

8-2015

Regulation of receptor tyrosine kinases by Ubiquilin 1.

Zimple Kurlawala
University of Louisville

Follow this and additional works at: <https://ir.library.louisville.edu/etd>

Part of the [Pharmacy and Pharmaceutical Sciences Commons](#)

Recommended Citation

Kurlawala, Zimple, "Regulation of receptor tyrosine kinases by Ubiquilin1." (2015). *Electronic Theses and Dissertations*. Paper 2214.
<https://doi.org/10.18297/etd/2214>

This Master's Thesis is brought to you for free and open access by ThinkIR: The University of Louisville's Institutional Repository. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of ThinkIR: The University of Louisville's Institutional Repository. This title appears here courtesy of the author, who has retained all other copyrights. For more information, please contact thinkir@louisville.edu.

REGULATION OF RECEPTOR TYROSINE KINASES BY UBIQUILIN1

By

Zimple Kurlawala

A Thesis Submitted

to the Faculty of the School of Medicine of the University of Louisville
in Partial Fulfillment of the Requirements for the Degree of

Master of Science

in Pharmacology and Toxicology

Department of Pharmacology and Toxicology

University of Louisville

Louisville, KY

August 2015

Copyright 2015

By

Zimple Kurlawala

All rights reserved.

REGULATION OF RECEPTOR TYROSINE KINASES BY UBIQUILIN1

By

Zimple Kurlawala
M.P.H Western Kentucky University, 2011
M.D. University of Seychelles-American Institute of Medicine, 2008

Thesis Approved on

July 25th, 2015

by following Thesis Committee

Levi Beverly, Ph.D.

Leah Siskind, PhD

Brian Ceresa, Ph.D.

Geoffrey Clark, PhD

Jesse Roman, M.D. PhD

ACKNOWLEDGEMENTS

I would like to thank Dr. Levi Beverly for his unmatched mentorship and patience and for introducing me to the world of scientific research and making me fall in love with it. I would also like to thank Dr. Brian Ceresa for sharing his professional expertise and always being available to discuss about the fascinating world of receptor tyrosine kinases. I would like to express my gratitude towards Dr. Leah Siskind for her endless support and guidance and finally Dr. Geoffery Clark for all of his valuable inputs to my project.

ABSTRACT

REGULATION OF RECEPTOR TYROSINE KINASES BY UBIQUILIN1

Zimple Kurlawala

June 25, 2015

Receptor Tyrosine Kinases (RTK's) like EGFR, Insulin Receptor, IGF1R and others mediate growth, proliferation and balance between apoptosis and survival. Dysregulation of RTK activity has been identified in a wide range of cancers and contributes to initiation, progression and metastasis of tumors. Ubiquilins (UBQLN) are fairly novel proteins and are studied mostly in the field of neurodegenerative disorders. Our laboratory recently discovered a link between UBQLN1 function and tumorigenesis. UBQLN1 is lost in about 50% of non-small cell lung cancer (NSCLC) cases. We have identified an interaction between UBQLN1 and IGF1R. Furthermore, UBQLN1 regulates activity of IGF1R and EGFR. Following loss of UBQLN1 in lung adenocarcinoma cells, there is decreased expression of IGF1R and EGFR at the mRNA and protein levels. Fewer receptors are available for ligand binding on plasma membrane of UBQLN1 deficient cells. Despite this decrease in total number of receptors, the ratio of active:inactive (phosphorylated:non-phosphorylated) receptor is higher in cells that have loss of UBQLN1 function. We conclude that UBQLN1 is essential in normal regulation of RTKs. Loss of UBQLN1 leads to persistent stimulation of IGF1R and EGFR which may contribute to transforming events in UBQLN1

deficient cells. Understanding the role of UBQLN1 in regulating receptor tyrosine kinase activity can facilitate development of pharmacological drugs targeting novel pathways in diseases that have altered function of UBQLN1.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
ABSTRACT.....	iv
LIST OF FIGURES.....	vii
Chapter 1: INTRODUCTION.....	1
Chapter 2: METHODS AND MATERIALS.....	14
Chapter 3: RESULTS.....	20
Chapter 4: DISCUSSION AND CONCLUSIONS.....	46
REFERENCES.....	51
CURRICULUM VITA.....	55

LIST OF FIGURES

1. Schematic of structural domains of Ubiquilin1.....	1
2. Schematic of structure of IGF1R.....	6
3. Structure-Function relationship of IGF1R.....	9
4. Signaling pathways of IGF1R.....	10
5. Receptor Trafficking.....	11
6. Schematic of clathrin-mediated endocytosis.....	12
7. Ubiquilin1 is lost and under-expressed in human cancers.....	21
8. Ubiquilin1 interacting partners	
8.1. Immunoprecipitation/Mass Spectrometry.....	23
8.2. Immunoprecipitation/Western Blot (IGF1R).....	24
8.3. Immunoprecipitation/Western Blot (IGF1R and INSR).....	25
9. Cancer mutants of Ubiquilin1 lose interaction with IGF1R.....	27
10. Loss of Ubiquilin1 decreases expression of IGF1R	
10.1. A549.....	29
10.2. H358, HPL1D.....	30
11. Saturation binding assay using I ¹²⁵ IGF1	
11.1. Binding activities.....	32
11.2. Scatchard plots.....	33

12. Immunofluorescence	
12.1. Ubiquilin1.....	35
12.2. IGF1R in HPL1D.....	36
12.3. IGF1R in A549 and H358.....	37
13. Ubiquilin1 decreases mRNA levels of IGF1R and EGFR.....	39
14. Loss of Ubiquilin1 alters degradation of RTK	
14.1. IGF1R.....	41
14.2. EGFR.....	42
15. IGF1R degradation assay.....	45

CHAPTER 1: INTRODUCTION

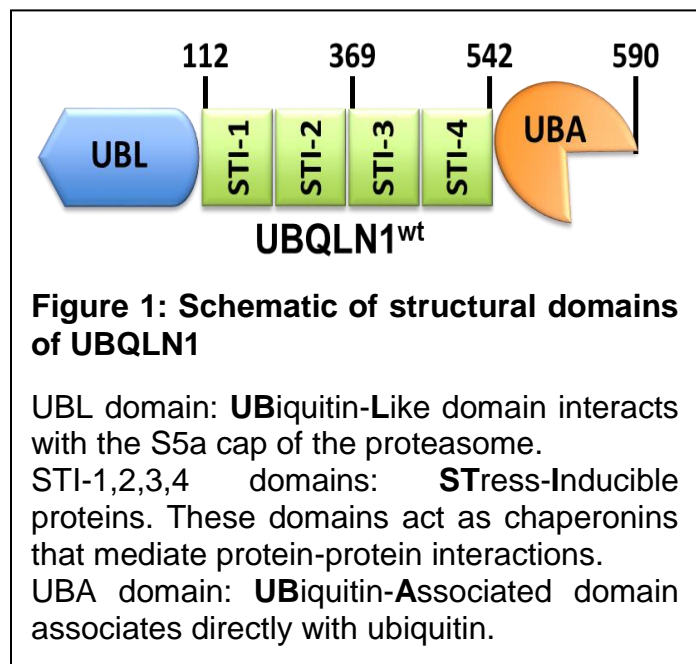
Ubiquilins (PLICs)

The Ubiquilin family of proteins (UBQLN1-4, UBQLNL) are evolutionarily conserved and structurally similar to each other. These proteins are encoded by genes on different chromosomes: UBQLN1 (9q22, 589 or 561 amino acids), UBQLN2 (Xp11.21, 624 amino acids), UBQLN3 (11p15, 654 amino acids), UBQLN4 (1q21, 601 amino acids) and UBQLN-L (11p15.4, 475 amino acids) [1].

Ubiquilins are also known as **P**roteins **L**inking **I**AP with **C**ytoskeleton (PLICs) as these were originally discovered as proteins that anchor vimentin filaments to the plasma membrane through

interaction with CD47, an integrin-associated protein (IAP) [2]. UBQLN1 is

approximately 63 kDa protein and all its family members have 3 main domains: ubiquitin-like domain (UBL) at the N-terminus, ubiquitin-associated domain (UBA) at



the C-terminus and STI chaperone-like regions in the middle (Figure 1). UBQLN1

maintains all the functionally important motifs present in this group [3]. Expression of Ubiquilin varies based on tissue type. UBQLN1 is ubiquitously expressed in all tissues, UBQLN2 and UBQLN4 show relatively higher expression in the brain, spleen, heart, liver and pancreas over other tissues, UBQLN3 is exclusively expressed in the testis [3] and UBQLNL is mostly found in plasma, platelet and liver.

Proposed Functions

Owing to their structural orientation, Ubiquilins are categorized as ubiquitin binding proteins (UBPs). UBQLN1 assists in proteasomal degradation of ubiquitinated substrates and also participates in ERAD (Endoplasmic Reticulum Associated Degradation), autophagy, apoptosis and receptor trafficking [4]. The UBL domain of UBQLN is structurally similar to ubiquitin and interacts with the proteasomal cap (S5a) [5] while the UBA domain associates directly with ubiquitin [6, 7]. The central region consisting of STI domains are called so because of their similarity to STI-1 proteins (**ST**ress **I**nducible proteins or Hsp70-Hsp90 organizing protein, HOP). This region acts as a co-chaperone and mediates hydrophobic interactions with other proteins [8, 9]. Together, the UBL domain of Ubiquilin1 binds to the proteasome while the UBA domain binds to ubiquitin on the substrate protein and aids in the substrate's degradation [5].

Several studies have reported that UBQLN1 facilitates proteolysis of substrates that it binds to. Dsk2p, the yeast homolog of UBQLN1, forms a trimeric complex

with Rad23p and Cdc48 and enables degradation of misfolded proteins via the proteasomal system [10]. In human cells, the UBL domain of UBQLN1 has been shown to regulate degradation of misfolded proteins like Ataxin3, HSP1a and EPS15 [11]. UBQLN1 expression aids in poly-ubiquitination of viral polymerase NS5B, decreasing its half-life in HCV infected hepatocytes [12]. In contrast, UBQLN1 stabilizes proteins like Presenilin1 and 2 [3, 13], Rad23 [14], IκB [15], and Bclb [1] when bound to them.

UBQLN1 and UBQLN2 were originally (1999) identified in ovarian cancer cells and showed to increase cell spreading and integrin mediated interactions with the extracellular matrix. Identifying functions of Ubiquilin proteins is an ongoing area of research. UBQLN1 is a stress-response protein; its expression protects astrocytes from hypoxia [16] and HeLa cells from starvation-induced death [17]. In 2010, Rothenberg et al showed that UBQLN1 interacts with autophagy marker LC3 in a complex with other proteins and reduction in UBQLN1 expression decreases autophagy levels in HeLa cells, brain and liver tissue of mice [18]. UBQLN1 is also reported to interact with the metabolite mTOR; however, the consequences of this interaction are not known [19]. In superior cervical ganglion neurons, UBQLN1 expression limits the expression of unassembled nicotinic acetylcholine receptors in the absence of nicotine stimulation [20].

Ubiquilin in Neurodegenerative Disorders

Presenilin proteins (PS1 and 2) are well-established Alzheimer's disease-associated proteins [21]. UBQLN1 binds to and stabilizes PS1 and 2 and co-localize in a vesicular-like or ER-like pattern [3, 12]. A meta-analysis in 2014 confirmed that a single intronic C/T polymorphism in UBQLN1 (UBQ-8i) significantly increased risk of Alzheimer's disease [22, 23]. In the presence of this polymorphism, the STI domains of UBQLN1 are unable to bind to PS proteins as a result of which a gamma secretase complex is formed generating A β 40 and A β 42 amyloid aggregations [13, 24, 25]. Ford et al (2006) hypothesize that UBQ-8i polymorphism favors formation of UBQLN dimers and loses its ability to bind to PS proteins. Neurofibrillary tangles and Lewy bodies in brains of Alzheimer's disease and Parkinson's patients robustly stained for anti-UBQLN antibodies provide more evidence to the role of UBQLN in these diseases [13].

UBQLN1 is also implicated in pathogenesis of triplet repeat neurodegenerative diseases especially ones characterized by expanded polyglutamine (PolyQ) tracts. Of the nine different types of PolyQ diseases, UBQLN1 plays a role in Huntington's disease (HD) and spinocerebellar ataxia type1 [26]. The expansion of glutamine tracts leads to aggregation of the affected protein as it is unable to get degraded effectively in absence of UBQLN1. In an animal model of Huntington's disease, UBQLN4 expression was identified to be protective as it decreased polyQ-induced protein aggregation through its interaction with Ataxin1 and UBQLN1 [27].

UBQLN2 mutations have been identified in X-linked Amyotrophic Lateral Sclerosis (ALS) and Fronto-Temporal Dementia (FTD). UBQLN2-positive inclusions are detected in spinal cord of ALS patients and in hippocampus of ALS patients with dementia proposing a role of mutant UBQLN2 aggregation in neurodegeneration [28].

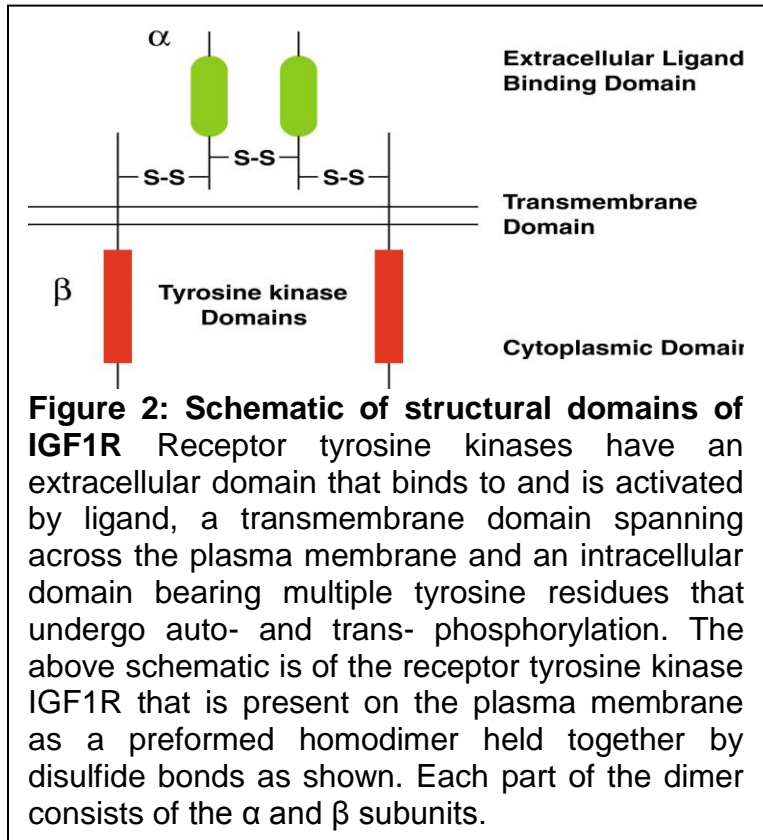
Ubiquilin in Cancer

First evidence of role of Ubiquilin proteins in cancer development was reported in 1999, when Funakoshi and colleagues found that *XDRP1*, a *Xenopus* orthologue 60% similar to Ubiquilin1 interacted with cyclin A, preventing its degradation and arresting cell division. Another way UBQLN1 has been proposed to regulate cell division is by binding to a tumor suppressor protein DAN and S(1-5) which modulates DNA synthesis. In contrast, UBQLN2 expression enhanced tumor progression in osteosarcoma [29].

