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Molecular Mechanisms of Insulin Receptor Homeostasis

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By

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Abstract of the Dissertation

Molecular Mechanisms of Insulin Receptor Homeostasis

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Receptor tyrosine kinases (RTKs) play a fundamental role in cell growth, metabolism and survival. Altered cell surface expression of RTKs has profound effects on organismal physiology and is associated with both metabolic disease and neoplasia. The insulin receptor (IR) is a prototypical RTK that regulates metabolism and lifespan and as such, insights from studies on IR homeostasis may be extended to other members of the RTK family.

I have discovered that eliminating AKT, a critical kinase downstream of the IR, increased IR expression and decreased its degradation. Interestingly, expression of other RTKs such as EGFR and IGF-1R was also upregulated when PI3K/AKT/mTOR signaling was inhibited, suggesting common regulatory mechanisms to control RTK expression and degradation. In addition, inhibiting PI3K signaling attenuated degradation of the insulin receptor induced by metabolic inhibitors. These data suggest that negative feedback mechanisms exist for the highly conserved PI3K/AKT/mTOR pathway at both transcriptional and post-translational levels to regulate RTK stability, in coordination with nutrient status, in mammalian systems.

To further investigate regulation of RTK expression at the earliest steps of its biogenesis, I have also examined how molecular chaperones that are partitioned in the ER luminal surface and cytosol collaborate to ensure protein maturation under the strict quality control system in the ER. I found that the ER chaperone calreticulin (CRT) and the cytosolic chaperone Hsp90 stabilized receptor expression through distinct pathways. In addition, live cell imaging using novel green fluorescent protein (GFP) chimeras of the IR revealed that movement of the receptor through the ER was accelerated by misfolding or by overexpression of either CRT or Hsp90.

Our results indicate that both CRT and Hsp90 control IR expression at its earliest maturation stages and modulate its movement within the ER. Together, these studies elucidate the distinct molecular mechanisms involved in the dynamic regulation of IR homeostasis and suggest reciprocal interactions between insulin signaling, glucose metabolism and IR expression.

Acknowledgments

The road to my PhD was a long, sinuous path and I would not have made it without the encouragement of a lot of people both here in Chicago and support from friends and family halfway around the world.

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Of course, I would also like to thank my advisor, Joe, without whose encouragement and support, I would not have been able to grow as a scientist and as a scientific communicator.

I feel very lucky to have also been surrounded by wonderful people outside of the lab whose friendship and support made a big difference in helping me enjoy grad school life to the fullest. My old friends from back home who are now based in Chicago, along with new friends I've made here in Evanston, were my constant buddies when it came to exploring restaurants, museums, plays and everything else that Chicago had to offer.

I would also like to thank my adopted families here, whose support and companionship really helped me feel at home in a strange new city. I feel very blessed to have sat beside Tita Lucy Rita† that one Suday morning at Sheil Center which soon paved the way for her welcoming me into her home and into her family despite my being a complete stranger to her then. And of couse, I would not be here now if not for the boundless supply of love, support and encouragement from the Credos. They continue to amaze me with their extreme generosity and sincere love and concern for those around them. I only hope that I can be half as kind to others as they have been to me and my family.

My family back home in the Philippines – Mom, Dad, my sister Ria and my brother Archie – constantly provided encouragement through e-mail, phone calls and yummy Pinoy goodies. I thank them for their prayers and for rallying support for me from other friends and family, too!

Last but not least, I would like to thank my husband, Kenneth, for his love, understanding and commitment, despite having an ocean between us for most of the last 10 years that we've been together. His constant faith and support helped me see it through to the very end.

Dedication

To my parents and to my husband, Kenneth,
who always supported all my endeavors and kept their unwavering faith in me.

List of abbreviations

BFA	brefeldin A
BiP	binding protein
CHX	cycloheximide
CNX	calnexin
CRT	calreticulin
EGFR	epidermal growth factor receptor
eIF4e	elongation initiation factor 4e
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ErbB2	erythroblastic leukemia viral oncogene homolog 2
ERQC	endoplasmic reticulum quality control
FGF	fibroblast growth factor
FnIII	fibronectin type III domain
FOXO	forkhead box containing protein, O subfamily
FRAP	fluorescence recovery after photobleaching
FLIP	fluorescence loss in photobleaching
GFP	green fluorescent protein
Grb2	growth factor receptor-bound protein 2
Hsp90	heat shock protein of 90 kDa
IGF1-R	insulin-like growth factor-1 receptor
INS	insulin

IR	insulin receptor
IRES	internal ribosome entry site
IRS	Insulin receptor substrate
MAPK	mitogen activated protein kinase
mTOR	mammalian target of rapamycin
PDGF	platelet derived growth factor
PDK	3-phosphoinositide dependent protein kinase-1
PH	pleckstrin homology domain
PI3K	phosphatidyl inositol 3-kinase
PIP	phosphoinositide 3-phosphate
PTEN	*p*hosphatase and *ten*sin homolog deleted on chromosome *ten*
PTP1B	protein tyrosine phosphatase 1B
RTK	receptor tyrosine kinase
SCR	scrambled siRNA sequence
SH2	Src homology 2 domain
TGFβ	transforming growth factor β
UTR	untranslated region
VEGF	vascular endothelial growth factor

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Chapter 1. General Introduction

Multicellular organisms have developed complex mechanisms to facilitate their growth and development in response to extracellular stimuli. A crucial strategy involves binding of secreted ligands to their cognate receptors on the surface of target cells to facilitate intercellular communication. Excessive signaling activity initiated by these cell surface receptors results in unrestricted cell growth and often leads to tumor formation. Conversely, reduced signaling from these receptors leads to insufficient growth and development of metabolic disorders.

One of the most important classes of surface proteins that mediate this intercellular communication is the receptor tyrosine kinase (RTK) superfamily. RTKs represent a class of integral membrane proteins that are involved in numerous signaling pathways within the cell, including cell proliferation, differentiation, migration and metabolism. Aberrant upregulation or downregulation of these RTKs has been associated with neoplasia or metabolic disorders, respectively, highlighting the need to understand the complex mechanisms that control RTK expression and degradation.

