, and an icing period of 10 minutes. 1.5μL of the Dpn1 treated PCR reaction was added, followed by icing the mixture for 30 minutes. Reactions were then heat shocked at 42°C for 30 seconds and iced for 2 minutes. 900μL of prewarmed 37°C LB broth was added and the cells were grown for 1.5 hours at 37°C on a shaker at 250rpm. Following incubation, the cells were spun down at 1200rpm for 10 minutes. 750μL of supernatant was removed leaving approximately 200μL which were used to resuspend the cells that were plated on LB ampicillin agar plates and incubated at 37°C overnight.

II.iv Sequencing and DNA preparation

Single colonies obtained from the transformations were picked and restreaked onto LB ampicillin plates and incubated overnight at 37°C. The following day these colonies were inoculated into 2mL LB ampicillin liquid cultures and grown overnight under identical conditions. The liquid cultures were harvested the next day and subjected to an alkaline lysis miniprep in order to prepare DNA for sequencing reactions. DNA concentration was determined using a Thermo Scientific Genesys 10S UV-Vis spectrophotometer. Sequencing reactions were prepared and submitted according to Genewiz protocol.

After confirming the success of the constructs via sequencing we inoculated 2mL LB ampicillin cultures that were grown for approximately 8 hours, then used to inoculate 250mL LB ampicillin cultures in erlenmeyer flasks which were allowed to grow overnight. We ultimately performed maxipreps of each DNA sample as per Qiagen maxiprep protocol.

II.v Cell Culture and Transfection

R- cells were cultured using DMEM (Dulbecco's Modified Eagle's Medium) (Sigma Aldrich) containing 4.5mg/mL high glucose. We supplemented the DMEM with 50mL FBS (VWR) and 5mL of 100X Antibiotic antimycotic (Sigma Aldrich). 0.25% Trypsin (Sigma Aldrich) was used to dissociate the cells before splitting. The cells were plated at a 1:10 dilution and were split 2 times per week to ensure appropriate growth. Plates were incubated using a Thermo Scientific Napco Series 8000 WJ CO2 incubator maintained at 37°C and 5%CO2/95% O2.

Transient transfection protocol was as follows:

On day 1 of transfection, cells were split as usual and the remaining cell suspension was counted to determine cell quantity. 1 million cells were plated with 10mL DMEM per 10cm dish. 2 10cm dishes were prepared for each condition R-, WT, A1206T, S1282I, E1289K, and R1337C, where one dish would ultimately be stimulated with IGF1 ligand, the other unstimulated. Day 2 of transfection consisted of preparing the master mix (for both plates per condition) transfection mixtures using X-tremeGene (Sigma Aldrich) at 1µL per 1µg DNA. We transfected each dish using a mixture of 10µg DNA, the corresponding volume of X-tremeGene, and 500µL unsupplemented (no protein or antibiotic antimycotic) DMEM. The transfection mixtures were added dropwise to both dishes of each condition and returned to the incubator overnight. On day 3 of transfection we removed the media and replaced it with 5 mL 1.0mg/mL low glucose DMEM, our starvation media. The plates were returned to the incubator overnight. On day 4 of transfection we stimulated 1 plate per condition with 40ng/mL IGF1 ligand and incubated at room temperature for 10 minutes. Following stimulation we removed the media and replaced it with 1mL 1X PBS. Cell lysates were prepared with R- lysis buffer consisting of 25mM Tris pH 8.0, 2mM EDTA pH 8.0, 140mM NaCl and 1% NP40, supplemented with 10 mg/mL aprotinin, 10mg/mL leupeptin, and 0.2M Sodium orthovanadate. Cell pellets were suspended in the complete R- lysis buffer, iced for 1 hour, and spun at 4°C at max 15000rpm 10 minutes using a TOMY centrifuge. The clarified supernatants were collected and protein concentration was determined using a VERSA Max Tunable microplate reader prior to immunoprecipitation.

II.vi Immunoprecipitation

Immunoprecipitation was performed using 1mg of protein per reaction. Protein A beads in a 50% slurry were prepared by washing in R- lysis buffer 4 times, each time removing the supernatant and replacing it with fresh buffer. Lysis buffer was added to 1mg of lysate for a total volume of 1mL. The antibody used for the pull down was JBW902 (Millipore), an IGF1R beta subunit specific antibody. 2μL antibody was added to the lysates and mixed appropriately. 40μL of the 50% protein A bead slurry was added to each reaction and the reaction was rocked at 4°C overnight. The following day the reactions were removed and spun at 10000rpm for 5 minutes to pellet the beads. The supernatant was removed and replaced with lysis buffer, and this process was repeated for a total of 4 times to ensure proper washing of the beads and removal of any background. The final wash was completed and all supernatant was removed so only beads remained. 25μL of 5X laemmli buffer was added to the samples which were then subjected to boiling at 94°C for SDS PAGE analysis.