Previous work from our lab has identified UBQLN1 as a regulator of anti-apoptotic protein Bcl2L10/Bclb [1]. We have reported that UBQLN1 is lost in about 50% of lung adenocarcinomas and tissue samples have varying UBQLN1 mRNA levels. We have also shown that loss of UBQLN1 or UBQLN2 promotes epithelial to mesenchymal transition (EMT) in lung adenocarcinoma cell lines [4].

Receptor Tyrosine Kinases (RTK's)

Receptor Tyrosine Kinases are cell surface proteins that have an N-terminus extracellular domain, a transmembrane domain and a C-terminus intracellular domain possessing intrinsic tyrosine kinase activity (Figure 2). These



receptors are present on the cell surface as preformed dimers as in case of Insulin-like Growth Factor 1 Receptor (IGF1R) or undergo dimerization upon ligand binding for example Epidermal Growth Factor Receptor (EGFR). There are approximately thirteen subfamilies of RTK's and sixty members in all [30]. Receptor activation occurs upon binding to specific ligands, following which the receptor gets auto- and trans-phosphorylated at their intracellular domain and stimulates downstream signaling pathways that mediate growth, proliferation, survival, tissue homeostasis, and metabolism. Dysregulation of RTK's have been identified in a number of cancers and can be responsible for initiation, progression and metastasis of tumors. Receptor tyrosine kinases like EGFR, IGF1R, Insulin receptor (INSR), FGFR and non receptor tyrosine kinases like

SRC, Abl, FAK are upregulated in a wide range of cancers [31]. In normal cells, the activity of RTK's is tightly regulated. However, these can become oncogenic following gain of function mutations, gene rearrangement, gene amplification, loss of genomic imprinting or abnormal receptor or ligand activation. RTKs like EGFR and analogues HER2, Erb2; VEGF and VEGFR, IGF1R and others are major targets of current cancer therapies.

EGFR and IGF1R

The epidermal growth factor receptor family comprises of many subtypes of receptors: EGFR (ErbB1), ErbB2/HER2/neu, ErbB3/HER3 and ErbB4/HER4. Ligands of these receptors include EGF, amphiregulin, betacellulin, neuregulins, and others. Activation of EGFR stimulates growth and proliferative pathways. Mutations and overexpression of EGFR family of proteins are largely implicated in lung adenocarcinoma and breast cancers [32].

Insulin-like growth factor 1 receptor (IGF1R), IGF2R and IR (insulin receptor) are RTKs that are ubiquitously expressed in cells and bind to their ligands IGF1, IGF2, insulin with differing affinities. Their basic function is to stimulate growth and survival. IGFBPs (IGF binding proteins 1-6) also bind these ligands and limit their bioavailability to bind to RTKs [33]. Increased IGF1R expression is found in a number of cancers, including breast, prostate, colorectal, liver, lung and melanoma. In a study by Nakagawa et al in 2012, IGF1R expression was associated with an increased risk of recurrence of non-small cell lung cancer

(NSCLC). A meta-analysis of 17 studies comprising 3,294 NSCLC patients concluded that IGF1R expression positively correlated with smoking status, tumor size and is negatively correlated with disease free survival [34]. Increased IGF1 levels increase the risk of development of breast, prostate, colorectal cancers. Normally, cells are dependent on circulating levels of IGF1 produced by the liver but cancer cells show increased IGF1R activity by producing their own IGF1 through autocrine and paracrine processes. IGF1R activation stimulates PI3K/AKT (survival) and RAS/MAPK (proliferation) pathways [35].

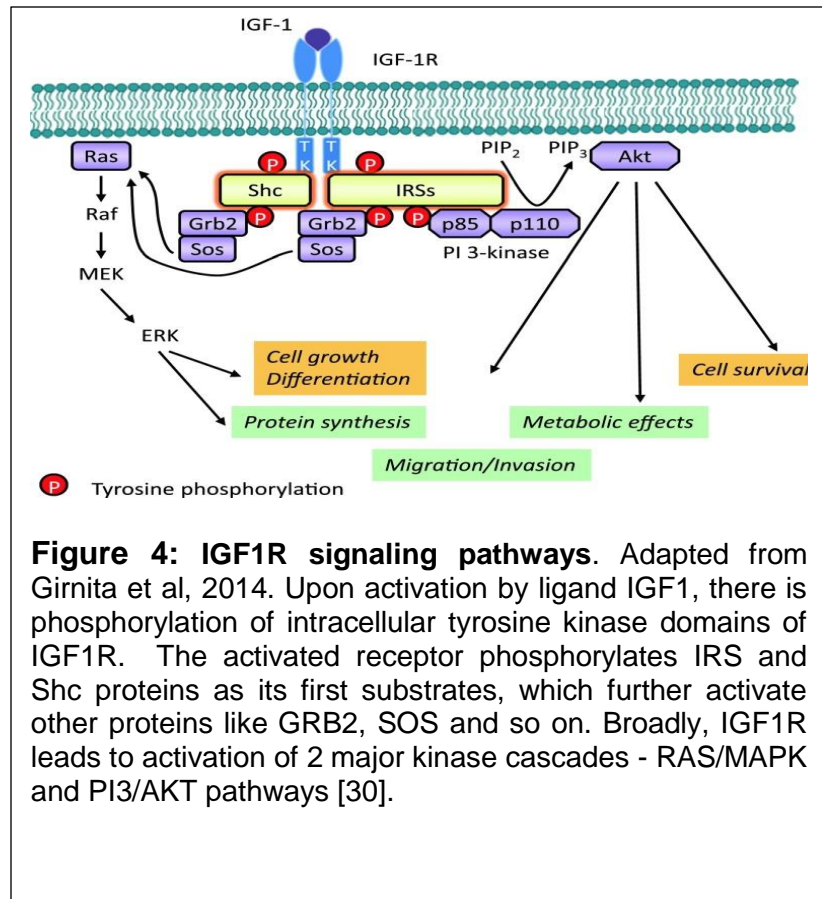
IGF1R Structure and Trafficking IGF1R is synthesized as a monomer consisting of α and β chains. This monomeric pro-receptor undergoes maturation through proteolysis and glycosylation to form dimers. IGF1 receptors are present on the cell surface as preformed dimers unlike EGFR that form dimers upon ligand binding (Figure 3). The β subunit consists of 627 amino acids, has a small extracellular portion, transmembrane portion and the intracellular C-terminal. The juxtamembranous region in the β subunit hosts the NPXY motif and the catalytic region. The NPXY motif participates in the process of receptor internalization while the catalytic region is crucial for ATP binding. Tyrosine 1131, 1135 and 1136 in the intracellular tyrosine kinase domains are critical for receptor autophosphorylation. The intracellular domains hold crucial signals that decide the fate of receptor trafficking once it is activated. For example, even though overall there is 70% amino acid homology between IGF1R and Insulin receptor, there is only 44% homology in their C-terminus sequence which dictates the

IRS proteins interact with IGF1R on the cell surface through their pleckstrin homology domains (PH) and PTB domains are present in their N-terminus. The C-terminus region of IRS proteins are variable and control the variety of interactions with other signaling molecules. The C-terminal of IRS proteins have a high affinity for proteins that have an SH2 domain like PI3K, Grb2, SH-PTP2 (phosphatase), adaptor proteins like CRK, NCK1 which act as docking proteins for interaction of IGF1R with other proteins like β 1 integrins on the cell surface.

Like IRS proteins, SHC proteins (SHC A-D) also consist of a PTB domain at their N-terminal and a SH2 domain at their C-terminal and

interact with proteins in a similar fashion. These domains, their characteristics and

the interactions they are capable of control further trafficking events of the activated receptor. Internalization of IGF1R is known to occur through clathrin mediated endocytosis (CME) and also through calveolin mediated endocytosis at high levels of IGF1 [37]. Internalized RTK's in early endosomes proceed to late



endosomes and continue to send signals through their C-terminus in the cytoplasm. In the late endosome, the RTK either gets recycled back to the plasma membrane through a recycling endosome or gets degraded via lysosomal or proteasomal pathways (Figure 5). Multi-protein complexes called

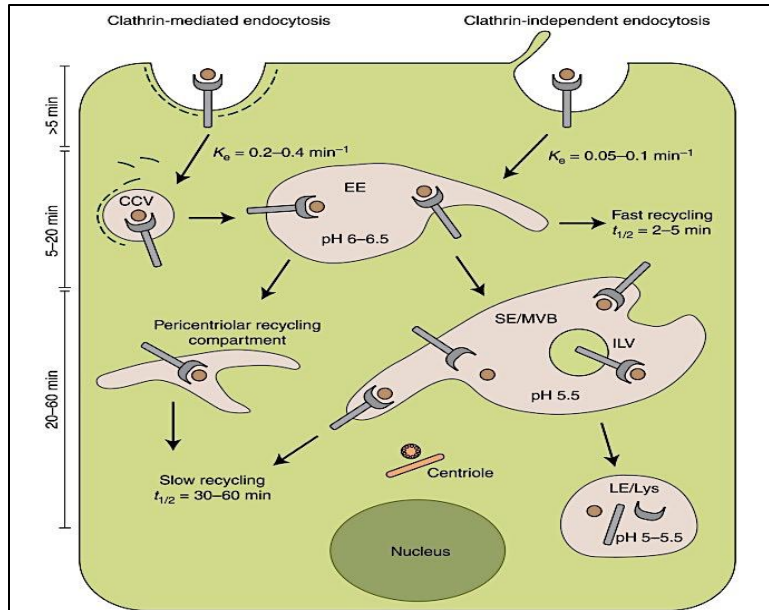
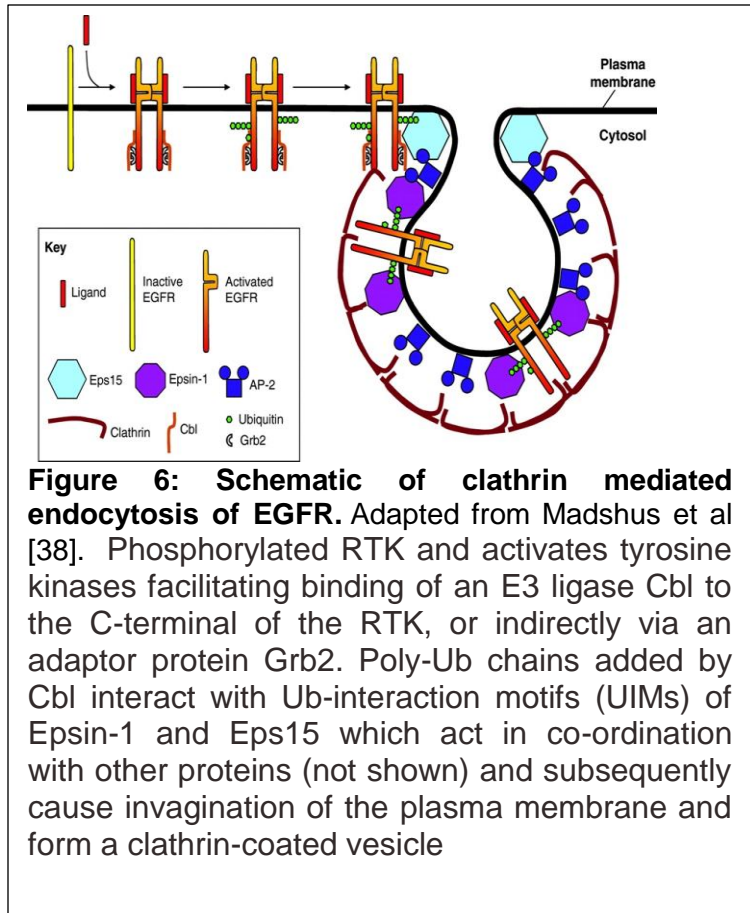


Figure 5: Endocytic Trafficking of RTK

Adapted from Goh & Sorkin [37]. This figure shows 2 pathways of endocytosis of ligand bound RTK: clathrin mediated and calveolin mediated. The internalized receptor in clathrin-coated vesicles (CCV) is transported to the early endosome (EE) to the sorting endosome (SE) or the multivesicular body (MVB) and from here either to the recycle endosome to be recycled back to the plasma membrane or degraded in the lysosomal endosome (LE).

Endosomal Sorting Complex Required for Transport (ESCRT) are critical for cellular transport of activated receptors [37]. These processes occur by systematic co-operation of a variety of adaptor proteins and in absence of an interacting partner can potentially disturb trafficking and alter biological consequences. Ubiquitin interacting motif (UIM) containing adaptor proteins like Epsin, Eps15, Eps15R recognize ubiquitin on RTKs, bind to clathrin and AP2 simultaneously and are critical for clathrin mediated endocytosis (CME) of some RTKs like EGFR (Figure 6). The NPXY motif not only regulates internalization of

IGF1R but also its downregulation. All RTKs possess a ubiquitin binding motif and ubiquitination has been well established as a crucial regulatory process of RTK trafficking. Ubiquitin is a small protein (7 kD) that is added to IGF1R in 3 consecutive steps via the E1, E2 and E3 ligases. The first two



enzymes, E1 and E2 work to load the last enzyme E3 on to IGF1R to add ubiquitin molecules on the receptor. IGF1R is a known substrate of three E3 ligases: Mdm2 [38] Nedd4 [39] and c-Cbl [40]. Ubiquitination of IGF1R occurs before entering the endocytic vessels during internalization [39]. Mdm2 poly-ubiquitinates IGF1R with K63-type chains and β -arrestins have been identified as crucial adaptor proteins to recruit Mdm2 to IGF1R [41]. Similarly, Grb10 is a key adaptor protein that recruits Nedd4 to IGF1R which multi mono-ubiquitinates the receptor [39]. At higher doses of IGF1, c-Cbl poly-ubiquitinates IGF1R with K48-type chains. Mdm2 recruitment favors stimulation of MAPK over PI3K pathways. Like IGF1R, EGFR is also poly-ubiquitinated with K63-type chains [42]. K48 poly-

ubiquitination signals target substrates for proteasomal degradation while K63 mono- and poly-ubiquitination are recognized by other proteins through their ubiquitin binding domains (UBD) and activate enzyme cascades like kinases, phosphatases, phospholipases and so on that feed into and activate multiple downstream pathways like PI3K/AKT, RAS/MAPK, metabolic pathways and others [43, 44]. Ubiquitination is a major molecular signal responsible for endocytic sorting such that ubiquitination of IGF1R is essential for its internalization while its not for other RTKs like EGFR and FGFR2. However, degradation of these 3 RTK's is highly dependent on their ubiquitin-conjugation [42, 45, 46].