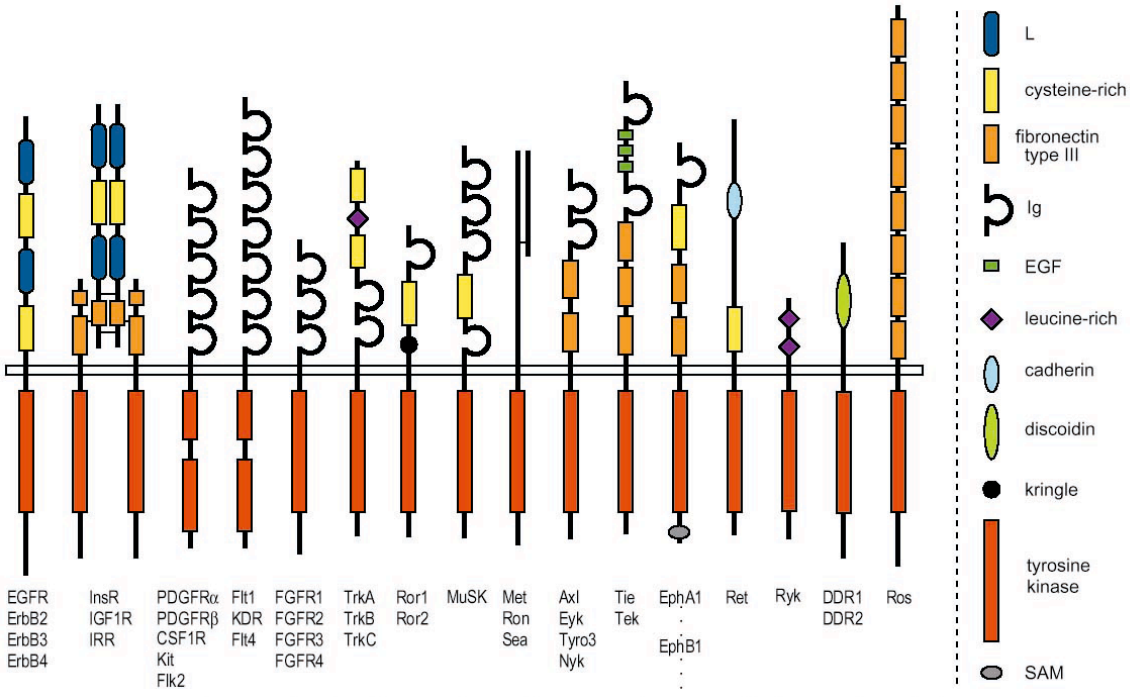
In order to investigate how RTK homeostasis is regulated, a major goal of my thesis research has been to investigate the role of PI3K/AKT/mTOR signaling, a pathway common to various RTKs, in receptor expression and degradation. This signaling cassette is the primary pathway involved in the metabolic functions of the insulin receptor (IR), a prototypical RTK, and as such, studies of IR turnover may have more general implications in understanding the regulation of other RTK family members, including the epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGF1-R). Given the important function of the IR in glucose metabolism, I have also explored the role of nutrient sensing in RTK homeostasis to determine whether coordinated regulatory mechanisms exist between receptor signaling and

nutrient availability. To further dissect the molecular components involved in RTK expression, I examined the earliest steps of receptor biogenesis to investigate how interactions between transmembrane RTKs and chaperones found on either sides of the ER membrane regulate receptor stability and maturation. Insights from these studies will provide a more comprehensive understanding of the steps involved in RTK maturation and turnover and help enable the design of better strategies to target neoplasia and metabolic disease.

1.1. Receptor tyrosine kinase (RTK) superfamily

The important role RTKs play in development is evidenced by the diversity of RTK ligands which include growth factors such as the epidermal growth factor (EGF), the fibroblast growth factor (FGF) and the platelet-derived growth factor (PDGF), among others (Figure 1.1). Given the centrality of these factors to growth and development, aberrant regulation of RTK expression and activity is associated with a broad variety of diseases including human tumor syndromes (Blume-Jensen and Hunter, 2001). RTKs are also involved in other metabolic disorders such as insulin resistance and type 2 diabetes.

Several mechanisms have been identified that increase the catalytic activity of RTKs, including chromosomal translocation, receptor amplification and point mutations (Blume-Jensen and Hunter, 2001; Lamorte and Park, 2001). Negative feedback control mechanisms that balance RTK expression and function with cellular growth and metabolic requirements are important to ensure signaling thresholds compatible with the induction of a physiological response. Since overactivation of RTK signaling has been implicated in the onset and progression of cancer, it is essential to understand how RTKs are downregulated and deactivated. Moreover, insufficient RTK expression and function leads to metabolic disorders and obesity,



(Hubbard and Till, 2000)

Figure 1.1. The receptor tyrosine kinase (RTK) superfamily. All RTKs contain an intrinsic tyrosine kinase domain that is activated upon binding their cognate growth factor ligand. RTK extracellular domains vary widely among the different receptors and determine the specificity of ligand binding.

thus I have been equally interested in understanding mechanisms that may lead to aberrant downregulation of RTKs.

1.2. Structural organization of RTK proteins

Members of the RTK family contain three major domains: an extracellular ligand binding domain, a transmembrane helical domain and an intracellular tyrosine kinase domain. The extracellular domains of RTKs consist of a diverse range of discrete globular domains such as immunoglobulin (Ig)-like domains, fibronectin type III-like domains, cysteine-rich domains, and EGF-like domains and form the basis for further subdivision of this superfamily into sub-classes. For example, class I RTKs such as the EGF receptor and the highly related ErbB2 or HER2 proteins, have cysteine-rich sequences. On the other hand, Class VII RTKs like the neurotrophin receptors, contain no or few cysteine-rich domains. A partial list of important RTK sub-classes is shown in Table 1.1.

In contrast, the cytoplasmic portion of RTKs has a much simpler organization, consisting of a juxtamembrane region, followed by the tyrosine kinase catalytic domain and a carboxy-terminal region. The intracellular domain may also contain additional regulatory sequences that are phosphorylated by other protein kinases (Hubbard et al., 1998). Some receptors, such as members of the PDGF receptor family, also contain a large ~100 residue insert in the tyrosine kinase domain, which is solely thought to play a role in the mitogenic response to PDGF (Escobedo and Williams, 1988).

Several growth factors exist as homodimers, such as VEGF, PDGF, and thus it is no surprise that most RTKs are expressed as as a single polypeptide on the cell surface and only

Table 1.1. Sub-classification of Receptor Tyrosine Kinases

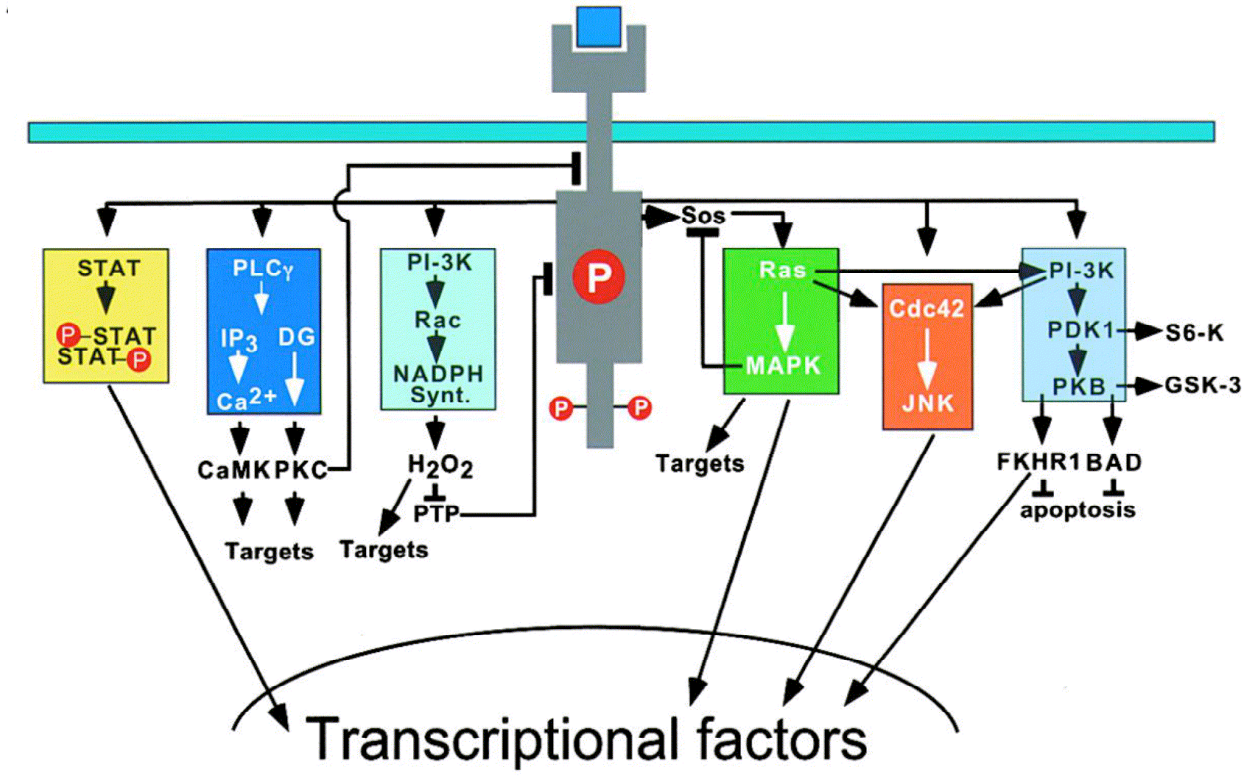
Class	Common structural features	Examples
I	Cysteine-rich sequences	EGF receptor, NEU/HER2, HER3
II	Cysteine-rich sequences; disulfide-linked heterotetramers	Insulin receptor, IGF-1 receptor
III	Contain 5 immunoglobulin-like domains, kinase insert	PDGF receptor, c-Kit
IV	Contain 3 immunoglobulin-like domains, kinase insert, acidic domain	FGF receptor
V	Contain 7 immunoglobulin-like domains, kinase insert	VEGF receptor
VI	Heterodimeric (like class II) except one of the two protein subunits is completely extracellular	Hepatocyte growth factor (HGF) receptor
VII	Contain none or few cysteine-rich domains	Neurotrophin receptor family and NGF receptor