II.vii Western Blot Analysis

10% SDS polyacrylamide gels were utilized using the mini Hoeffer system. A Precision Plus protein marker (Bio-Rad) was used for reference. The gel was run for approximately 1.5 hours at 100V in 1X running buffer consisting of 125mM tris hydroxymethlyamine methane, 960 mM glycine and 17.3mM SDS. Once completed, the apparatus was taken apart and the gels were transferred onto a PVDF membrane using a transfer cassette submerged in 1X transfer buffer consisting of 25 mM tris hydroxymethylamine methane and 192mM glycine run at a constant 350mA for 1 hour. After transfer the membranes were removed from the cassette and placed in 1X TBST to wash while the Bovine Serum Albumin (VWR) and antibody dilutions were prepared. 5% BSA was prepared by adding 2.5g powdered BSA to 50mL 1X TBST. The mixture was rocked on a Lab-Line 3D Rotator to ensure complete dissolution. The membranes were blocked with 25mL 5%BSA and rocked for 1 hour. After blocking, the membranes were probed with 10mL of the antibody pYpY 1162/1163 1:5000 (Transduction Labs) at 4°C on a rotator overnight to test for activation indicated by tyrosine phosphorylation. The following day the

membranes were removed and washed in 1X TBST for 15 minutes. The secondary antibody used following anti-pYpY was Anti-Rabbit (GE Healthcare) at 1:10,000 in 1% TBST for 30 minutes rotating at room temperature. Following probing the membranes were washed well with 1% TBST for 30 minutes, the TBST replaced every 10 minutes. The washed membranes were incubated as per manufacturer's directions in a working solution of SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific), an enhanced chemiluminescent (ECL) substrate, for 1 minute. The membranes were placed in a cassette between two polyethylene sheets to eliminate bubbles and taken to the dark room where CL-XPosure Film (Thermo Scientific) was placed atop the covered membranes for various lengths of time and was later exposed using a developer.

Following the initial probing with pYpY 1162/1163 antibodies bound to the membrane were removed by incubating the membranes with 10mL stripping buffer consisting of Tris pH 6.7, 2% SDS, and 100mM BME for 30 minutes at 55°C. The membranes were washed thoroughly and blocked with 5% BSA as previously described. We then re-probed the membranes to detect receptor expression using the antibody IGF-IRβC20 (SantaCruz) which interacts with the C-terminal of the receptor. The membrane was incubated overnight at 4°C on a rotator in 1:2500 dilution of IGF-IRβC20 in 1%TBST. The following day the membrane was removed and washed thoroughly. Identical protocols previously described were used for secondary antibody probing, membrane washing, ECL substrate incubation, and film exposure.

III. Results

C-terminal mutations A1206T, S1282I, E1289K, and R1337C were selected from the COSMIC database and primers were designed accordingly. PCR reactions were prepared for each mutant with a previously cloned pBPV IGF1R WT plasmid in addition to buffers, dNTPs, and polymerase. Dpn1-digested PCR products were transformed into E. coli cells. DNA products were submitted for sequencing to confirm the mutations. To observe effects of each mutated construct, transfection experiments were conducted using 10μg DNA prepared with each point mutation. R- cells were transiently transfected with each respective sample and

stimulated with IGF1. Receptor was isolated via immunoprecipitation and the resulting samples were subjected to SDS-PAGE. Gels were transferred to a PVDF membrane to asses activity using western blotting with an antibody that recognizes the pYpY 1162/1163 phosphorylated (activated) form of the receptor. Western blot analysis showed that the A1206T, S1282I, E1289K, and R1337C mutant forms of IGF1R were still activated in response to IGF1 and inactive in the absence of IGF1. Figure 6 shows activation of A1206T and S1282I. Figure 7 shows activation of E1289K and R1337C (Note that in Figure 7, the S1282I construct was poorly expressed). If any of the mutations had caused constitutive activation of IGF1R, we expected to see a positive signal in the Western blot for unstimulated cells.