Aim

To study regulation of receptor tyrosine kinases by Ubiquilin1.

CHAPTER 2: MATERIALS AND METHODS

Cell culture

Human normal lung epithelial cell line HPL1D and human non-small cell lung carcinoma cell line A549 and H358 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic/antimycotic (Sigma, St Louis, MO, USA). The 3 cell lines were routinely subcultured every 3–4 days. All siRNA transfections were performed using Dharmafect1 #T-2001-03 (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA) as per manufacturer's protocol. After total 48 hours of transfection, cells were serum starved for 12 hours. After which IGF1 50ng/ml or 10ng/ml of EGF was added to stimulate the cells. For protein stability studies, 20uM of Cycloheximide was added an hour prior to adding IGF1. MG132 (25uM) and NH₄Cl (20uM) were also added an hour prior to adding IGF1 for experiments investigating route of degradation of RTKs. At the end of stimulation, 6 hours for IGF1 and 3 hours for EGF, cells were harvested in CHAPS lysis buffer (1% CHAPS detergent, 150mM NaCl, 50mM Tris pH 7, 5mM EDTA). Protein was quantitated by using Pierce's BCA Protein Assay Reagent Kit (# 23227) from Pierce Biotechnology, Rockford, IL, USA as per manufacturer's protocol.

Immunoprecipitation and Western Blot Analysis

293T cells were cultured in DMEM supplemented with 10% FBS. DNA transfections were done using PEI (PEI 2.5:1 DNA). All cell extracts were prepared following scrape harvesting of 293T cells using CHAPS lysis buffer (1% CHAPS detergent, 150 mM NaCl, 50 mM Tris pH 7, 5 mM EDTA), For immunoprecipitations, 200 ug of protein was incubated in 400 uL of total CHAPS buffer and incubated with indicated affinity matrix (Anti-FLAG beads) for 1 h at 4 °C. Following incubation, the matrix was washed three times in CHAPS buffer and then SDS loading buffer was added directly to washed matrix, boiled, and loaded directly into the wells of a PAGE gel. Drug treatments were performed as described in the text using 20 uM cycloheximide or 25 uM proteasome inhibitor MG132.

Immunofluorescence Staining

Cells were dry seeded in 35 mm IF plates culture and transfected with either non targeting siRNA (siNT) or with siRNAs targeting UBQLN1 (siU1 and siU1-2) the following day. After 48 hrs of transfection, cells were fixed with 4.0% paraformaldehyde in PBS for 15-20 min and then permeabilized with 0.1% Saponin for 60 minutes at room temperature. Cells were rinsed thrice with PBS, and then incubated overnight with anti-UBQLN1 or anti-IGF1R antibody (at a dilution of 1:1000). Next day, after three successive washes with PBS, cells were then incubated with Alexa Fluor 488 goat anti-rabbit IgG (A11034: Molecular Probes, Invitrogen detection technologies, Eugene, OR, USA) at a dilution of

1:1000. After incubation with secondary antibody for 60 minutes, cells were rinsed with PBS and incubated with Alexa Fluor 568 Phalloidin (A12380: Life technologies Eugene, OR, USA) for 10 minutes at the dilution of 1:1000. After 3 successive washes with PBS, nuclei were counterstained with DAPI (diluted 1:000) for 10 min at room temperature followed by three washes (5–10 min each) with PBS. The cells were then imaged under Nikon A1R confocal laser scanning microscope. Multiple images were acquired from multiple experiments and representative images are presented.

Radioligand Binding

Cells were incubated for 2 hours with increasing concentrations (0.001 nM to 0.05 nM) of ^{125}I -IGF1 (Perkin Elmer, Waltham, MA) at 4°C in binding buffer (DMEM/20 mM HEPES/0.1% BSA, pH 7.3). Two fractions were collected – total ligand binding (^{125}I -IGF1) and non-specific binding (mixture of radiolabeled and non-radiolabeled ligand). After 2 hours incubation on ice, cells were washed four times with binding buffer to remove unbound radioligand. Solubilizing buffer (0.5N SDS, 1N NaCl) was used to collect the cells and the radioactivity of each fraction was determined using a Beckman Coulter (Brea, CA) gamma counter. Specific binding for each concentration of ^{125}I -IGF1 was calculated by subtracting non-specific binding from total binding for that concentration of ^{125}I -IGF1. Scatchard graphs were plotted to determine K_d and B_{max} .

Total RNA Extraction and Real Time PCR

Total RNA was isolated from the A549 cells after washing twice with phosphate-buffered saline (PBS) and harvested with E.Z.N.A Total RNA Extraction Kit (Omega, USA) according to the supplier's protocol followed by DNase digestion. RNA quality and quantity were determined by photometry. Total RNA (1µg) was reverse-transcribed to cDNA using Thermo Script RT-PCR kit. Briefly, RNA was reverse-transcribed in cDNA with oligo (dT) primers and 200 U of Superscript II (Invitrogen) following manufacturer's instructions. Real-time analysis for IGF1R, EGFR, and normalizing gene human β 2Microglobulin was performed using SYBR Green Master Mix as per the manufacturer's instruction (Applied Biosystems). This technique continuously monitors the cycle-by-cycle accumulation of fluorescently labeled PCR product. Briefly, cDNA corresponding to 25 ng of RNA served as a template in a 10µl reaction mixture containing, 0.2 nM (each) primer, and 5 µl FastStart DNA Master SYBR Green mix (ABI). Samples were loaded into 96-well plate format and incubated in the fluorescence thermocycler 7500 (ABI System). Initial denaturation at 95 °C for 10 min was followed by 45 cycles, each cycle consisting of 95 °C for 15 s, touchdown of 1 °C/cycle from the primer-specific start to end annealing temperatures for 5 seconds, and 60 °C for 10 seconds. The primer sequences used for specific genes are listed in Table. All quantifications were normalized to the housekeeping HPRT gene, which showed a very stable expression in A549 cells. Fold changes in gene expression were calculated using $2^{-\Delta\Delta CT}$ method. Following are the primer sequences used for the reaction:

IGF1R F: GCCAAGCTAAACCGGCTAA
R: TATCCTCTTTTGGCCTGGACATA
EGFR F: GGCTCTGGAGGAAAAGAAAGTTT
R: CACCTCACAGTTATTGAACATCCTCT
hβ2Microglobulin F: TGACTTTGTCACAGCCCAAGATA
R: AATGCGGCATCTTCAAACCT

Statistical analysis

All data were analyzed. Differences between two groups were statistically analyzed using unpaired Student's *t*-test. Differences were considered significant when $p < 0.05$.

Reagents

IGF1R-beta #3027, IGF1R Beta XP #9750, p-IGF1R beta #3918, EGFR #2232, p-EGFR #2304, AKT #9272, p-AKT #9271 (Cell Signaling Technologies Inc. Danvers, MA 01923); Tubulin #B512 (Sigma); GAPDH #FL335 (Santa Cruz); Ubiquilin polyclonal Ab was made by inoculating rabbits with a peptide specific to UbqIn1 (Yenzym Antibodies LLC); Alexa Fluor 488 goat anti-rabbit IgG #A11034 (Molecular Probes, Invitrogen detection technologies, Eugene, OR. USA); Alexa Fluor 568 Phalloidin #A12380 (Life technologies Eugene, OR. USA); Anti-FLAG Affinity Gel (Sigma A2220).

RNAi Sequences

All RNAi (siRNAs) used for study were ordered from Thermo Fisher Scientific Biosciences Inc. Lafayette, CO 80026, USA and transfections were done using Dharmafect1 as per the suppliers instructions.

NonTargeting	siNT	UAAGGCUAUGAAGAGAUACAA
Ubiquilin1	siUBQLN1	GAAGAAAUCUCUAAACGUUUUUU
	siUBQLN1-2	GUACUACUGCGCCAAUUU

CHAPTER 3: RESULTS

UBQLN1 is lost and under-expressed in cancers (*Previous work*) (Figure 7)

Previously, our lab has shown that UBQLN1 is lost, mutated and under-expressed in human cancers. We analyzed 877 cancer cell lines from the Cancer Cell Line Encyclopedia and found that 243 (27.7%) had loss or deletion of UBQLN1. Furthermore, these cancer cell lines demonstrated significantly lower expression of UBQLN1 ($P < 0.0001$) than those without loss or deletion suggesting that copy number alteration is a mechanism of UBQLN1 inactivation in cancer cells. Similarly, examination of 230 lung adenocarcinoma tumors from the Cancer Genome Atlas revealed a high frequency of copy number loss (50.8%) with a corresponding significant decrease in gene expression levels ($P < 0.001$)[4]. Our lab has identified 343 genes by microarray analyses that are expressed differently between tumors with high or low levels of UBQLN1. By Ingenuity pathway analysis, we found that UBQLN1 played a major role in proteins associated with molecular transport and protein trafficking. Among these are proteins involved in signaling pathways like MYC and AKT signaling and other proteins involved in cell cycle regulation [1].

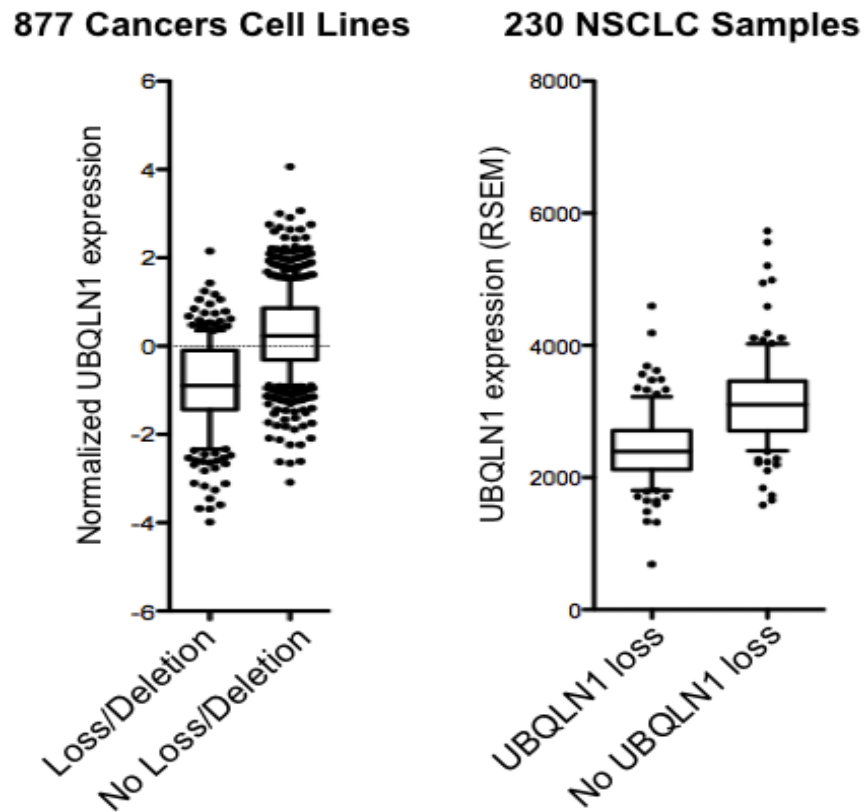


Figure 7: Ubiquilin1 is lost and under-expressed in cancers [4]

UBQLN1 is frequently lost and under-expressed in cancer cell lines. Cancer cell lines are separated by UBQLN1 copy number status and their corresponding UBQLN1 expression plotted. Box plots depict the median group expression (middle line), the 25th and 75th percentiles (bottom and top of box, respectively), with the whiskers indicating the 10th and 90th percentiles. Values for all samples outside this range are represented by dots. The UBQLN1 locus is lost in 243 of the 877 cell lines examined. UBQLN1 is significantly under-expressed in samples with UBQLN1 loss or deletion ($P < 0.0001$, Mann–Whitney U-test).

UBQLN1 interacts with IGF1R and INSR (Figure 8)

To detect potential interacting partners of UBQLN1, we over-expressed FLAG epitope-tagged UBQLN1 followed by immunoprecipitation and mass spectrometry (MS) analysis to identify FLAG conjugated proteins. Interaction was detected for proteins ESYT1 and ESYT2 (calcium-mediated intrinsic membrane proteins), IGF1R, IGF2R and INSR (receptor tyrosine kinases) and BAT3/BAG6 proteins (a cytosolic multi-protein complex involved in the post-translational delivery of tail-anchored membrane proteins to the endoplasmic reticulum membrane) (Figure 8.1, *Previous Work*). We confirmed the interaction between UBQLN1 and IGF1R by Western Blot analysis of immunoprecipitated FLAG-tagged UBQLN1 in HEK-293T cells in conditions of complete media with vehicle (DMSO), proteasomal inhibitor MG132 and serum starvation for 12 hours (Figure 8.2, *Previous Work*). UBQLN1 interacted with IGF1R in all 3 conditions. However, an increased interaction was detected when the proteasome was blocked with MG132. We then investigated if interaction of UBQLN1 with IGF1R was a phenomenon applicable to other receptor tyrosine kinases and performed similar experiments to pull down FLAG conjugated proteins in 293T cells and probed for other RTKs by Western Blot analysis. We found that insulin receptor (INSR), a protein 60% homologous to IGF1R also interacts with UBQLN1 but EGFR exhibited a weak, almost no interaction with UBQLN1 (Figure 8.3).

Unique Peptide Count		Gene Name
MIG	UBQLN1	
	48	ESYT1
	34	BAT3/BAG6
	30	ESYT2
	26	UBQLN1
	22	IGF2R
	10	IGF1R
	10	SELS
	8	UBLA
	8	INSR

Figure 8.1: Immunoprecipitation/Mass Spectrometry Data

HEK-293T cells were transfected with FLAG epitope-tagged UBQLN1 followed by immunoprecipitation and mass spectrometry (MS) analysis to identify FLAG conjugated proteins. MIG is the empty vector used as a control for overexpression. These proteins are some of the top interacting partners of UBQLN1. The strongest interaction was detected for ESYT1, then BAT3/BAG6 and so on. Receptor Tyrosine Kinases IGF2R, IGF1R and INSR interact with UBQLN1. Larger number indicates stronger interaction.