dimerize upon ligand binding. However, one exception is the class II RTKs, the insulin receptor (IR) and the highly homologous insulin-like growth factor 1 receptor (IGF1-R), which are both expressed on the cell surface as a pre-existing dimer of two α chains disulfide-linked to two β chains, forming an $\alpha_2\beta_2$ heterotetramer. Another notable exception is the hepatocyte growth factor receptor, which is comprised of two polypeptides, a short α chain that is disulfide-linked to a membrane-spanning β chain.

1.3. Mechanisms of activation of receptor tyrosine kinases

The RTK superfamily is involved in a wide range of intracellular signaling pathways to control cell growth, survival and differentation (Figure 1.2). Examples of these pathways include phosphoinsoitol metabolism mediated by PLCγ and JAK/STAT signaling (reviewed in (Schlessinger, 2000)). However, the pathways mediated by Ras-Erk/MAP kinase and phosphatidylinositide-3 kinase (PI3K)-AKT represent the two critical signaling cascades induced upon the activation of RTKs by growth factors.

Upon binding its cognate ligand, the intrinsic intracellular tyrosine kinase domain of an RTK is activated and transduces the extracellular signal to the cytoplasm through both autophosphorylation and phosphorylation of downstream components of the signaling cascade. While the extracellular domains of each RTK are diverse and confer specificity for the cognate ligand, the intracellular kinase domains are highly conserved and are homologous to the ATP-binding regions of cAMP-dependent protein kinases (PKA) (Hubbard and Till, 2000; Schlessinger, 2000). The catalytic kinase domain enables transfer of the γ phosphate of ATP to hydroxyl groups of tyrosines on target proteins. In contrast, non-RTK cell surface receptors that



(Schlessinger, 2000)

Figure 1.2. Signaling Pathways Activated by RTKs. Different signaling pathways are presented as distinct signaling cassettes (colored boxes). In several cases the signaling cassettes do not include all the known components of a given pathway. Also shown, examples of stimulatory and inhibitory signals for the different pathways. For example, in addition to activation of the MAP kinase signaling cascade, Ras activates PI-3 kinase and Cdc42. Stimulation of PI-3 kinase leads to activation of PDK1 and PKB, two kinases that regulate various metabolic processes and prevent apoptotic death. In addition, PI-3 kinase activation stimulates generation of hydrogen peroxide which in turn oxidizes and blocks the action of an inhibitory protein tyrosine phosphatase (PTP). The signaling cassettes presented in the figure regulate the activity of multiple cytoplasmic targets. However, the Ras/MAP, STAT, JNK, and PI-3 kinase signaling pathways also regulate the activity of transcriptional factors by phosphorylation and by other mechanisms.

also mediate their response by tyrosine phosphorylation usually have a shorter cytoplasmic domain due to the absence of a tyrosine kinase region. Thus, noncovalent interactions with nonreceptor tyrosine kinases such as Jak or Src are needed for signal transduction.

Ligand binding results in a conformational change in the tyrosine kinase domain of the RTK. This releases inherent stearic hindrance and allows binding of ATP and results in the proper positioning of residues involved in substrate binding and catalysis. Aside from relieving the inhibitory conformation to enable full kinase activation, phosphorylation of specific tyrosine residues also creates binding sites for Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain containing proteins (Pawson, 1995). Signaling proteins containing SH2 and PTB domains, such as SHC, IRS-1 and Grb2, are modular in nature and often have no intrinsic enzymatic activity of their own. However, these adaptor proteins link RTK activation to downstream signal transduction pathways by recruiting other enzymes in the signaling pathway and putting them in close proximity to their target substrate. The following example reflects how RTK signaling mediates the cell's response to physiological conditions. When blood vessels are injured, PDGF is often released. Attachment of PDGF to its cognate receptor expressed on the surface of smooth muscle cells in the blood vessel leads to activation of this RTK. Activated PDGFR specifically recruits the adaptor protein, Grb2, which contains an SH2 domain flanked on either side by another linker domain, SH3, which binds to proline residues and recruits an enzyme-containing protein called SOS to the receptor. SOS is now low localized near the membrane and can activate a membrane-associated protein, Ras, which continues the signal transduction cascade. Ultimately, this chain of events stimulates transcription of genes that cause mitogenesis and promote wound healing.

In other cases, linker proteins may contain their own enzymatic domain, aside from possessing an SH2 domain. Examples of such proteins include phospholipase C γ and phosphoinositide 3 kinase (PI3K). Upon binding of the SH2 domain to the phosphotyrosine on the RTK, the enzymatic domain of the linker protein then undergoes a structural change and becomes active and enables phosphorylation of its own target protein, the next step in the signaling chain of events.

Even though RTKs share the same basic tyrosine kinase domain, differences in non-catalytic residues located within this tyrosine kinase domain determines which adaptor molecule is recruited to the receptor. Phosphotyrosine-binding SH2 domains are found in over 100 separate proteins but RTKs have a remarkable ability to recruit its own specific set of SH2-containing proteins. This is because SH2-containing proteins also contain a second domain that recognizes a particular sequence of three or so amino acids adjacent to the phosphotyrosine that is present in only a specific type of RTK. For example, for the above-mentioned Grb2, it will only bind to a phosphotyrosine as long as the adjacent residues follow this binding motif: pY-X-I/V-I/L/V (Rodriguez et al., 2004; Songyang et al., 1993). Thus, all SH2 domains can bind to phosphorylated tyrosines but the difference in binding affinity to adjacent residues found in the tyrosine kinase domain enables great specificity in binding to particular RTKs.