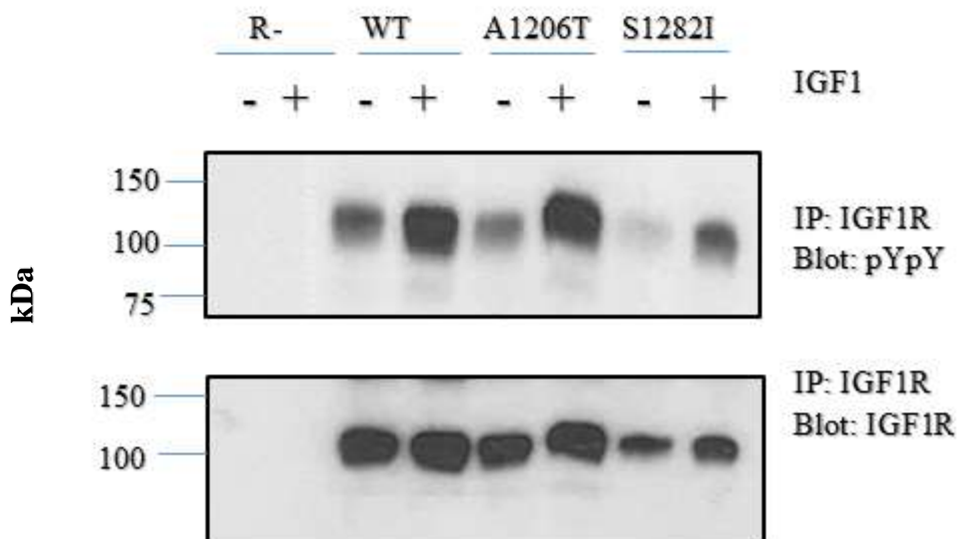

Figure 6. Western blot analysis of IGF1R autophosphorylation in R- cells after transient transfection with 10ug *WT IGF1R*, *IGF1R A1206T*, and *IGF1R S1282I* with(+) and without(-) addition of IGF1 ligand.

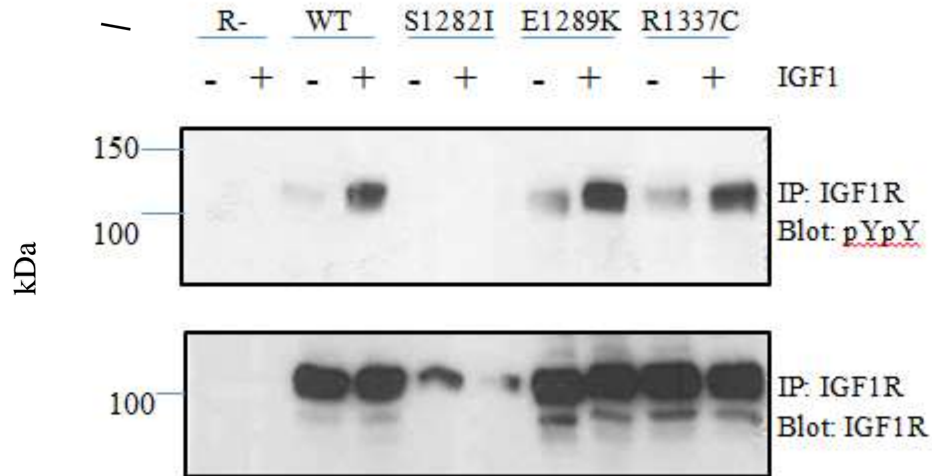

Figure 7. Western blot analysis of IGF1R autophosphorylation in R- cells after transient transfection with 10ug *WT IGF1R*, *IGF1R S1882I, IGF1R E1289K,* and *IGF1R R1337C* with(+) and without(-) addition of IGF1 ligand.

IV. Discussion

Many kinases have been shown to have their activity regulated by their C-terminal domain. This regulation is often seen in studies that address the activity of kinases when the C-terminal tail is mutated. We know that IGF1R activation is responsible for promoting cellular proliferation and inhibiting apoptosis through the MAPK and PI3K pathways[17]. The COSMIC database shows that C-terminal IGF1R mutations are present in a variety of cancers. These facts suggest that the mutant receptors present in cancer patients could contribute to a cancer cell's ability to thrive through the sustenance of proliferative signaling in addition to the avoidance of apoptosis. The high occurrence of C-terminal mutations documented in IGF1R and the knowledge of C-terminal regulation found in other kinases led us to the hypothesis that IGF1R could be regulated by its C-terminal domain.

Though we anticipated results suggesting that the mutations did alter kinase activity, our results prove otherwise. All four mutants, A1206T, S1282I, E1289K and R1337C showed autophosphorylation patterns similar to that of the wild type receptor. Figure 6 and Figure 7 show that each mutant receptor, in the presence of IGF1, has autophosphorylation consistent with a receptor present in healthy cells. These results are very much like a similar experiment

that recreated C-terminal mutations to elucidate the function of the C-terminal tail of IGF1R.[5] Autophosphorylation was found to be similar to wild type in these mutants as well. Here, instead of serving as a regulatory domain, the C-terminal tail has been found significant for downstream signaling. Other studies show that the C-terminal domain is necessary for receptor degradation[6]. No other studies have shown that the C-terminal tail participates in regulation.