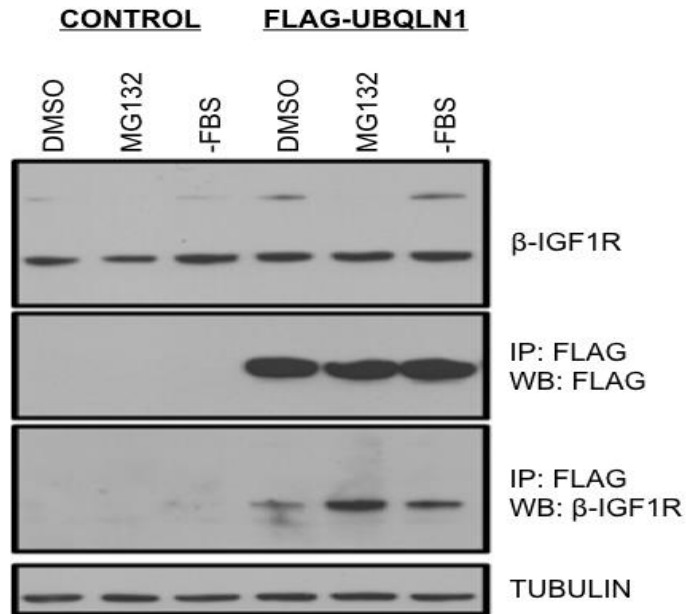


Figure 8.2: UBQLN1 interacts with IGF1R (Previous work)

HEK-293T cells were transfected with either empty vector (control) or FLAG-epitope tagged UBQLN1 (FLAG-UBQLN1) followed by immunoprecipitation and Western Blot analysis to detect interaction between UBQLN1 and IGF1R. Post transfection, cells were cultured in 3 conditions: complete media with vehicle (DMSO), proteasomal inhibitor MG132 and serum starvation for 12 hours. UBQLN1 interacts with IGF1R in all 3 conditions. An increased interaction was detected when the proteasome was blocked with MG132.

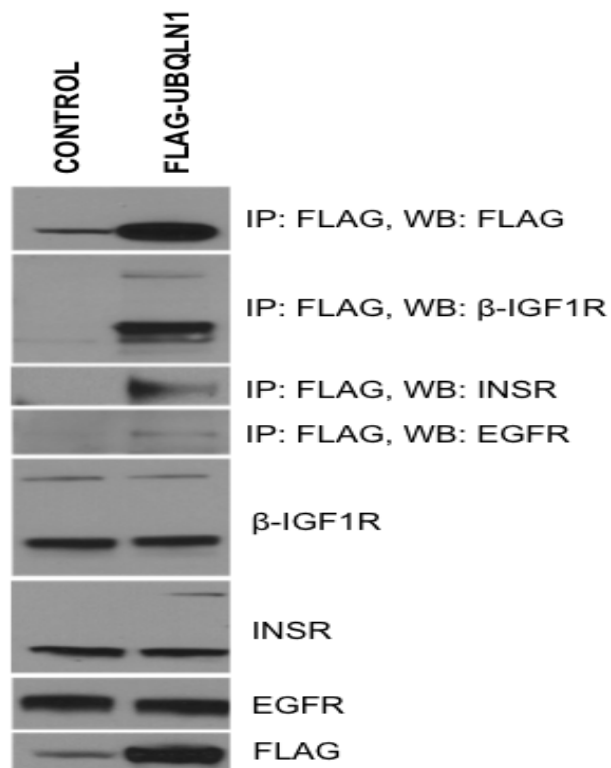


Figure 8.3: UBQLN1 interacts with IGF1R and INSR

HEK-293T cells were transfected with either empty vector (control) or FLAG-epitope tagged UBQLN1 (FLAG-UBQLN1) followed by immunoprecipitation and Western Blot analysis. UBQLN1 interacts with IGF1R and INSR, confirming the IP/MS data in Figures 8.1 and 8.2 but only weakly interacts with EGFR.

Cancer Mutants of UBQLN1 may lose interaction with IGF1R (Figure 9)

HEK-293T cells were transfected with FLAG-epitope tagged constructs of mutants of UBQLN1 found in lung adenocarcinomas to test for an alteration in ability of these mutations to interact with IGF1R (Figure 9). Eight constructs were chosen from different domains of wild type UBQLN1 protein. P44Q and I84F are mutations found in the UBL domain; Q174X, Y276D, A286T, Q433H, R313S are mutations found in the STI domains while R562S are mutations found in the UBA domain of UBQLN1. Post transfection, all mutants showed stable expression of the FLAG-tagged constructs. Wild type UBQLN1 interacts with IGF1R as also seen in previous figures, however some mutations cause a loss of this interaction. Mutations in the UBL domain (P44Q, I84F), UBA domain (R562S) and a nonsense mutation in the first STI domain (Q174X) lead to a loss of interaction with IGF1R.

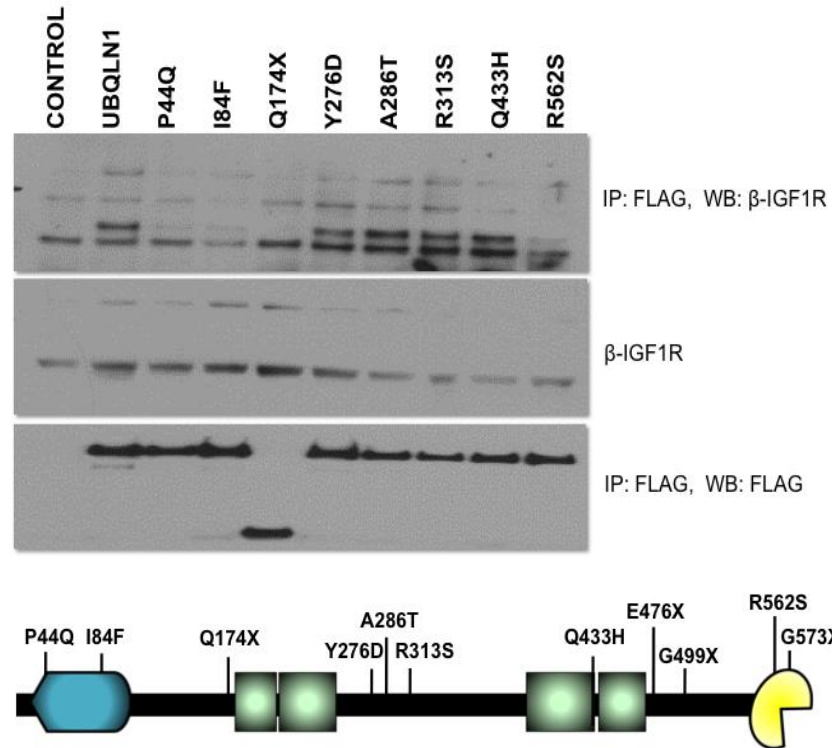


Figure 9: Cancer mutants of UBQLN1 lose interaction with IGF1R

HEK-293T cells were transfected with FLAG-epitope tagged constructs of mutants of UBQLN1 followed by immunoprecipitation and Western Blot analysis. P44Q and I84F are mutations found in the UBL domain; Q174X, Y276D, A286T, Q433H, R313S are mutations found in the STI domains while R562S mutation is found in the UBA domain of UBQLN1. While all transfected mutant constructs stably express in these cells, mutations in the UBL domain (P44Q, I84F), UBA domain (R562S) and a nonsense mutation in the first STI domain (Q174X) lead to a loss of interaction with IGF1R.

UBQLN1 loss decreases IGF1R protein level expression (Figure 10)

Following confirmation of interaction between UBQLN1 and IGF1R, steady state expression of IGF1R were tested in lung adenocarcinoma cell lines A549 and H358 and also in HPL1D lung epithelial cells. UBQLN1 protein expression was downregulated using 2 different siRNAs for UBQLN1 (siU1 and siU1_2) (Figure 10). A549 cells were cultured in 3 different conditions: complete media, serum starvation for 12 hours and stimulation by ligand IGF1 (6h) following 12 hours of serum starvation. Cycloheximide (20uM) was added to cells in all 3 conditions to prevent new protein synthesis. Post treatment, cells were harvested, lysed and analyzed by Western Blot for total and phosphorylated receptor (IGF1R or EGFR) expression levels. While the total IGF1R expression was decreased in UBQLN1 deficient cells in all 3 conditions as seen in Figure 10.1, differences were glaringly evident in post stimulation conditions for both receptors. Phosphorylated IGF1R levels were undetectable in A549 cells in complete and serum deficient media, however post stimulation with IGF1, the ratio of phosphorylated to total IGF1R levels was greatly increased in siUBQLN1 cells (1.46 vs. 1.05) compared to control. Results are normalized to non-treated control in complete media. In H358 cells exposed to conditions of serum starvation and IGF1 stimulation, phosphorylated/total IGF1R is higher in siU1 cells compared to siNT cells (Figure 10.2). We detected phosphorylated IGF1R even in serum starved siU1 cells but absent in control. Similar results were observed in IGF1 stimulated HPL1D cells (Figure 10.2).

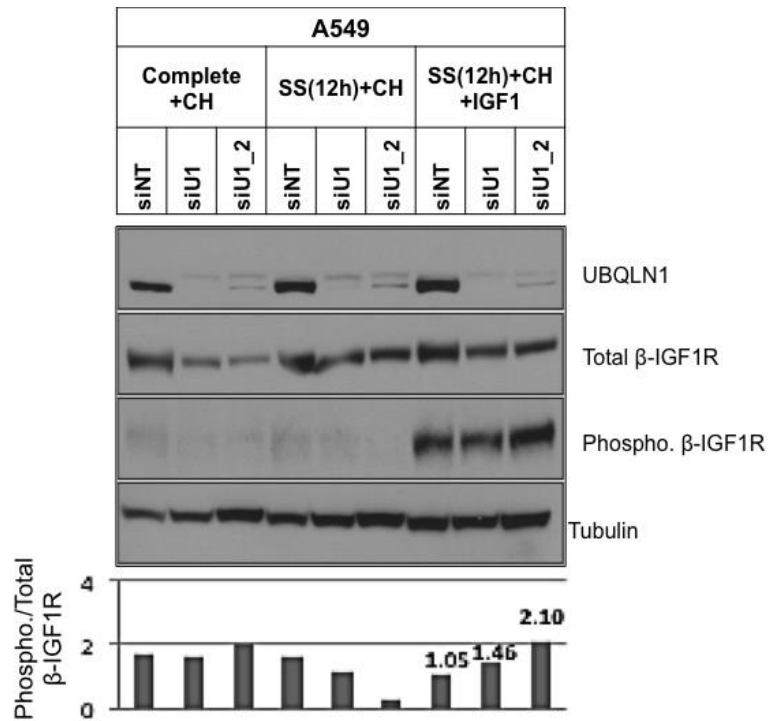


Figure 10.1: Loss of UBQLN1 decreases total IGF1R expression in A549 cells

A549 cells were transfected with siRNA (NT: non targeting control, U1 and U1_2: UBQLN1) and post transfection, cultured in 3 different conditions in the presence of cycloheximide (translation inhibitor): Complete media, Serum Starvation (SS) or stimulation with IGF1 for 6 hours, post 12 hours of serum starvation. Total IGF1R levels were decreased in UBQLN1 knock down cells and results are more pronounced post stimulation with IGF1. Phosphorylated IGF1R levels were undetectable in A549 cells in complete and serum deficient media, however post stimulation with IGF1, the ratio of phosphorylated to total IGF1R levels was greatly increased in siUBQLN1 cells (1.46, 2.10 vs. 1.05) compared to control.

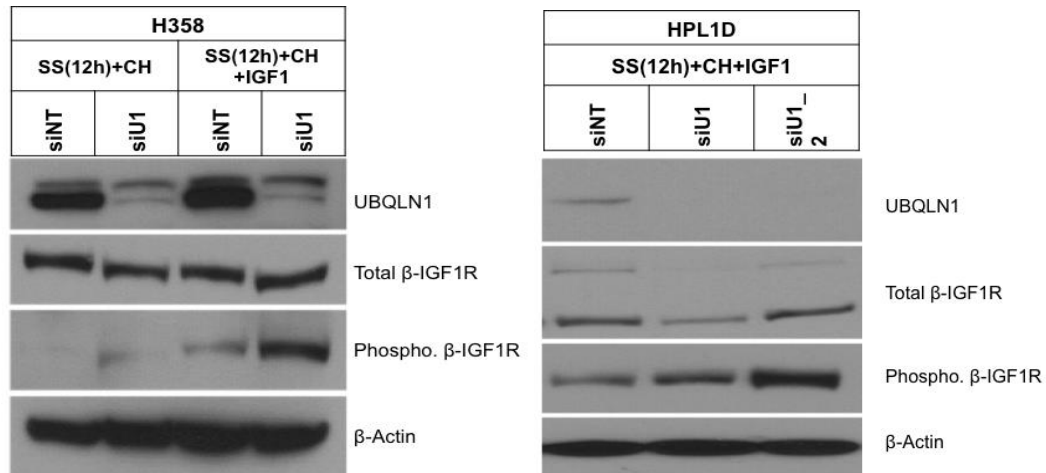


Figure 10.2: Loss of UBQLN1 decreases total IGF1R expression in H358 and HPL1D cells

H358 cells were transfected with siRNA (NT: non targeting control, U1: UBQLN1) and post transfection, cultured in 2 different conditions in the presence of cycloheximide (translation inhibitor): Serum Starvation (SS) or stimulation with IGF1 for 6 hours, post 12 hours of serum starvation. Total IGF1R levels were decreased in UBQLN1 knock down cells. There is increased expression of phosphorylated IGF1R in UBQLN1 knock down cells in serum starved cells as well as post stimulation with IGF1. Similarly, in lung epithelial cell line HPL1D, following siRNA transfection, total receptor levels were decreased, but there is an increase in phosphorylated IGF1R expression.

**Loss of UBQLN1 results in decreased cell surface expression of IGF1R
(Figure 11)**

Following Western Blot data that showed decreased total IGF1R expression in cells, saturation binding assays were performed to test if the overall decrease in total IGF1R expression also reflected as a decrease in receptor number on the cell surface. Radioligand binding assays were performed utilizing radioactive ligand (I^{125} -IGF1) to test for differences in the number of binding sites (number of cell surface receptors) in HPL1D cells between UBQLN1 deficient cells and control (Figure 9). In these experiments, HPL1D cells were transfected with UBQLN1 siRNA (siU1) or control (siNT) and 48 hours post-transfection, cells were incubated with increasing concentrations of the radiolabeled ligand (I^{125} -IGF1) with the intention to saturate the receptors. The purpose of performing saturation binding assays was to determine the differences in maximum binding capacity B_{max} (number of cell surface receptors) between UBQLN1 deficient cells and control. Plotting saturation curves showed that although the amount of radioactive ligand used did not saturate the receptors, the total binding activities for I^{125} -IGF1 were considerably lower in cells with loss of UBQLN1 compared to control (Figures 11.1). Scatchard plot analysis suggests that IGF1 receptors exhibit properties of positive cooperativity i.e. binding of ligand to one site increases the affinity at other binding sites. Overall, B_{max} or number of cell surface IGF1 receptors was 2-fold lower in UBQLN1 deficient cells while the K_d i.e. affinity is almost the same (0.4×10^{-10} M vs. 0.6×10^{-10} M) (Figure 11.2). Data are representative of 3 independent experiments.