Inactive monomers are thought to be in equilibrium with inactive and active dimers and ligand binding stabilizes the active dimer formation and allows signal activation to proceed. Inactive pre-formed dimers, on the other hand, only become active after ligand binding triggers a conformational change. As the active forms of the insulin receptor and monomeric RTKs are both dimeric, their signaling mechanisms upon activation remain similar. It has been proposed that active dimers exist even in the absence of ligand binding since RTK activity can be

enhanced by inhibitors of protein tyrosine phosphatases or by receptor overexpression (Schlessinger, 2000). In addition, a recent study has discovered that a triad of residues within the kinase hinge regions of most RTKs form a network of hydrogen bonds that keeps the kinase in an autoinhibited conformation (Chen et al., 2007). Pathogenic mutation of these residues lead to increased receptor activity and tumor formation.

1.4. Downregulation of RTK activity

Temporal and spatial regulation of RTK activity is crucial to mediating their intracellular tasks. Signaling cascades initiated by RTKs are tightly regulated and properly balanced in order to promote the proper physiological response. RTKs coordinate a wide variety of biological processes and are thus subject to multiple levels of control to ensure effective regulation.

1.4.1. Inhibition by protein tyrosine phosphatases

Protein tyrosine phosphatases (PTP) play an important role in regulating RTK activity. PTPs such as SHP-1 or PTP1B dephosphorylate the phospho-tyrosine residues in activated RTKs and inhibit their activity. Due to the dynamic equilibrium between active and inactive RTKs, virtually all RTKs can be activated, even in the absence of ligand binding, by treatment with PTP inhibitors. This demonstrates that RTK activity is continuously being kept in check by the activity of these phosphatases. Perturbations to the balance between RTK and phosphatase activity can lead to serious physiological consequences. Overexpression of the phosphatase PTP1B has been observed in *erbB2*-transformed cell lines and overexpression *in vivo* was found to impair insulin sensitivity and contributed to the development of insulin resistance (Zabolotny et al., 2004), suggesting that upregulation of this phosphatase has important ramifications in

tumor progression as well as in metabolic disease. Activity of phosphatases downstream of RTKs also plays an important role in regulating signaling activity. The tumor suppressor PTEN (*p*hosphatase and *ten*sin homolog deleted on chromosome *ten*) is a lipid phosphatase that dephosphorylates PIP3 at the plasma membrane and thereby inhibits PI3K-mediated signals for cell growth and development. Homozygous inactivation of PTEN occurs in a large fraction of glioblastomas (Liu et al., 1997) and a significant percentage (approx 10%) of breast cancer cell lines (Li et al., 1997; Teng et al., 1997). Overall, PTEN is one of the most common targets of mutation in human cancer, with a mutation frequency approaching that of p53.

I.4.2. Receptor internalization

Attenuation of growth factor signaling can also be attained by endocytosis of the RTK and its bound ligand via clathrin-coated pits or through a caveolar pathway. The endocytic vesicles fuse with endosomes which deliver the receptors and their ligands to various intracellular destinations. Ubiquitination has recently been shown to play a role in internalization of activated RTKs. Phosphorylated tyrosines on RTKs may target the receptor to the ubiquitin (Ub) pathway by mediating interactions either with SH2-domain-containing ubiquitin ligases (E3s) or SH2-containing adaptors that recruit E3s (Joazeiro et al., 1999). Cbl is a tyrosine kinase-specific E3 ligase that contains both a catalytic RING finger domain in addition to an SH2 domain. Phosphorylation may result either in the direct recruitment of Cbl to the RTK, or the indirect recruitment of Cbl by Grb2 (Joazeiro et al., 1999). Once localized in proximity to the receptor, or directly bound to pY residues, Cbl becomes phosphorylated and recruits ubiquitin-loaded E2 enzyme that covalently tags the receptor. Ubiquitinated receptor molecules are then recognized by a multi-protein complex that sorts them to an invaginating

clathrin-coated pit. These 'loaded' clathrin-coated regions engulf the activated receptor in a dynamin-dependent manner to form a clathrin-coated vesicle. The shedding of clathrin, accompanied by a reduction in internal pH and the accumulation of hydrolytic enzymes, give rise to the early endosome. The intravesicular pH drops along the endocytic pathway, from 6.0-6.5 in early endosomes to 4.5-5.5 in late endosomes and lysosomes, which causes dissociation of many ligand-receptor complexes. Proteins destined for degradation proceed to the lysosome or the proteasome. Internalized molecules can be either recycled back from endosomes to the plasma membrane and participate in several rounds of endocytosis. However, as the receptor proceeds along the endocytic pathway, the efficiency of recycling declines. This process of Cbl-mediated mono-ubiquitination has been shown to be necessary and sufficient for endocytosis and degradation of EGFR and PDGFR (Haglund et al., 2003; Mosesson et al., 2003).

The caveolar pathway of endocytosis (reviewed in (Mayor and Pagano, 2007; Mukherjee et al., 2006)) is less characterized and involves caveolin-1, which serves as the coat protein, and dynamin-2a, which promotes vesicle budding. Caveolae are 50–80 nm flask-shaped pits that form in the plasma membrane and are enriched in caveolins, sphingolipids and cholesterol. Activated receptors are first released from immobile, actin-tethered caveolar invaginations and internalized via small transport vesicles called cavicles. Adapter proteins such as Grb10 and Eps15, and the ubiquitin ligase Nedd4, are all involved in this internalization process. The cavicles are the principal shuttle that transports cargo along microtubules to and from pericentrosomally located caveosomes that are devoid of classic endosomal markers. Molecules internalized via the caveolar pathway may be targeted to the Golgi, the ER or the nucleus for signaling or the caveosomes may fuse with the classic endocytic pathway in a Rab5-dependent manner for lysosomal or proteasomal degradation.

1.4.3. Inhibitory serine/threonine phosphorylation

Negative feedback is one of the mechanisms that provide an effective control of RTK signaling. However, the process of ligand-induced down-regulation occurs over several hours. For a more immediate time frame, phosphorylation of the receptor on serine and threonine residues is thought to be one of the primary mechanisms for attenuation of its kinase activity. Activation of protein kinase C (PKC)-dependent signaling pathways by phorbol ester leads to inhibition of RTK activity due to site-specific phosphorylation of threonine 654 in the juxtamembrane region (Countaway et al., 1990). Similarly, mutation of serine phosphorylation sites in EGFR resulted in its increased oncogenic potential (Theroux et al., 1992), highlighting the importance of these residues in downregulating receptor activity. Thus, serine phosphorylation is required for EGF receptor desensitization in EGF-treated cells. This regulatory phosphorylation site is located at the carboxyl terminus of the EGF receptor within the subdomain that binds SH2 regions of signaling molecules (Countaway et al., 1992).

Another example of regulatory serine phosphorylation for RTKs is the observed increase in serine phosphorylation of the insulin receptor during chronic insulin treatment (Qiao et al., 1999). Similarly, excessive serine phosphorylation of the insulin receptor has been hypothesized to be one of the causes of insulin resistance observed in patients suffering from polycystic ovary syndrome (PCOS) (Dunaif et al., 1995).