These studies solely observed autophosphorylation in cells transfected with mutant forms of IGF1R. Additional experiments could be performed to confirm these results. A beneficial and supportive experiment would be a kinase assay to observe the transfer of radiolabeled phosphate from radioactive ATP to a substrate of the receptor.

Additional mutational studies are necessary in order to make a definitive conclusion regarding whether or not the C-terminal domain plays a regulatory role. COSMIC shows a considerable number of mutations that have yet to be evaluated in regards to autophosphorylation studies *in vitro.* Though unlikely due to an array of evidence suggesting otherwise, it is possible that a mutant within this unstudied group yields constitutive activation of the receptor suggesting a C-terminal regulatory role. Despite the lack of evidence to support C-terminal tail regulation, other studies have shown that these C-terminal mutations affect downstream signaling pathways that contribute to cancer growth and survival[5]. It would be beneficial to study these mutations further, perhaps by repeating western blot analyses where the activation of downstream targets is probed, in order to develop therapeutic strategies to target these receptors for those who suffer from hyperactive pathways downstream of IGF1R.

The results of this research along with those of other studies suggest that we should consider the C-terminal to have various roles other than a regulatory function. The more information gained regarding the C-terminal and its function, the more information regarding receptor biochemistry and possible therapeutic targets we will have.

References

1. Manning, G. (2002). The Protein Kinase Complement of the Human Genome. *Science*, 298(5600), 1912-1934.

2. Robinson, D. R., Wu, Y., & Lin, S. (2000). The protein tyrosine kinase family of the human genome. *Oncogene*, 19(49), 5548-5557.

3. Lemmon, M. A., & Schlessinger, J. (2010). Cell Signaling by Receptor Tyrosine Kinases. *Cell*, 141(7), 1117-1134.

4. Craddock, B. P., Cotter, C., & Miller, W. T. (2007). Autoinhibition of the insulin-like growth factor I receptor by the juxtamembrane region. *FEBS Letters, 581*(17), 3235-3240.

5. Craddock, B. P., & Miller, W. T. (2012). Effects of Somatic Mutations in the C-Terminus of Insulin-Like Growth Factor 1 Receptor on Activity and Signaling. *Journal of Signal Transduction, 2012*, 1-7.

6. Sehat, B., Andersson, S., Vasilcanu, R., Girnita, L., & Larsson, O. (2007). Role of Ubiquitination in IGF-1 Receptor Signaling and Degradation. *PLoS ONE, 2*(4).

7. Chiara, F., Bishayee, S., Heldin, C., & Demoulin, J. (2004). Autoinhibition of the Platelet-derived Growth Factor β-Receptor Tyrosine Kinase by Its C-terminal Tail. *Journal of Biological Chemistry, 279*(19), 19732-19738.

8. Hubbard, S. R., & Miller, W. T. (2014). Closing in on a mechanism for activation. *ELife, 3*.

9. Kavran, J. M., Mccabe, J. M., Byrne, P. O., Connacher, M. K., Wang, Z., Ramek, A., . . . Leahy, D. J. (2014). How IGF-1 activates its receptor. *ELife, 3*.

10. Lee, J., Miyazaki, M., Romeo, G. R., & Shoelson, S. E. (2014). Insulin Receptor Activation with Transmembrane Domain Ligands. *Journal of Biological Chemistry, 289*(28), 19769-19777.

11. Martins, A. S., Olmos, D., Missiaglia, E., & Shipley, J. (2011). Targeting the Insulin-Like Growth Factor Pathway in Rhabdomyosarcomas: Rationale and Future Perspectives. *Sarcoma, 2011*, 1-11.

12. Hanahan, D., & Weinberg, R. (2011). Hallmarks of Cancer: The Next Generation. *Cell, 144*(5), 646-674.

13. Grimberg, A. (2003). Mechanisms by which IGF-I May Promote Cancer. *Cancer Biology & Therapy, 2*(6), 628-633.

14. Rosenzweig, S. A., & Atreya, H. S. (2010). Defining the pathway to insulin-like growth factor system targeting in cancer. *Biochemical Pharmacology, 80*(8), 1115-1124.

15

15. Kucab, J. E., & Dunn, S. E. (2003). Role of IGF-1R in Mediating Breast Cancer Invasion and Metastasis. *Breast Disease, 17*(1), 41-47.

16. Chen, H. X., & Sharon, E. (2013). IGF-1R as an anti-cancer target--trials and tribulations. *Chinese Journal of Cancer, 32*(5), 242-252.

17. Tao, Y., Pinzi, V., Bourhis, J., & Deutsch, E. (2007). Mechanisms of Disease: Signaling of the insulin-like growth factor 1 receptor pathway—therapeutic perspectives in cancer. *Nature Clinical Practice Oncology, 4*(10), 591-602.