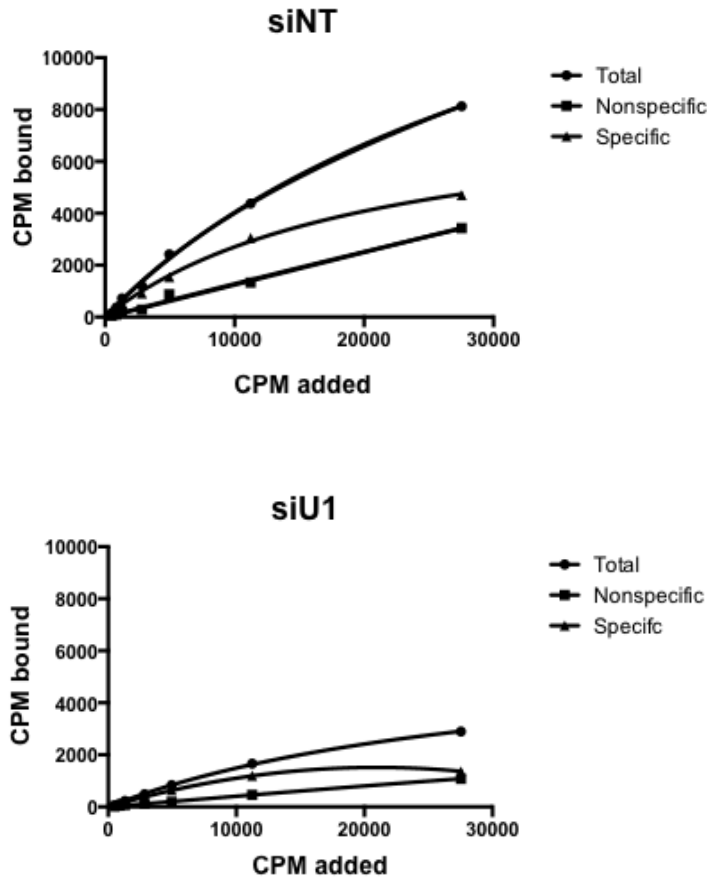
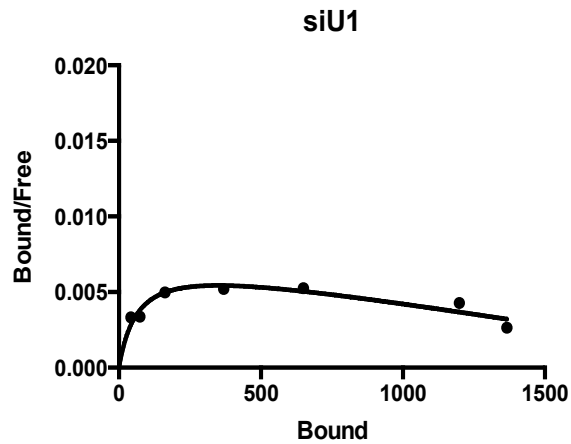
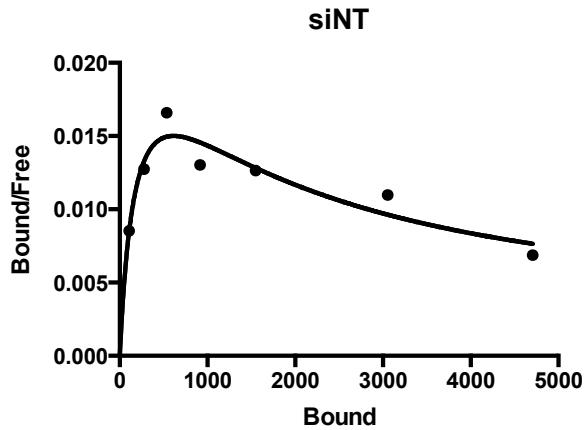


Figure 11.1: Saturation binding of I¹²⁵-IGF1 in HPL1D cells

HPL1D cells were transfected with UBQLN1 siRNA (siU1) or control (siNT) and 48 hours post-transfection, cells were incubated with 7 increasing concentrations of the radiolabeled ligand I¹²⁵-IGF1 (counts per minute, **CPM added**) and cells were processed after 2-hour incubation to determine the amount of radioligand bound to the cell surface receptors (**CPM bound**). Plotting saturation curves showed that the total binding activities for I¹²⁵-IGF1 were considerably lower in cells with loss of UBQLN1 (siU1) compared to control (siNT). Total: Total binding of I¹²⁵-IGF1 to cell surface proteins. Non-Specific: Binding of I¹²⁵-IGF1 to cell surface proteins other than IGF1R. Specific: Binding of I¹²⁵-IGF1 to IGF1R and is calculated by subtracting Non-Specific CPM from Total CPM for each dose of I¹²⁵-IGF1. Radioactivity was measured as CPM in a Beckman gamma counter (efficiency=0.45). Specific activity of I¹²⁵-IGF1 = 2200 Ci/mmol.



	siNT	siUBQLN1
Bmax	4.56×10^7 receptors/cell	2.54×10^7 receptors/cell
	23 fmol/mg total protein	11 fmol/mg total protein
Kd	0.6×10^{-10} M	0.4×10^{-10} M

Figure 11.2: Scatchard plot analysis

Scatchard plots were graphed using binding data to determine the number of ligand-binding sites (Bmax) on the cell surface and to calculate ligand affinity (Kd) for IGF1R. Data are plotted as bound receptors versus bound/free receptors. Scatchard plot for IGF1R exhibits positive cooperativity for I¹²⁵-IGF1. The table represents the differences in Bmax in HPL1D cells that have loss of UBQLN1. There were fewer binding sites on surface of UBQLN1 deficient cells while the Kd remained constant. Radioactivity was measured as counts per minute (CPM) in a Beckman gamma counter (efficiency=0.45). Specific activity of I¹²⁵-IGF1 = 2200 Ci/mmol.

Immunofluorescence (Figure 12)

Confocal microscopy imaging was performed to test for any gross detectable changes in overall expression of IGF1R between UBQLN1 deficient cells and control. Figure 12.1 demonstrates confirmation of UBQLN1 knock down using siU1 in HPL1D cells. HPL1D, A549 and H358 cells were transfected with siRNA (siUBQLN1 and siNT) and 48 hours post-transfection, cells were fixed and permeabilized and probed with indirect fluorescence targeting β -IGF1R in these cells. Overall, IGF1R expression was higher in lung adenocarcinoma cell lines A549 and H358 (Figure 12.3) compared to lung epithelial cell line HPL1D (Figure 12.2). In HPL1D cells, we observed slight decrease in cell surface expression of IGF1R in siUBQLN1 cells compared to control.

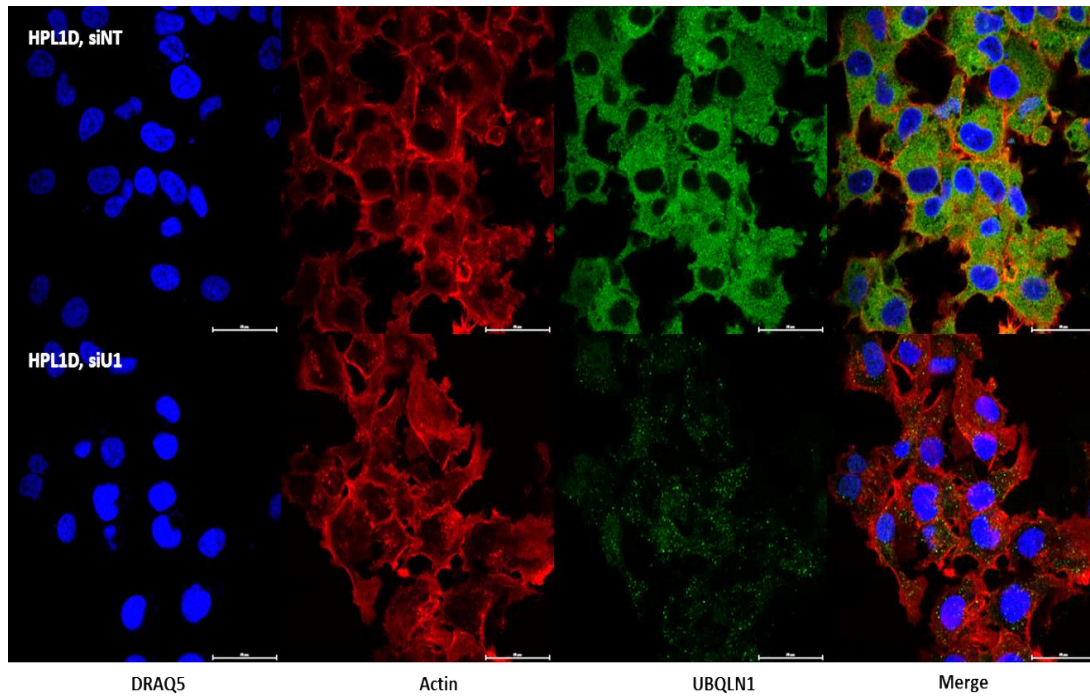


Figure 12.1: Confocal microscopy image of UBQLN1

Cells were incubated overnight in complete media at 4C with primary antibody against UBQLN1. Alexa 488- conjugated secondary antibody was used for indirect fluorescence detection. DRAQ5 was used to stain nuclei and Phalloidin was used for staining of cytoskeletal β -Actin. HPL1D cells with siRNA against UBQLN1 clearly show a decreased signal for UBQLN1 staining. The images were captured using Nikon A1R confocal microscope. The scale represents 20 μ M.

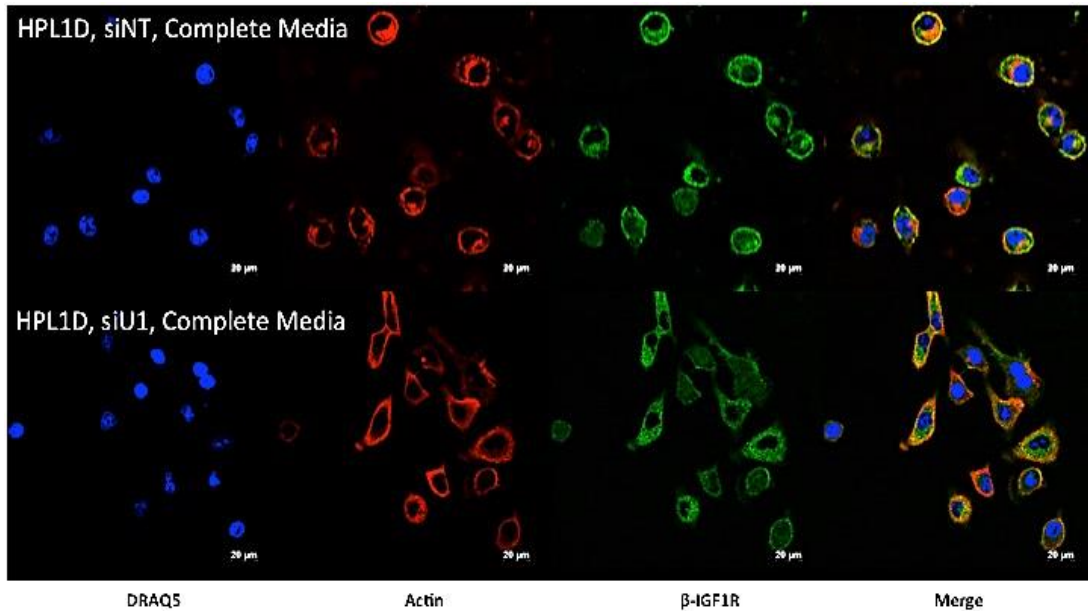


Figure 12.2: Immunofluorescence imaging of β -IGF1R in lung epithelial cell line.

Cells were incubated overnight in complete media at 4C with primary antibody against β -IGF1R. Alexa 488- conjugated secondary antibody was used for indirect fluorescence detection. DRAQ5 was used to stain nuclei and Phalloidin was used for staining of cytoskeletal β -Actin. In HPL1D cells, there clearly appears to be lower expression of IGF1R on the plasma membrane of cells that have loss of UBQLN1 (siU1) compared to control. The images were captured using Nikon A1R confocal microscope. The scale represents 20 μ M.