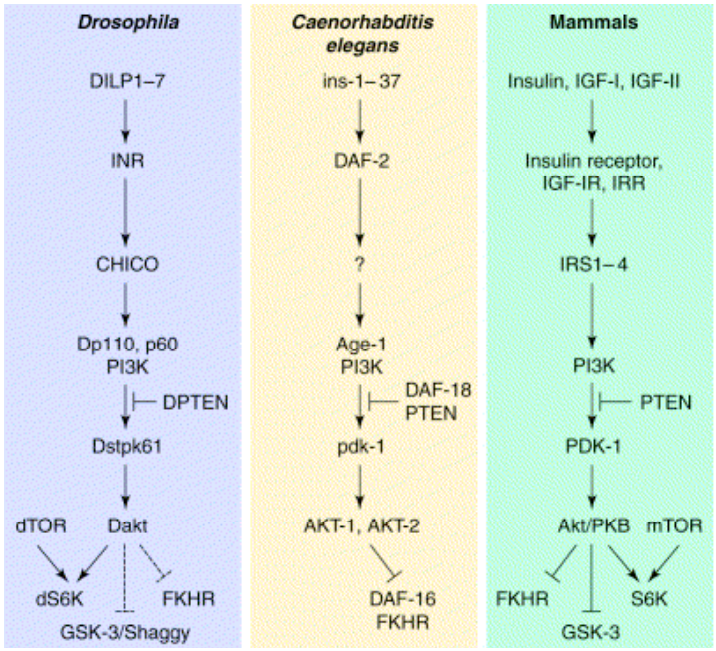
Inhibitory serine phosphorylation may also occur on proteins downstream of the RTKs. Previous reports have shown that mTOR activation of p70S6K mediates phosphorylation of IRS-1 at the inhibitory serine sites (S312 and/or S636/639) which leads to proteasomal IRS-1 degradation (Easton et al., 2006). Glycogen synthase kinase 3 (GSK-3) has also been shown to

phosphorylate IRS-1 on serine residues, facilitating downregulation of the adaptor protein (Eldar-Finkelman and Krebs, 1997).

1.5. Receptor tyrosine kinases in other metazoans

Genome sequencing, in addition to more specific investigations, points to the early appearance of RTK in metazoans and intense diversification within some RTK subfamilies. The first RTK outside of metazoans was found in a unicellular choanoflagellate, *Monosiga brevicollis*, called MBRTK1. It includes multiple extracellular ligand-binding domains which resemble those of RTKs in sponges and humans and suggests the ability to receive and transduce signals. The first RTK likely arose from fusion of an epidermal growth factor (EGF)–like domain and a cytoplasmic tyrosine kinase before the appearance of animals (King and Carroll, 2001). In the freshwater sponge *Ephydatia fluviatilis*, nine putative RTK genes were identified following reverse transcription–polymerase chain reaction amplification, of which four are related to the RTK genes found in the fruitfly *Drosophila melanogaster* and vertebrates: the Musk, ephrin (Eph), Ros, and EGF receptors (Suga et al., 2001).

Other RTK genes have been found in early metazoans, including the fruitfly *D. melanogaster* and the nematode, *Caenorhabditis elegans*. Genetic screens in these organisms have revealed that components of the signaling pathway of the insulin receptor, in particular, have been highly conserved through evolution (Figure 1.3) (Garofalo, 2002). Screens for *d*auer *a*rrest *f*ormation in *C. elegans* have lead to the identification of orthologs of the insulin receptor (*daf-2*) and post-receptor signaling pathway components, including the catalytic subunit of PI-3-kinase (*age-1*), serine-threonine kinases Akt/PKB (*akt-1* and *akt-2*) and a forkhead transcription factor (*daf-16*).



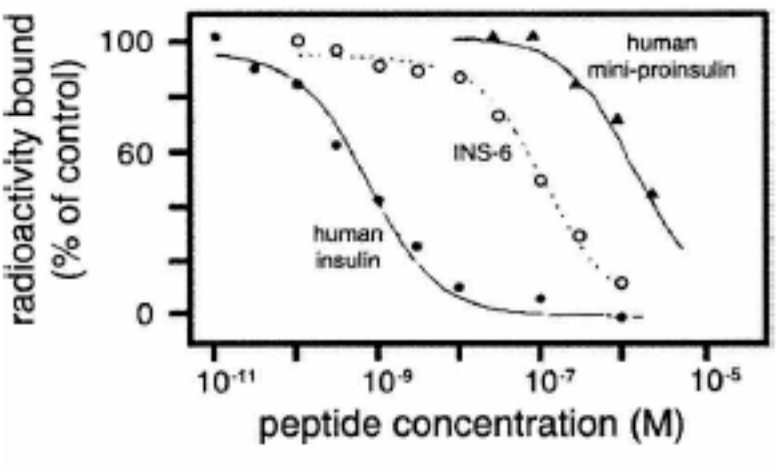
(Garofalo, 2002)

Figure 1.3. Evolutionary conservation of insulin signaling pathways across metazoans. Mammalian insulin-like peptides are present in the fruitfly *Drosophila* and the nematode *C. elegans*, and activate homologous receptors to facilitate insulin signaling in the organism to promote glucose metabolism and cell growth and development.

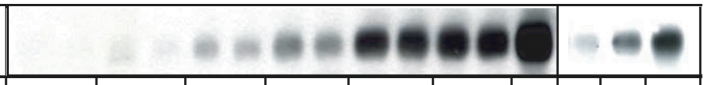
Elucidation of the complete structure of the insulin receptor from the *daf-2* gene in *Drosophila* (Fernandez et al., 1995) revealed that it had a similar structural organization as its mammalian counterpart. Although genetic screens for diapause or long-lived phenotypes did not reveal DAF-2 ligands, search of the *C. elegans* genome by sequence- and structure-based algorithms has uncovered a family of 37 putative insulin-related (*ins*) genes (Pierce et al., 2001). These sequences are classified into three classes, designated α, β, and γ, based on predicted arrangements of disulfide bonds. Type γ proteins contain three canonical disulfide bridges found in vertebrate insulins and insulin-like growth factors (IGFs). Type α proteins are similar to type γ except they lack an A6-A11 disulfide bridge, an invariant feature of vertebrate insulins and IGFs. Type β proteins appear to retain insulin's three canonical disulfide bridges, plus a fourth, whose location is variable. Since these sequences diverge from mammalian insulins at conserved sites required for biological activity, their classification as insulin-like has been presumptive. However, there has been evidence that some of these *ins* genes, such as the divergent ligand daf-28, functions in the insulin signaling pathway (Li et al., 2003).

In addition, work from our lab in collaboration with others, described insulin receptor binding affinity of another related *C.elegans* ligand, INS-6 (Hua et al., 2003). This β-class INS protein forms folds to form a well-defined structure with four disulfide bridges. Despite marked sequence divergence, an insulin-like fold is stabilized by reorganization of the hydrophobic core. Although the topography of the surface differs radically from that of insulin, the *C. elegans* protein can bind to and activate the human insulin receptor (hIR) (Figure 1.4). Interestingly, the affinity of INS-6 for the hIR is at least 10-fold greater than that of the corresponding single-chain analog of human insulin ("mini-proinsulin") (Hua et al., 1998; Markussen et al., 1985). Our

A



B

IP: anti-IRα IB: anti-pY										
Concentration (nM)	0	0.1	0.5	1	5	10	50	100	500	1000
Type of insulin	Bovine insulin							*C. elegans* INS-6		

(Hua et al., 2003)

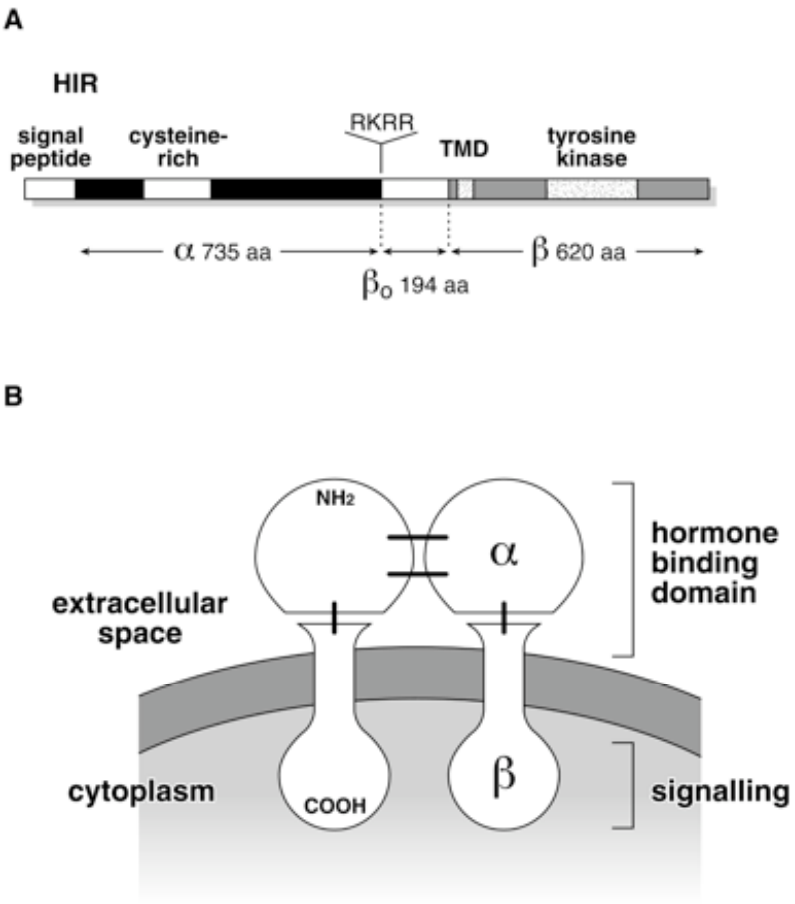
Figure. 1.4. *C. elegans* INS-6 binds to and activates the human insulin receptor (IR). *A*. Competitive displacement of ^{125}I-human insulin by INS-6 (◯), unlabeled human insulin (●), or human mini-proinsulin (▲). *B*. Relative activity of bovine insulin and *C. elegans* INS-6 in stimulating autophosphorylation of the human insulin receptor.

results validate assignment of this *ins* gene to an insulin-like superfamily and broaden the scope of structure-function relationships in design of human insulin agonists.

I.6. The insulin receptor as a model RTK

The insulin receptor is an ideal protein to study RTK homeostasis based on several qualifications. First, the IR was among the first members of the RTK family to be isolated and cloned over twenty years ago, enabling detailed molecular analysis of its structure and function (Ebina et al., 1985; Ullrich et al., 1985) (Figure 1.5). Extensive studies have been performed to analyze its signaling pathways and biogenesis. In particular, the PI3K/AKT/mTOR pathway, the primary signaling cascade downstream of the insulin receptor, is also involved in the function of other RTK members, including EGFR and PDGFR. Second, the insulin receptor is involved in cell growth and glucose metabolism and aberrant function or expression of this protein is related to development of type 2 diabetes, a growing worldwide epidemic. In addition, it has also been recently implicated in a variety of neoplasias, similar to other more commonly known diseases such as breast cancer, etc. wherein dysregulated expression of RTKs plays an important role in development and progression of the disease. Third, its mode of synthesis involves production of a preproprotein in the ER prior to polypeptide cleavage in the Golgi, which enables a unique opportunity to more easily differentiate immature forms from the mature receptor found on the cell surface when investigating regulatory mechanisms involved in RTK biogenesis.

Thus, aside from possibly contributing to better therapeutics for metabolic disease, insights gained from studying mechanisms of IR homeostasis may also be extended to other members of the RTK family and enable development of more effective inhibitors of tumor formation and growth.



(Bass et al., 1998)

Figure 1.5. Structural organization of the human insulin receptor. *A*. Insulin preproreceptor with furin cleavage site (RKRR) indicated. *B*. Mature insulin receptor on cell surface.

I.7. Insulin signaling in mammalian systems

Insulin mediates intracellular glucose metabolism by inducing a conformational change upon binding the receptor, enabling *trans* phosphorylation of the IR β-subunit. Kinase domain activation involves seven tyrosine autophosphorylation sites in three distinct regions of the β subunit, including two juxtamembranous residues (Y^{965}, Y^{972}), three within the catalytic loop (Y^{1158}, Y^{1162}, Y^{1163}) and two within the COOH-terminus (Y^{1328}, Y^{1334}) (Myers and White, 2002). Upon insulin binding, Y^{1162} is displaced from the catalytic site allowing *trans* phosphorylation of the tyrosines in the catalytic loop followed by phosphorylation of the juxtamembranous tyrosines located within a canonical NPXY motif (Eck et al., 1996; Hubbard et al., 1994). Activation of the insulin receptor enables recruitment of a variety of SH2-containing proteins which serve to initiate different signaling pathways such as the phophatidylinositol 3 kinase (PI3K) and mitogen-activated pathway kinase (MAPK) signaling cascades. Each of these pathways plays a distinct role in mediating the cellular effects of insulin (Figure 1.6).

Activated insulin receptor can also phosphorylate the substrates Cbl and APS. Cbl interacts with Cbl-associated protein (CAP), which can bind to the lipid raft protein flotillin. This interaction recruits phosphorylated Cbl into the lipid raft, resulting in the recruitment of the SH2/SH3 adaptor protein CrkII through an interaction of the SH2 domain of CrkII with phosphorylated Cbl. CrkII binds constitutively to the GDP–GTP factor C3G, which can catalyze the exchange of GTP for GDP on the lipid-raft-associated protein TC10. Upon activation, TC10 interacts with one or more effectors to initiate a separate signaling pathway that, along with the PIP3-dependent protein kinases, can stimulate the trafficking of Glut4 vesicles, their docking and their fusion with the plasma membrane. Potential TC10 effectors include other Rho-family small