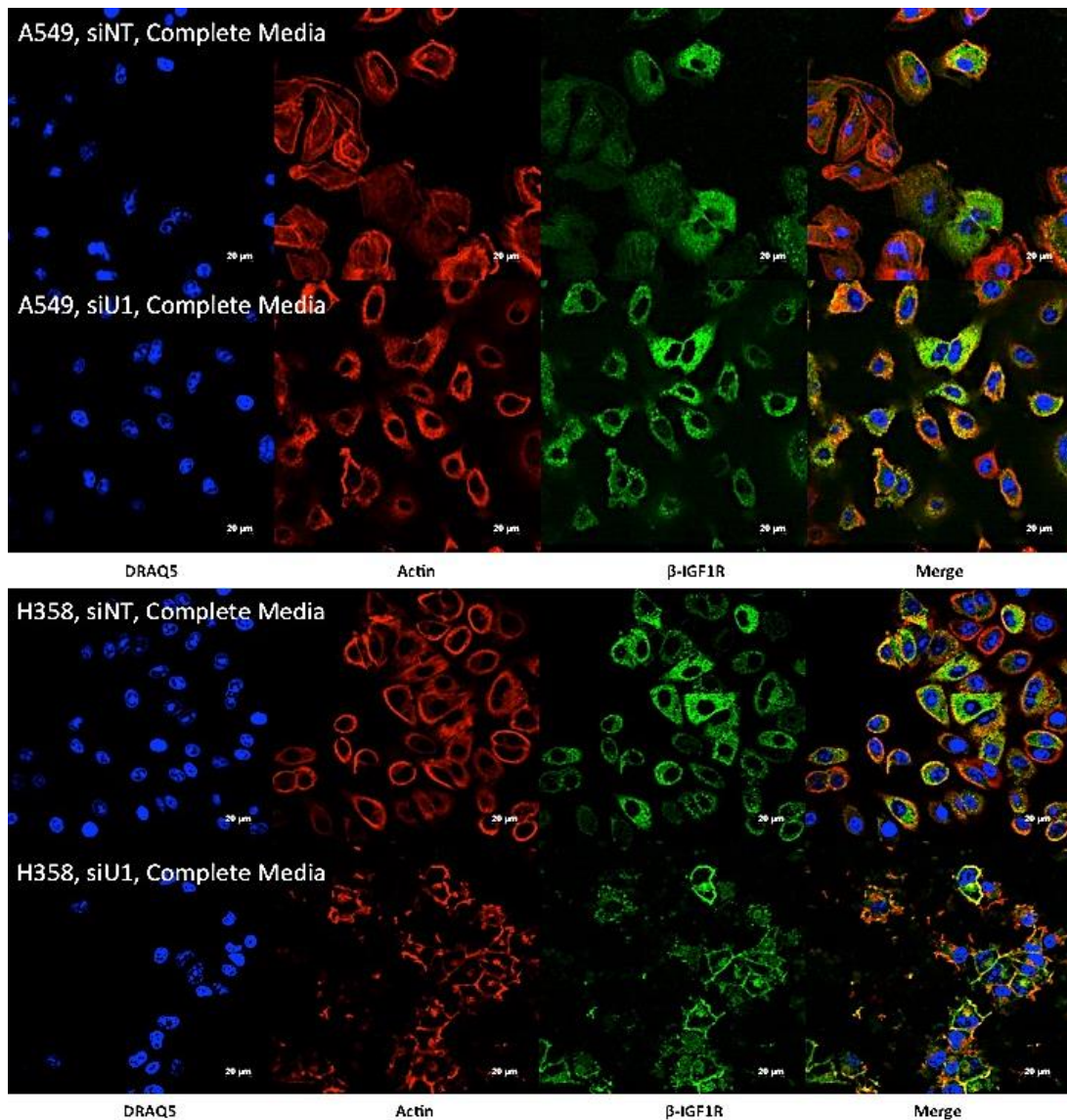


Figure 12.3: Immunofluorescence imaging of β -IGF1R in hLAC cell lines.

Cells were incubated overnight in complete media at 4C with primary antibody against β -IGF1R. Alexa 488- conjugated secondary antibody was used for indirect fluorescence detection. DRAQ5 was used to stain nuclei and Phalloidin was used for staining of cytoskeletal β -Actin. Overall, there is higher expression of IGF1R in hLAC cells (A549, H358) than lung epithelial cells (HPL1D) but there are no grossly detectable differences in its expression in UBQLN1 deficient cells (siU1). The images were captured using Nikon A1R confocal microscope. The scale represents 20 μ M.

Loss UBQLN1 leads to decreased IGF1R and EGFR mRNA levels in A549 cells (Figure 13)

Quantitative real-time PCR (qRT-PCR) were performed in A549 cells to test for relative mRNA levels of IGF1R and EGFR in cells that have siRNA mediated loss of UBQLN1 (Figure 13). Results were normalized to housekeeping gene human beta-2-microglobulin. We found a 2-fold decrease in IGF1R mRNA levels ($p=0.0015$, $SEM=0.04$ for siUBQLN1, $p=0.0094$, $SEM=0.06$ for siUBQLN1-2) and almost 30% decrease in EGFR mRNA levels ($p=0.0261$, $SEM=0.08$ for siUBQLN1) in UBQLN1 deficient cells. Data are representative of 3 independent experiments done in triplicates.

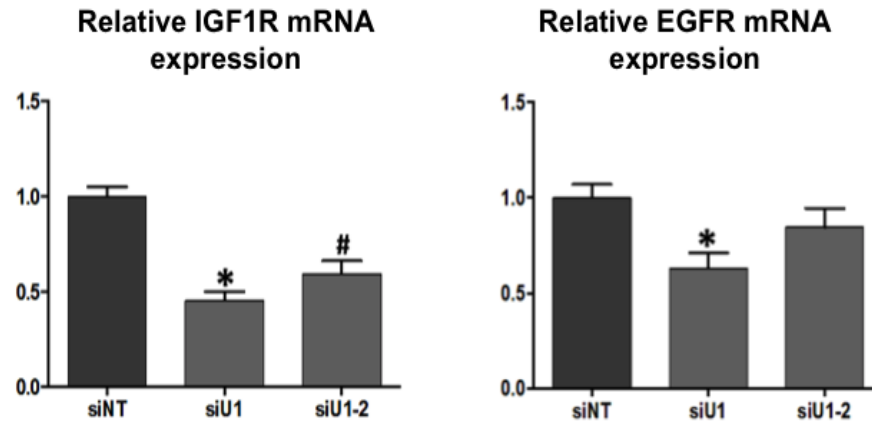


Figure 13: Relative mRNA levels of IGF1R and EGFR in A549 cells

A549 cells were transfected with siRNA (siNT: control, siU1, siU1_2: UBQLN1) and cultured in complete media. 48 hours post transfection, qRT-PCR was performed. A 50% decrease in IGF1R mRNA levels (* $p=0.0015$, SEM=0.04 for siUBQLN1, # $p=0.0094$, SEM=0.06 for siUBQLN1-2) and almost 30% decrease in EGFR mRNA levels (* $p=0.0261$, SEM=0.08 for siUBQLN1) in UBQLN1 deficient cells.

Loss of UBQLN1 affects degradation of RTKs (Figure 14)

These set of experiments were performed to test for role of UBQLN1 in degradation of IGF1R and EGFR. A549 cells were transfected with respective siRNA (siUBQLN1 or siNT) (Figure 14). 48 hours post-transfection, cells were incubated with either a proteasomal (MG132) or lysosomal inhibitor (NH₄Cl) or both for an hour prior to stimulation with ligand IGF1 (50ng/ml) or EGF (10ng/ml). Therefore, in all, there were 5 experimental conditions as observed in the figure. Cells were harvested (6 hours after IGF1 stimulation or 3 hours after EGF stimulation) and analyzed by Western Blot. In the absence of lysosomal and proteasomal inhibitors, our results were consistent with our previous data. In UBQLN1 deficient cells, we observed decreased expression of total IGF1R and EGFR while phosphorylated IGF1R and EGFR levels were increased when stimulated with respective ligands. Presence of MG132 and NH₄Cl, rescued some amount of total receptor levels otherwise degraded in the absence of UBQLN1 while stabilized phosphorylated IGF1R and EGFR in UBQLN1 deficient cells compared to control. These data imply that loss of UBQLN1 accelerates degradation of non-phosphorylated IGF1R and EGFR and therefore resulting in higher expression of phosphorylated form of the 2 receptors. It also appears that in A549 cells, UBQLN1 may be essential for lysosomal degradation of phosphorylated IGF1R as loss of UBQLN1 almost completely prevents its degradation via this pathway. Under these experimental conditions, proteasomal and lysosomal pathways degrade EGFR equally and UBQLN1 may play an important role in degradation of proteins via both these pathways.

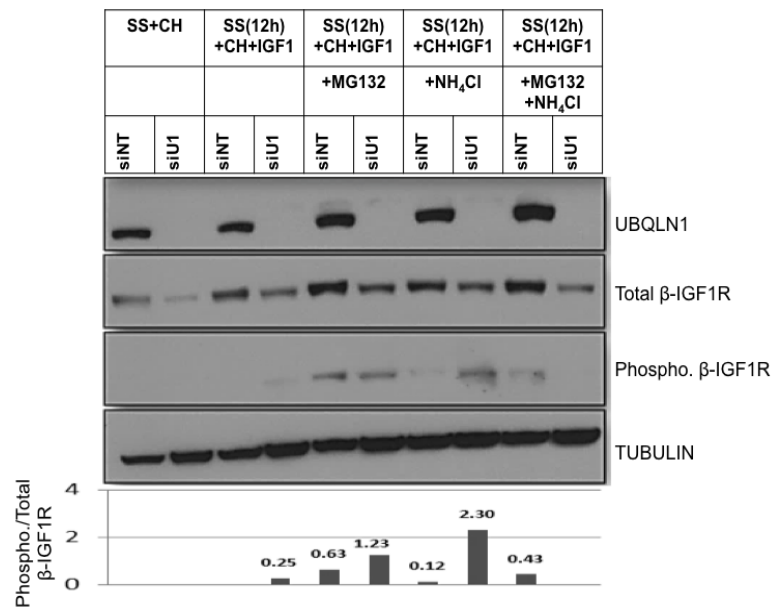


Figure 14.1: Loss of UBQLN1 affects degradation of IGF1R

These experiments were performed in A549 cells following siRNA mediated knock down of UBQLN1 (siU1). 48 hours post siRNA transfections; cells were exposed to 5 different conditions in the presence of cycloheximide. Serum starvation (SS) for 12 hours, SS for 12h and stimulation by 50ng/ml recombinant IGF1 for 6 h, addition of MG132 an hour prior to ligand stimulation, addition of NH₄Cl, a lysosomal inhibitor an hour prior to stimulation and addition of both inhibitors. There is decreased total IGF1R in siU1 lanes in all conditions. MG132 and NH₄Cl, both rescue IGF1R from degradation implying that under these experimental conditions, IGF1R is degraded via both routes. Higher phosphorylated receptor levels are observed consistently across all siU1 cells in all conditions indicating that loss of UBQLN1 prevents dephosphorylation of IGF1R and stabilizes the active receptor. In addition, use of MG132 and NH₄Cl further stabilize the active receptor. In conclusion, loss of UBQLN1 accelerates degradation of non-phosphorylated IGF1R while stabilizes the phosphorylated form. Semiquantitative densitometry analysis was performed using ImageJ software.

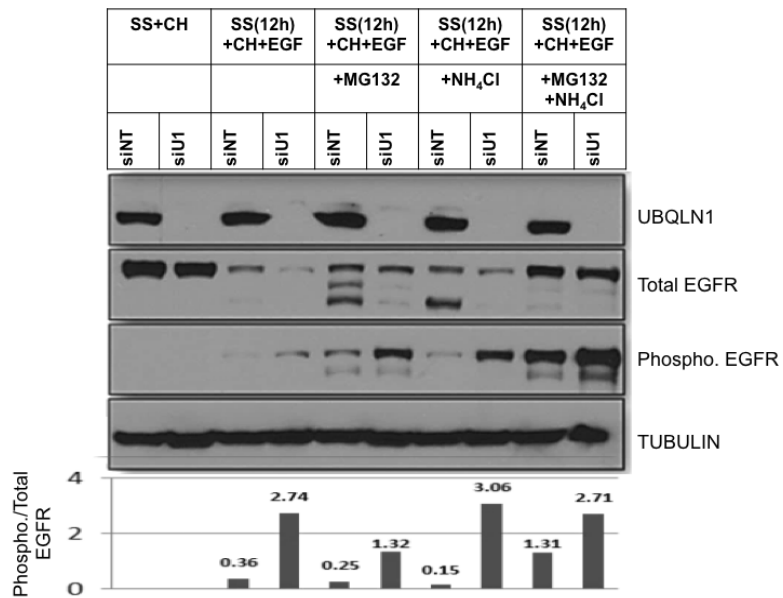


Figure 14.2: Loss of UBQLN1 affects degradation of EGFR

These experiments were performed in A549 cells following siRNA mediated knock down of UBQLN1 (siU1). 48 hours post siRNA transfections; cells were exposed to 5 different conditions in the presence of cycloheximide. Serum starvation (SS) for 12 hours, SS for 12h and stimulation by 50ng/ml recombinant EGF for 3 hours, addition of MG132 an hour prior to ligand stimulation, addition of NH₄Cl, a lysosomal inhibitor an hour prior to stimulation and addition of both inhibitors. There is decreased total EGFR in siU1 lanes in all conditions. MG132 and NH₄Cl, both rescue EGFR from degradation implying that under these experimental conditions, EGFR is degraded via both routes. Higher phosphorylated receptor levels are observed consistently across all siU1 cells in all conditions indicating that loss of UBQLN1 prevents dephosphorylation of EGFR and stabilizes the active receptor. In addition, use of MG132 and NH₄Cl further stabilize the active receptor. In conclusion, loss of UBQLN1 accelerates degradation of non-phosphorylated IGF1R while stabilizes the phosphorylated form. Semiquantitative densitometry analysis was performed using ImageJ software.

IGF1R degradation assay (Figure 15)

Our data showed that UBQLN1 regulates steady state expression and degradation of IGF1R and EGFR. UBQLN1 deficient cells have lower mRNA and protein levels of IGF1R and EGFR, but higher expression of phosphorylated form of the receptor 6 and 3 hours post stimulation with ligand. To test if the increased expression phosphorylated receptor was sustained at longer time points, a degradation assay of the receptor was performed. In this experiment, A549 cells were cultured in complete media and transfected with siRNA for UBQLN1 or control. Cycloheximide (20uM) was added to the media to prevent biosynthesis of new protein levels. The ligand IGF1 (50ng/ml) was spiked into the serum free media after 60 minutes exposure of cells to cycloheximide and followed for 5 different time points: 0h, 6h, 12h, 18h, 27h. At the end of each time point, cells were harvested, lysed and analyzed by Western Blot to test for protein expression. For every time point, expression of total IGF1R was decreased in UBQLN1 deficient cells and the differences became more apparent with longer time points. Phosphorylated receptor was detected only in IGF1 stimulated cells with highest expression at 6h and decreased phosphorylation at longer time points. Most importantly, phosphorylated receptor levels were detected even at 27 hours post ligand stimulation. IGF1R activation stimulates PI3K/AKT pathway and expression of AKT was tested for effects of UBQLN1 knockdown on this pathway. A similar trend was observed for both IGF1R and AKT proteins such that total protein level expression in UBQLN1 deficient cells were decreased while the phosphorylated protein levels were increased at 0h in absence of IGF1

stimulation and differences in protein expression were more pronounced post ligand stimulation even at 27 hours. In conclusion, IGF1R protein was not fully degraded even at the longest time point chosen for this experiment (27 hours). However, IGF1R half-life studies have not been published in lung adenocarcinoma cell lines before and therefore this may not necessarily be an unusual finding.