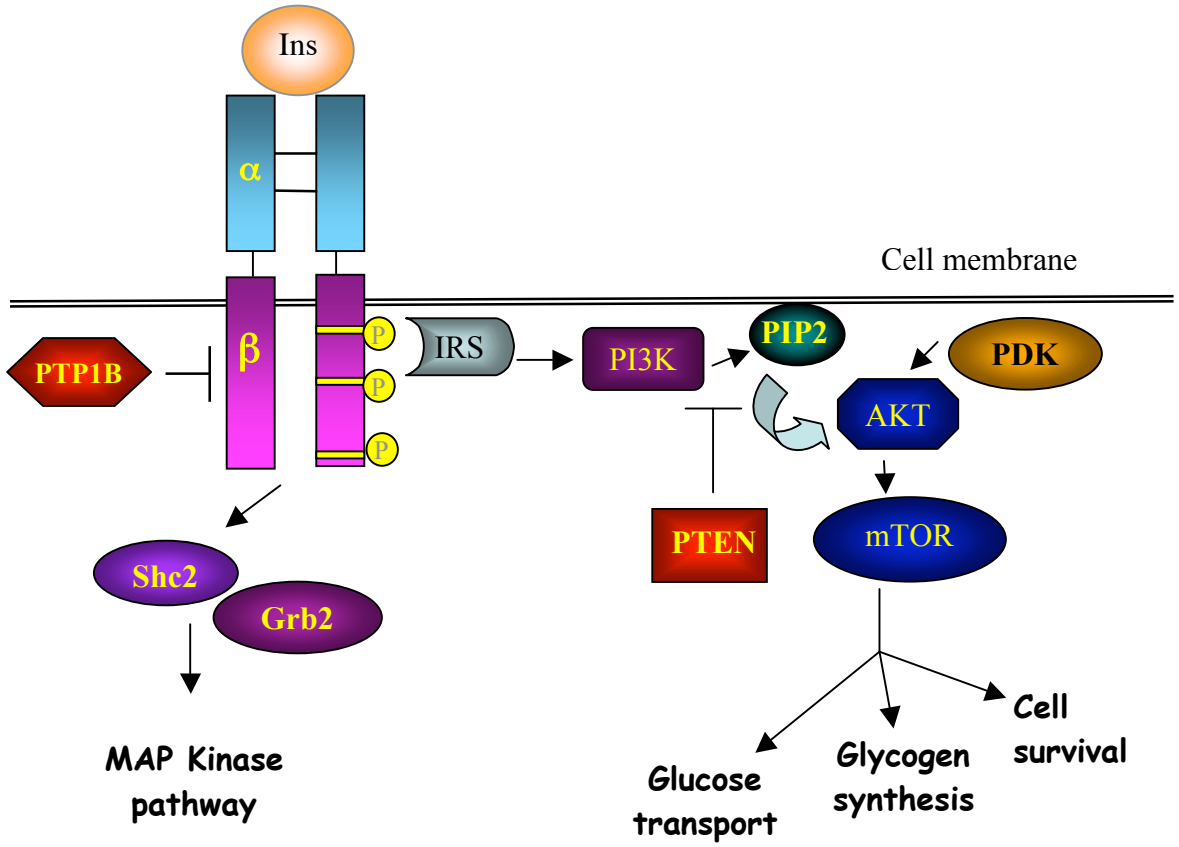


Figure 1.6. Insulin signaling pathways. Upon binding insulin, the receptor undergoes trans autophosphorylation and recruits SH2-containing domains such as Shc2, Grb2 which transduce mitogenic signals via the MAP kinase pathway, or IRS, which mediates glucose metabolism and cell survival through PI3K/AKT/mTOR. Phosphatases such as PTP1B and PTEN dephosphorylate the receptor or lipid substrates, respectively, and negatively regulate insulin signaling.

GTPases, proteins involved in the polymerization of actin and proteins involved in the regulation of SNARE (SNAP receptor) complexes.

1.7.1. PI3K pathway

The PI3K/AKT/mTOR signaling cassette is the primary insulin signaling pathway involved in glucose metabolism. Phosphorylation of the IR on key tyrosine residues leads to recruitment and phosphorylation of insulin-related substrates 1-4 (IRS 1-4), adaptor proteins which bind via a phosphotyrosine binding domain (PTB) to pY^{972} (Eck et al., 1996). IRS proteins then recruit and activate SH2-containing effector proteins, including the regulatory subunit of the lipid kinase PI3K. The IRS signaling scaffold provides an additional level of specificity in contrast to the PDGF and EGF receptors which directly bind and activate SH2-containing proteins (Saltiel and Pessin, 2002). Recruitment and activation of PI3K to the cell surface results in phosphorylation of phosphatidyl inositol 4,5-bisphosphate (PIP2) and recruitment and phosphorylation of the serine-threonine kinase Akt via its pleckstrin homology (PH) domain. AKT is phosphorylated following translocation by phospholipid-associated PDK1 (Alessi, 2001; Myers and White, 2002; Vanhaesebroeck and Alessi, 2000). Akt was originally discovered in the retrovirus AKT8 (Bellacosa et al., 1991) and is also known as PKB (protein kinase B). Akt mediates many of the metabolic actions of insulin, including stimulation of glucose transport, glycogen synthesis, protein translation, inhibition of apoptosis and promotion of cell survival (Barber et al., 2001; Pap and Cooper, 1998; Summers and Birnbaum, 1997). AKT homologues have been identified in a wide range of metazoans from yeast and nematodes to insects and birds. In mammalian systems, there are three major isoforms of AKT, all of which contain a lipid-binding PH domain in its N-terminus, followed by a serine/threonine kinase

catalytic domain and a putative regulatory C-terminal region (Figure 1.7A). Figure 1.7B shows a multiple sequence alignment of the three human AKT isoforms I generated by using the online application, Clustal W (Chenna et al., 2003). All three isoforms exhibit greater than 85% sequence identity with the greatest difference found in their PH domain. The significant difference between these isoforms is in their tissue expression and the roles they play in growth and development. AKT1 is the most ubiquitously expressed isoform and implicated in organismal growth (Cho et al., 2001). AKT2 expression is highest in insulin responsive tissues such as skeletal muscle, liver and fat (Altomare et al., 1998). AKT3 is expressed in the testes and brain and is essential for growth but not glucose homeostasis (Easton et al., 2005).

AKT can directly activate mTOR, another serine/threonine kinase, by phosphorylating it at Ser2448 (Nave et al., 1999). AKT can also indirectly activate mTOR by phosphorylating TSC2, an mTOR inhibitor, and enabling mTOR activation by the small GTPase, Rheb (reviewed in (Hay and Sonenberg, 2004)). Recently, a third mechanism of action has been demonstrated which involves PRAS40 (proline-rich AKT substrate 40 kDa) phosphorylation by AKT, which releases PRAS40 from mTOR resulting in mTOR activation (Vander Haar et al., 2007). Active mTOR then phosphorylates both p70S6K and the translational repressor 4EBP-1, enabling cap-dependent translation to occur. mTOR plays a crucial role in regulating growth in response to nutrient status of the cell as its activity has been shown to be influenced by the intracellular concentration of ATP (Dennis et al., 2001).

Insulin signaling through the PI3K/AKT/mTOR pathway results in translocation of the glucose transporter, GLUT4, to the cell surface to enable uptake of glucose from the extracellular environment. Intracellular glucose production is also inhibited while increasing glycogen and protein synthesis.