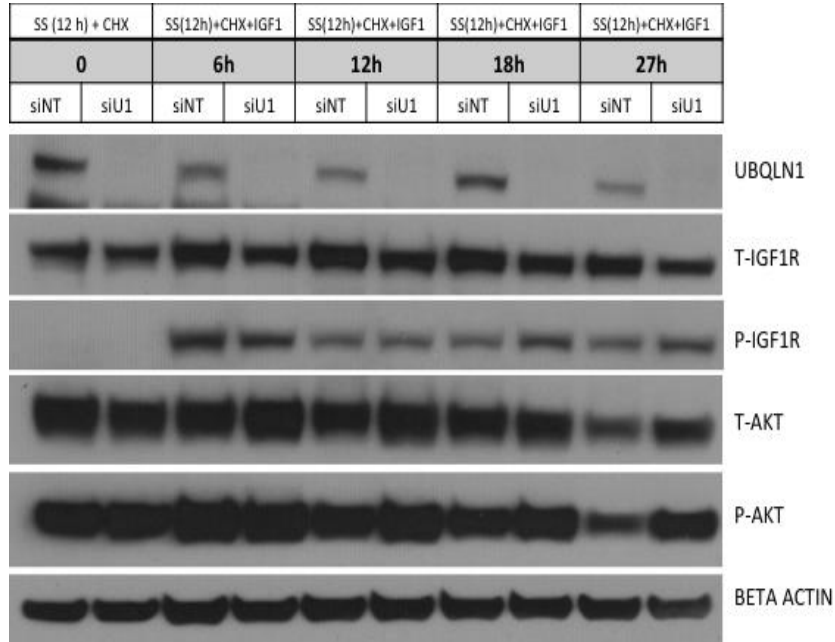


Figure 15: IGF1R degradation assay

A549 cells were transfected with siRNA (NT: non targeting control, U1 and U1_2: UBQLN1). 48 hours post transfection, cells were serum starved for 12 hours and exposed to translational inhibitor cycloheximide before stimulating with ligand IGF1 and chased for the following time points: 0h, 6h, 12h, 18, 27h. Total IGF1R levels were decreased in UBQLN1 knock down cells at all time points and results are more pronounced at later time points. Phosphorylated IGF1R levels were undetectable 0h without ligand stimulation and at every other time point, in complete and serum deficient media, however post stimulation with IGF1, the ratio of phosphorylated to total IGF1R levels was greatly increased in siUBQLN1 cells (1.46, 2.10 vs. 1.05) compared to control.

CHAPTER 4: DISCUSSION

The role of Ubiquilin proteins is largely studied in the field neurodegenerative disorders and is slowly emerging in the field of cancer. We have demonstrated role of UBQLN1 in lung cancer and how UBQLN1 regulates expression and activity of receptor tyrosine kinases like IGF1R and EGFR. Our lab has previously shown that UBQLN1 is lost and under-expressed in lung adenocarcinomas cell lines and samples [1] and cells that have loss of UBQLN1 develop clonogenic potential and migratory and invasive properties [4].

We have identified an interaction between UBQLN1 and IGF1R and INSR. Some mutations in UBL, UBA and STI domains of UBQLN1 led to loss of interaction with IGF1R. UBQLN1 interacts with and regulates expression and activity of IGF1R in lung cell lines. When UBQLN1 is lost, there is decreased overall expression of IGF1 receptors. Similarly, loss of UBQLN1 also leads to a decrease in expression of EGFR, despite only weakly interacting with it, which indicates that UBQLN1 regulates expression of receptor tyrosine kinases independent of interaction. Research studying the role of UBQLN1 in receptor trafficking have only been conducted in neurons where expression of UBQLN1 stabilizes cell surface levels of GABA_A receptors [47] and upregulates nicotinic acetylcholine receptors [20]. Radioligand binding assays confirmed that decreased total receptor levels in UBQLN1 deficient cells also reflected as

decreased number of cell surface IGF1R in these cells. Despite fewer receptors being activated in UBQLN1 deficient cells, there is an increased ratio of phosphorylated to total receptor levels. Loss of UBQLN1 leads to higher levels of phosphorylated IGF1R, EGFR and AKT protein levels. Experiments in a lung adenocarcinoma cell line also showed that the higher expression of active IGF1R is also sustained at the longest time point tested—27 hours after ligand stimulation. Decreased levels of IGF1R and EGFR in UBQLN1 deficient cells can be a result of decreased biosynthesis of the protein or increased turnover or a variety of post-translational modifications or a combination of all. UBQLN1 deficient A549 cells showed 50% decrease in transcript levels of IGF1R and 30% decrease in EGFR levels demonstrating there is decreased biosynthesis of the RTKs. One of UBQLN1's well-known functions is facilitating degradation of proteins [18, 27, 48] When tested for degradation of the receptor, loss of UBQLN1 stabilized the phosphorylated receptor and accelerated degradation of the non-phosphorylated receptor. This suggests that when UBQLN1 is present, it directly or indirectly, mediates degradation of phosphorylated IGF1R and EGFR and plays an important role in RTK trafficking and turnover. Mechanisms underlying these phenomena are not currently known but we suggest that higher levels and/or longer duration of a continuously active protein can contribute to initiation and/or progression of transforming events in cells.

Ubiquitination is a crucial regulatory process in protein signaling and turnover. Ubiquitination of proteins is a multi-step process such that E1 and E2 ligases recruit protein specific E3 ligases to load ubiquitin molecules on it. Three E3

ligases have been identified to ubiquitinate IGF1R namely Mdm2[38], Nedd4[39], and c-Cbl[40]. Mdm2 mono-ubiquitinates IGF1R at multiple sites on the receptor, Nedd4 polyubiquitinates with K63 type chains and c-Cbl polyubiquitinates with K48 type chains. K48 poly-ubiquitination signals proteins towards the proteasome for degradation while K63 mono- and poly-ubiquitination signals for interactions with proteins for other cellular processes [30]. Normally, activated IGF1R and EGFR are internalized by endocytosis and subsequently downregulated by lysosomes or ubiquitin-proteasome system or recycled back to the plasma membrane thus tightly regulating the duration and intensity of downstream signaling events [37]. Studies have shown that mutations in proteins (ex: c-Cbl) that control RTK (ex: c-MET) trafficking and turnover can cause this regulation to be lost such that it leads to persistent activity of the receptor even in the absence of receptor overexpression or activating oncogenic mutations [49]. Based on data on UBQLN1's interaction with ubiquitinated proteins [6, 18], it is most likely that UBQLN1 acts as an adaptor protein aiding in ubiquitination of the phosphorylated receptor. There is no data currently on whether Ubiquilin1 binds E3 ligases. However, UBQLN1 binds ubiquitin through its UBA domain and may control type of ubiquitination in coordination with other proteins. As stabilization of the phosphorylated RTK is a hallmark finding of loss of UBQLN1 in lung cancer cell lines, we hypothesize that normally UBQLN1 facilitates K48 poly-ubiquitination of non-phosphorylated receptor and signals for its degradation. In absence of UBQLN1, the phosphorylated RTK is unable to get degraded leading to its stabilization and continuous stimulation of downstream signaling pathways.

An alternate theory is that UBQLN1 may be required for normal activity of phosphatases that act on RTK's. Timely downregulation of phosphorylated IGF1R and EGFR may be delayed in absence of UBQLN1, leading to stabilized activity of the phosphorylated RTK. The phosphatase protein PTP1B negatively regulates IGF1R phosphorylation in a ligand dependent manner[50]. Another phosphatase SHP2 associates simultaneously with phospho-tyrosines on IGF1R and SH2 domains and directly dephosphorylates IGF1R. An adaptor protein, SHPS2 is critical for recruitment of the phosphatase to the plasma membrane. An integrin protein $\alpha\text{V}\beta\text{3}$ acts via this phosphatase SHP2 to decrease phosphorylation of IGF1R [51]. Ubiquilin1 was originally discovered as an integrin associated protein [2] and through its action on integrins may control SHP2 phosphatases activity.

Overall, UBQLN1 regulates activity of IGF1R and EGFR and in cancers that have loss of UBQLN1, dysregulation of this activity may contribute to initiation and progression of tumorigenesis and it may also contribute to other pathologies associated with RTK aberrations. While RTK's has been shown to be a logical and valid target for many cancers including lung adenocarcinoma, targeting these receptors has not proven to be very successful in the long run. Possible reasons for failure include the complexity of the RTK system, parallel growth and survival pathways and lack of patient selection biomarkers. A variety of mechanisms may confer intrinsic or acquired resistance, highlighting the need for understanding the regulation of IGF1R in greater detail. Regulation of activity of receptors after ligand binding/activation can often be overlooked when

developing therapeutics against targeted receptor. We have observed that UBQLN1 regulates IGF1R activity such that lung adenocarcinoma cell lines that have loss of UBQLN1 have higher activity of the RTK. UBQLN1 loss in tumors can act as a biomarker for patient selection for treating with receptor tyrosine kinase pathway inhibitors. In conclusion, UBQLN1 plays a role in regulating RTK expression and activity and studying its mechanism of action is warranted and promising as a novel therapeutic approach in pathologies involving receptor tyrosine kinases.

REFERENCES

1. Beverly, L.J., et al., *Ubiquitination, localization, and stability of an anti-apoptotic BCL2-like protein, BCL2L10/BCLb, are regulated by Ubiquilin1*. Proc Natl Acad Sci U S A, 2012. **109**(3): p. E119-26.
2. Wu, A.L., et al., *Ubiquitin-related proteins regulate interaction of vimentin intermediate filaments with the plasma membrane*. Mol Cell, 1999. **4**(4): p. 619-25.
3. Massey, L.K., et al., *Overexpression of ubiquilin decreases ubiquitination and degradation of presenilin proteins*. J Alzheimers Dis, 2004. **6**(1): p. 79-92.
4. Shah, P.P., et al., *Ubiquilin1 represses migration and epithelial-to-mesenchymal transition of human non-small cell lung cancer cells*. Oncogene, 2015. **34**(13): p. 1709-17.
5. Buchberger, A., *From UBA to UBX: new words in the ubiquitin vocabulary*. Trends Cell Biol, 2002. **12**(5): p. 216-21.
6. Wilkinson, C.R., et al., *Proteins containing the UBA domain are able to bind to multi-ubiquitin chains*. Nat Cell Biol, 2001. **3**(10): p. 939-43.
7. Zhang, D., S. Raasi, and D. Fushman, *Affinity makes the difference: nonselective interaction of the UBA domain of Ubiquilin-1 with monomeric ubiquitin and polyubiquitin chains*. J Mol Biol, 2008. **377**(1): p. 162-80.
8. Mueller, T.D., M. Kamionka, and J. Feigon, *Specificity of the interaction between ubiquitin-associated domains and ubiquitin*. J Biol Chem, 2004. **279**(12): p. 11926-36.
9. Zhao, L. and S.L. Ackerman, *Endoplasmic reticulum stress in health and disease*. Curr Opin Cell Biol, 2006. **18**(4): p. 444-52.
10. Medicherla, B., et al., *A genomic screen identifies Dsk2p and Rad23p as essential components of ER-associated degradation*. EMBO Rep, 2004. **5**(7): p. 692-7.
11. Heir, R., et al., *The UBL domain of PLIC-1 regulates aggresome formation*. EMBO Rep, 2006. **7**(12): p. 1252-8.
12. Gao, L., et al., *Interaction with a ubiquitin-like protein enhances the ubiquitination and degradation of hepatitis C virus RNA-dependent RNA polymerase*. J Virol, 2003. **77**(7): p. 4149-59.
13. Mah, A.L., et al., *Identification of ubiquilin, a novel presenilin interactor that increases presenilin protein accumulation*. J Cell Biol, 2000. **151**(4): p. 847-62.
14. Hwang, G.W., D. Sasaki, and A. Naganuma, *Overexpression of Rad23 confers resistance to methylmercury in saccharomyces cerevisiae via*

- inhibition of the degradation of ubiquitinated proteins.* Mol Pharmacol, 2005. **68**(4): p. 1074-8.
15. Feng, P., et al., *Kaposi's sarcoma-associated herpesvirus K7 protein targets a ubiquitin-like/ubiquitin-associated domain-containing protein to promote protein degradation.* Mol Cell Biol, 2004. **24**(9): p. 3938-48.
 16. Ko, H.S., T. Uehara, and Y. Nomura, *Role of ubiquilin associated with protein-disulfide isomerase in the endoplasmic reticulum in stress-induced apoptotic cell death.* J Biol Chem, 2002. **277**(38): p. 35386-92.
 17. N'Diaye, E.N., et al., *PLIC proteins or ubiquilins regulate autophagy-dependent cell survival during nutrient starvation.* EMBO Rep, 2009. **10**(2): p. 173-9.
 18. Rothenberg, C., et al., *Ubiquilin functions in autophagy and is degraded by chaperone-mediated autophagy.* Hum Mol Genet, 2010. **19**(16): p. 3219-32.
 19. Wu, S., et al., *Characterization of ubiquilin 1, an mTOR-interacting protein.* Biochim Biophys Acta, 2002. **1542**(1-3): p. 41-56.
 20. Ficklin, M.B., S. Zhao, and G. Feng, *Ubiquilin-1 regulates nicotine-induced up-regulation of neuronal nicotinic acetylcholine receptors.* J Biol Chem, 2005. **280**(40): p. 34088-95.
 21. Tanzi, R.E. and L. Bertram, *Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective.* Cell, 2005. **120**(4): p. 545-55.
 22. Zhang, T. and Y. Jia, *Meta-analysis of Ubiquilin1 gene polymorphism and Alzheimer's disease risk.* Med Sci Monit, 2014. **20**: p. 2250-5.
 23. Bertram, L., et al., *Family-based association between Alzheimer's disease and variants in UBQLN1.* N Engl J Med, 2005. **352**(9): p. 884-94.
 24. Zhang, C. and A.J. Saunders, *An emerging role for Ubiquilin 1 in regulating protein quality control system and in disease pathogenesis.* Discov Med, 2009. **8**(40): p. 18-22.
 25. Ford, D.L. and M.J. Monteiro, *Studies of the role of ubiquitination in the interaction of ubiquilin with the loop and carboxyl terminal regions of presenilin-2.* Biochemistry, 2007. **46**(30): p. 8827-37.
 26. Riley, B.E. and H.T. Orr, *Polyglutamine neurodegenerative diseases and regulation of transcription: assembling the puzzle.* Genes Dev, 2006. **20**(16): p. 2183-92.
 27. Wang, H. and M.J. Monteiro, *Ubiquilin interacts and enhances the degradation of expanded-polyglutamine proteins.* Biochem Biophys Res Commun, 2007. **360**(2): p. 423-7.
 28. Deng, H.X., et al., *Mutations in UBQLN2 cause dominant X-linked juvenile and adult-onset ALS and ALS/dementia.* Nature, 2011. **477**(7363): p. 211-5.
 29. Tsukamoto, S., et al., *Ubiquilin 2 enhances osteosarcoma progression through resistance to hypoxic stress.* Oncol Rep, 2015. **33**(4): p. 1799-806.
 30. Girnita, L., et al., *Something old, something new and something borrowed: emerging paradigm of insulin-like growth factor type 1 receptor (IGF-1R) signaling regulation.* Cell Mol Life Sci, 2014. **71**(13): p. 2403-27.

31. Blume-Jensen, P. and T. Hunter, *Oncogenic kinase signalling*. Nature, 2001. **411**(6835): p. 355-65.
32. Ohlsson, C., et al., *The role of liver-derived insulin-like growth factor-I*. Endocr Rev, 2009. **30**(5): p. 494-535.
33. Yarden, Y., *The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities*. Eur J Cancer, 2001. **37 Suppl 4**: p. S3-8.
34. Zhao, S., et al., *Insulin-like growth factor receptor 1 (IGF1R) expression and survival in non-small cell lung cancer patients: a meta-analysis*. Int J Clin Exp Pathol, 2014. **7**(10): p. 6694-704.
35. Massoner, P., et al., *Insulin-like growth factors and insulin control a multifunctional signalling network of significant importance in cancer*. Br J Cancer, 2010. **103**(10): p. 1479-84.
36. Girnita, L., et al., *Something old, something new and something borrowed: emerging paradigm of insulin-like growth factor type 1 receptor (IGF-1R) signaling regulation*. Cell Mol Life Sci, 2014. **71**(13): p. 2403-27.
37. Goh, L.K. and A. Sorkin, *Endocytosis of receptor tyrosine kinases*. Cold Spring Harb Perspect Biol, 2013. **5**(5): p. a017459.
38. Girnita, L., A. Girnita, and O. Larsson, *Mdm2-dependent ubiquitination and degradation of the insulin-like growth factor 1 receptor*. Proc Natl Acad Sci U S A, 2003. **100**(14): p. 8247-52.
39. Vecchione, A., et al., *The Grb10/Nedd4 complex regulates ligand-induced ubiquitination and stability of the insulin-like growth factor I receptor*. Mol Cell Biol, 2003. **23**(9): p. 3363-72.
40. Sehat, B., et al., *Identification of c-Cbl as a new ligase for insulin-like growth factor-I receptor with distinct roles from Mdm2 in receptor ubiquitination and endocytosis*. Cancer Res, 2008. **68**(14): p. 5669-77.
41. Girnita, L., et al., *{beta}-Arrestin is crucial for ubiquitination and down-regulation of the insulin-like growth factor-1 receptor by acting as adaptor for the MDM2 E3 ligase*. J Biol Chem, 2005. **280**(26): p. 24412-9.
42. Huang, F., et al., *Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain*. Mol Cell, 2006. **21**(6): p. 737-48.
43. Adhikari, A. and Z.J. Chen, *Diversity of polyubiquitin chains*. Dev Cell, 2009. **16**(4): p. 485-6.
44. Varadan, R., et al., *Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling*. J Biol Chem, 2004. **279**(8): p. 7055-63.
45. Mao, Y., et al., *Polyubiquitination of insulin-like growth factor I receptor (IGF-IR) activation loop promotes antibody-induced receptor internalization and down-regulation*. J Biol Chem, 2011. **286**(48): p. 41852-61.
46. Haugsten, E.M., et al., *Ubiquitination of fibroblast growth factor receptor 1 is required for its intracellular sorting but not for its endocytosis*. Mol Biol Cell, 2008. **19**(8): p. 3390-403.

47. Saliba, R.S., M. Pangalos, and S.J. Moss, *The ubiquitin-like protein Plic-1 enhances the membrane insertion of GABAA receptors by increasing their stability within the endoplasmic reticulum*. J Biol Chem, 2008. **283**(27): p. 18538-44.
48. Lim, P.J., et al., *Ubiquilin and p97/VCP bind erasin, forming a complex involved in ERAD*. J Cell Biol, 2009. **187**(2): p. 201-17.
49. Peschard, P., et al., *Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein*. Mol Cell, 2001. **8**(5): p. 995-1004.
50. Buckley, D.A., et al., *Regulation of insulin-like growth factor type I (IGF-I) receptor kinase activity by protein tyrosine phosphatase 1B (PTP-1B) and enhanced IGF-I-mediated suppression of apoptosis and motility in PTP-1B-deficient fibroblasts*. Mol Cell Biol, 2002. **22**(7): p. 1998-2010.
51. Rocchi, S., et al., *Interaction of SH2-containing protein tyrosine phosphatase 2 with the insulin receptor and the insulin-like growth factor-I receptor: studies of the domains involved using the yeast two-hybrid system*. Endocrinology, 1996. **137**(11): p. 4944-52.

CURRICULUM VITAE

CONTACT INFORMATION

Zimple Kurlawala

Office Address:

Department of Pharmacology & Toxicology
University of Louisville School of Medicine
570 S. Preston Street
Louisville, KY 40202
Ph: 502-852-0664

GRADUATE EDUCATION

2013- **PhD Pharmacology & Toxicology**, University of Louisville, KY (08/2013-)
Current GPA: **3.72**

2011 **M.P.H.**, Western Kentucky University, Bowling Green, KY (01/2009 -
08/2011) GPA: **3.81**

MEDICAL EDUCATION

2008 **M.D.**, University of Seychelles - American Institute of Medicine (USAIM),
Victoria, Seychelles (08/2004 - 11/2008)

Honors: Internal Medicine, Family Medicine, Radiology, Ophthalmology,
Medical Terminology

President of the Student body at USAIM (2004 - 2005)

PRE-MED CURRICULUM

2004 Mithibai College, University of Mumbai, Mumbai, India (06/2002 - 04/2004)

U.S. CLINICAL EXPERIENCE

2012 Insured hands-on clinical experience supervised and evaluated by teaching attending physicians in a sub-internship-like atmosphere.

Psychiatry (4 weeks), PACT Atlanta, Decatur, GA (11/12)

Internal Medicine (4 weeks), Emory University Hospital Midtown, Atlanta, GA (10/12 - 10/12)

Neurology (4 weeks), Piedmont Hospital, Atlanta, GA (09/12 - 09/12)

Neurology (4 weeks), Piedmont Hospital, Fayetteville, GA (07- 08/12)

Family Medicine (4 weeks), Cascade Primary Care, Atlanta, GA (07/12)

- Individual Performance Evaluations available

Geriatric Medicine (6 weeks), Observership, Life Care Centers of America, Palm Bay, FL (05-06/2012)

U.S. EMPLOYMENT

2013 **Graduate Student**, Dept. of Pharmacology/Toxicology, University of Louisville, KY (08/2013--)

2012 **Part-time Faculty**, Department of Public Health, Western Kentucky University, Bowling Green, KY (06/2009 - 05/2012)

- Taught American Heart Association curriculum, 'Heartsaver First Aid and CPR'. Provided opportunity for students to obtain certification as CPR and first provider.

2009 **Master Control Operator**

WKYU-PBS, Western Kentucky University, KY (06 – 08/2009)

EXAMINATIONS

2012 **USMLE** Step 1: 209/79, Step 2: 207/77, Step 2 CS: Pass, all at first attempt.

2008 **GRE**: 1320 (Q: 740, V: 540)

2008 **TOEFL iBT**: 105

RESEARCH EXPERIENCE

- 2013 **Regulation of anti-apoptotic proteins in cancer progression and treatment**, University of Louisville, Kentucky
- Dr. Levi Beverly
- 2013 **Marcus Stroke and Neuroscience Center**, Grady Hospital
- Dr. Raul Nogueira
- 2011 **Annotations of genomes of bacteriophages: Wizard007 and BarrelRoll**, WKU
- Co-investigator
 - Dr. Claire Rinehart
- 2010 **Examining college students' perceptions about organ donation**, WKU
- Principal Investigator
 - Dr. Christine Nagy, Dr. Gary English, Dr. Darlene Shearer
- 2010 **Focus Groups for 'Eat more fruits and veggies' among college students**, WKU, BRDHD
- Co-investigator
 - Dr. Christine Nagy
- 2009 **Effects of radon gas emission in homes**, WKU
- Research Paper
 - Dr. Ritchie Taylor
- 2009 **Obesity Prevention**, WKU
- Research Paper
 - Dr. Steve Nagy
- 2009 **Evaluation of long term effects of LASIK surgeries**, WKU
- Research Proposal
 - Dr. Thomas Nicholson
- 2009 **Teenage Pregnancy Prevention**, WKU
- Co-investigator
 - Dr. Steve Nagy
- 2009 **A personalized programming approach towards sexual violence risk reduction**, WKU
- Co-Investigator
 - Dr. Steve Nagy
- 2008 **An analysis of urinary calculi in a suburb of Mumbai, India**, M.D. Thesis
- Dr. Anirudh Badade
- 2005 **Pharmacokinetics and Pharmacodynamics of Alcohol**, USAIM
- Dr. Madhav Mutalik

PUBLICATIONS & PRESENTATIONS

- PP Shah, WW Lockwood, K Saurabh, **Z Kurlawala**, SP Shannon, S Waigel, W Zacharia, and LJ Beverly. *Ubiquilin1 represses migration and epithelial-to-mesenchymal transition of human non-small cell lung cancer cells*. *Oncogene*.
- SF, Jones GM, Jones ML, **Kurlawala ZD**, Leet TA, Lovell L.J, Phillips KE, Shoup LE. *Complete Genome Sequences of 138 Mycobacteriophages*. *Journal of Virology*. 2012 Feb; 86(4): 2382-2384. Cited in PubMed; PMID: 22282335.

Poster Presentation

- Kurlawala, Z., Malik, D., Beverly, L., (2014) *Regulation of IGF1R by Ubiquilin1*, Mechanisms and Models of Cancer, Cold Spring Harbor Laboratories, NY.
- Kurlawala, Z., Malik, D., Beverly, L., (2014) *Regulation of IGF1R by Ubiquilin1*, Midwest Membrane Trafficking and Signaling Symposium, American Society for Cell Biology, Louisville, KY.
- Kurlawala, Z., Malik, D., Beverly, L., (2014) *Regulation of IGF1R by Ubiquilin1*, Research!Louisville, Louisville, KY.
- Kurlawala, Z., Nagy, C. (2011). *Examining college students' perceptions about organ donation*. Poster presented at: American Public Health Association; Washington, DC.

Oral Presentation

- **Kurlawala, Z.**, English, G. (2011). *Examining college students' perceptions about organ donation*. Oral Presentation presented at: WKU Research Conference; Bowling Green, KY.
- **Kurlawala, Z.**, Nagy C., English, G. (2011). *Examining college students' perceptions about organ donation*. Oral Presentation presented at: Kentucky Public Health Association; Louisville, KY.
- **Kurlawala, Z.**, Nagy, S. (2010). *A personalized programming approach towards sexual violence risk reduction*. Oral Presentation presented at: WKU Research Conference; Bowling Green, KY.
- **Kurlawala, Z.**, Nagy, S. (2010). *A personalized programming approach towards sexual violence risk reduction*. Oral Presentation presented at: Kentucky Public Health Association; Louisville, KY.
- **Kurlawala, Z.**, Nagy, S. (2010). *A personalized programming approach towards sexual violence risk reduction*. Oral Presentation presented at: American Public Health Association; Denver, CO.

CONFERENCES, WORKSHOPS AND SYMPOSIA

- 2014 Mechanisms and Models of Cancer, Cold Spring Harbor Laboratories, New York
Research!Louisville, University of Louisville, Kentucky
- 2013 Research!Louisville, University of Louisville, Kentucky
- 2011 American Public Health Association (APHA) Annual Conference, Washington, D.C. (10/2011)
Kentucky Public Health Association (KPHA) Annual Conference, Louisville, KY (03/2011)
Western Kentucky University Annual Research Conference, Bowling Green, KY (02/2011)
- 2010 APHA Annual Conference, Denver, CO (11/2010)
KPHA Annual Conference, Louisville, KY (03/2010)
WKU Annual Research Conference, Bowling Green, KY (02/2010)

PROFESSIONAL MEMBERSHIPS

- 2012 **American Heart Association**, Member and Instructor (since 2009)
AmeriClerkships Medical Society, Irvine, CA

CERTIFICATIONS

- 2012 **ECFMG** certified MD (10/2012)
- 2012 **Advanced Cardiac Life Support**, American Heart Association (06/2012)
- 2012 **Basic HIPAA Online Training Certificate**, Advanced Colleges of America (ACA), Irvine, CA (06/2012)
- 2012 **U.S. Pre-Clinical Healthcare Acculturation Certificate**, ACA, Irvine, CA (06/2012)
- 2012 **Basic Life Support**, American Heart Association (05/2012)
- 2012 **Blood Borne Pathogens Online Course** (General Portion), American Heart Association (05/2012)
- 2012 **Heartsaver First Aid & CPR**, American Heart Association (05/2012)
- 2009 **Basic Skills for College Teaching**, FaCET at Western Kentucky University (08/2009)
- 2009 **Forensic Epidemiology-Joint Training for Law Enforcement and Public Health Officials** Homeland Security Program (Extended Learning - West Virginia University) (10/2009)

COMMUNITY INVOLVEMENT

- 2012 **Volunteer**, Sickie Cell Foundation of Georgia
- 2012 **Public Health Intern**, Barren County Public Health Department, KY (01/2011 - 08/2011)
- Smoking coalition; prepared presentations on cardiovascular diseases and obesity which are now being used as health education materials in 8 counties in Kentucky; participated in school health fair for elementary school children, participated in a health education booth about CDC recommended food safety guidelines at Kroger grocery store in Bowling Green, KY
- 2009 **Instructor**, Juvenile Detention Center, Bowling Green, KY

AWARDS & ACCOMPLISHMENTS

- 2015 Graduate Student Council Research Fund Award
- 2014 Winner - **First Place**, Research!Louisville, Masters Level-Basic Science Research, Louisville, KY
- 2014 IMD3 Travel Award
- 2012 **Presenter**, "Lunch and Learn" presentation on congestive heart failure for the nursing staff at Life Care Centers of America, Palm Bay, FL (06/2012)
- 2012 **Athlete of the Week**, Western Kentucky University
- 2010 Recipient of the **American Humanics Emerging Non-Profit Award**, 2010, as a participant in the Future Selves Program, Western Kentucky University (2007-2010)
- 2010 **Best Student Chapter Award**, served as Vice President of KPHA, Western Kentucky University
- 2010 **Guest Lecturer**, First Aid for Injury Emergencies, Bowling Green Housing Authorities
- 2006 **Honors**: Internal Medicine, Family Medicine, Radiology, Ophthalmology, Medical Terminology
- 2005 **President of Student Body**, USAIM

HOBBIES, INTERESTS, AND OTHER ACCOMPLISHMENTS

- **Women Doubles Badminton Champion** - WKU Intramurals (Spring 2011 & 12)
- **PADI certified Open Water SCUBA Diver**
- Puzzletier (crosswords, Sudoku, brain teasers, etc.)
- Creative Arts (Calligraphy, Kirigami, Ceramic Painting, etc.)
- Teaching - a skill and interest