A

B

```
AKT1_HUMAN      ----------------------------MSDVAIVKEGWLHKRGEYIKTWRPRYFLLKND 32
AKT2_HUMAN      ----------------------------MNEVSVIKEGWLHKRGEYIKTWRPRYFLLKSD 32
AKT3_HUMAN      AAAAENPNPKADITKYHFSKLGAQRGVIMSDVTIVKEGWVQKRGEYIKNWRPRYFLLKTD 60
                                            *.:*:::****::*******.*********.*

AKT1_HUMAN      GTFIGYKERPQDVDQREAPLNNFSVAQCQLMKTERPRPNTFIIRCLQWTTVIERTFHVET 92
AKT2_HUMAN      GSFIGYKERPEAPDQTLPPLNNFSVAECQLMKTERPRPNTFVIRCLQWTTVIERTFHVDS 92
AKT3_HUMAN      GSFIGYKEKPQDVDLP-YPLNNFSVAKCQLMKTERPKPNTFIIRCLQWTTVIERTFHVDT 119
                *:******:*:  *    ********:*********:****:****************::

AKT1_HUMAN      PEEREEWTTAIQTVADGLKKQE--EEEMDFRSGSPSDNSGAEEMEVSLAKPKHRVTMNEF 150
AKT2_HUMAN      PDEREEWMRAIQMVANSLKQRAPGEDPMDYKCGSPSDSSTTEEMEVAVSKARAKVTMNDF 152
AKT3_HUMAN      PEEREEWTEAIQAVADRLQRQE--EERMNCSPTSQIDNIGEEEMDASTTHHK-RKTMNDF 176
                *:*****  *** **: *:::   *: *:    *  *.   ***:.: :: : : ***:*

AKT1_HUMAN      EYLKLLGKGTFGKVILVKEKATGRYYAMKILKKEVIVAKDEVAHTLTENRVLQNSRHPFL 210
AKT2_HUMAN      DYLKLLGKGTFGKVILVREKATGRYYAMKILRKEVIIAKDEVAHTVTESRVLQNTRHPFL 212
AKT3_HUMAN      DYLKLLGKGTFGKVILVREKASGKYYAMKILKKEVIIAKDEVAHTLTESRVLKNTRHPFL 236
                :****************:***:*:*******:****:********:**.***:*:*****

AKT1_HUMAN      TALKYSFQTHDRLCFVMEYANGGELFFHLSRERVFSEDRARFYGAEIVSALDYLHSEKNV 270
AKT2_HUMAN      TALKYAFQTHDRLCFVMEYANGGELFFHLSRERVFTEERARFYGAEIVSALEYLHSRD-V 271
AKT3_HUMAN      TSLKYSFQTKDRLCFVMEYVNGGELFFHLSRERVFSEDRTRFYGAEIVSALDYLHSGK-I 295
                *:***:***:*********.***************:*:*:***********:**** . :

AKT1_HUMAN      VYRDLKLENLMLDKDGHIKITDFGLCKEGIKDGATMKTFCGTPEYLAPEVLEDNDYGRAV 330
AKT2_HUMAN      VYRDIKLENLMLDKDGHIKITDFGLCKEGISDGATMKTFCGTPEYLAPEVLEDNDYGRAV 331
AKT3_HUMAN      VYRDLKLENLMLDKDGHIKITDFGLCKEGITDAATMKTFCGTPEYLAPEVLEDNDYGRAV 355
                ****:*************************.*.*************************

AKT1_HUMAN      DWWGLGVVMYEMMCGRLPFYNQDHEKLFELILMEEIRFPRTLGPEAKSLLSGLLKKDPKQ 390
AKT2_HUMAN      DWWGLGVVMYEMMCGRLPFYNQDHERLFELILMEEIRFPRTLSPEAKSLLAGLLKKDPKQ 391
AKT3_HUMAN      DWWGLGVVMYEMMCGRLPFYNQDHEKLFELILMEDIKFPRTLSSDAKSLLSGLLIKDPNK 415
                ************************:********:*:*****..:*****:*** ***::

AKT1_HUMAN      RLGGGSEDAKEIMQHRFFAGIVWQHVYEKKLSPPFKPQVTSETDTRYFDEEFTAQMITIT 450
AKT2_HUMAN      RLGGGPSDAKEVMEHRFFLSINWQDVVQKKLLPPFKPQVTSEVDTRYFDDEFTAQSITIT 451
AKT3_HUMAN      RLGGGPDDAKEIMRHSFFSGVNWQDVYDKKLVPPFKPQVTSETDTRYFDEEFTAQTITIT 475
                *****..****:*.* ** .: **.* :*** **********.******:***** ****

AKT1_HUMAN      PPD--QDDSMECVDSERRPHFPQFSYSASGTA 480
AKT2_HUMAN      PPD--RYDSLGLLELDQRTHFPQFSYSASIRE 481
AKT3_HUMAN      PPEKYDEDGMDCMDNERRPHFPQFSYSASGRE 507
                **:    *.:  :: ::*.**********
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Legend:

* residues are identical in all sequences

: conservative substitutions (according to color code below) are observed

. semi-conservative substitutions are observed

AVFPMILW	RED	Small (small+ hydrophobic (incl.aromatic -Y))
DE	BLUE	Acidic
RK	MAGENTA	Basic - H
STYHCNGQ	GREEN	Hydroxyl + sulfhydryl + amine + G
Others	Grey	Unusual amino/imino acids etc

Figure 1.7. Structural organization and protein homology of human AKT isoforms. *A*. Organization of common AKT protein domains *B*. Multiple protein sequence alignment of the three human AKT isoforms using Clustal W (1.83)

I.7.2. Regulation of insulin signaling

Each step in the insulin signaling cascade is regulated by both positive and negative factors resulting in either signal amplification or termination. Activated receptors can be endocytosed and either be recycled back to the cell surface or shunted to a degradative pathway. Signaling activity can also be negatively regulated by phosphatases such as protein tyrosine phosphatase 1B (PTP1B) and phosphoinositide 3-phosphatase (PTEN) which dephosphorylate key tyrosine residues in the insulin receptor and PIP3, respectively, and are important negative regulators (Nakashima et al., 2000). Other proteins, such as suppressor of cytokine signaling-1 (SOCS1), SOCS3 and growth-factor-receptor–bound protein 10 (Grb10) inhibit IR activity by sterically blocking its interaction with the IRS adaptor proteins, or by modifying the receptor's kinase activity directly (Ueki et al., 2004).

Another important mechanism for downregulating insulin signaling is through serine phosphorylation of the receptor itself, or of its adaptor proteins, IRS 1-4, which inhibits binding to the juxtamembrane region of the IR and impairs signal transduction (Paz et al., 1997; Pederson et al., 2001). In women exhibiting insulin resistance and suffering from polycystic ovarian syndrome (PCOS), increased serine phosphorylation of the receptor was observed, with a corresponding decrease in tyrosine phosphorylation (Dunaif et al., 1995). Serine phosphorylation of IRS1 is also increased in insulin-resistant states and is thought to contribute to the pathogenesis of insulin resistance. IRS proteins are serine phosphorylated by GSK3 or mTOR, both of which are activated downstream of IRS phosphorylation (Eldar-Finkelman and Krebs, 1997). Activity of serine kinases can be further stimulated by the chronic elevation of insulin levels, leading to a continuous chain reaction of insulin-induced insulin resistance.

I.8. Insulin receptor biogenesis

The IR gene is located on chromosome 19 in humans, spans over 120 kb and contains 22 exons (Seino and Bell, 1989). Exons 1-11 encode the α-subunit and exons 12-22 the β-subunit. There are two isoforms of the insulin receptor as a result of alternative splicing of the 36-nucleotide exon 11 in the carboxy-terminus of the α-subunit. The IR-A or exon 11- isoform is found predominantly in leukocytes while the IR-B or exon 11+ isoform is predominant in the liver. Similar amounts of both variants are present in skeletal muscle and adipose tissue (Benecke et al., 1992). Recently, differential expression of these two IR isoforms has been implicated in a wide range of cancers (Denley et al., 2003) and suggests that the presence or absence of exon 11 has important consequences in cell growth and development. The insulin receptor plays a pivotal role in regulating glucose homeostasis and its synthesis and maturation are regulated at the transcriptional, translational and post-translational levels.

The mature IR is initially synthesized as a single chain preproreceptor (proIR) containing a signal peptide that directs it co-translationally to the endoplasmic reticulum (ER) (Figure 1.8). The proreceptor is composed of an α-subunit, containing the insulin binding site, and the β-subunit, containing a single transmembrane domain and a tyrosine kinase domain. Upon translocation into the lumen of the ER and signal peptide cleavage, the proIR undergoes extensive asparagine-linked glycosylation at 17 sites within the α-subunit (Ullrich et al., 1985). Glycosylation is coordinated with binding to two ER-localized glycan-specific chaperones, calnexin (Cnx) and calreticulin (Crt). Inhibition of Cnx/Crt binding accelerates dimerization but reduces the efficiency of IR folding (Bass et al., 1998; Helenius and Aebi, 2001). Like other oligomeric membrane proteins, dimerization is necessary prior to export from the ER. Following

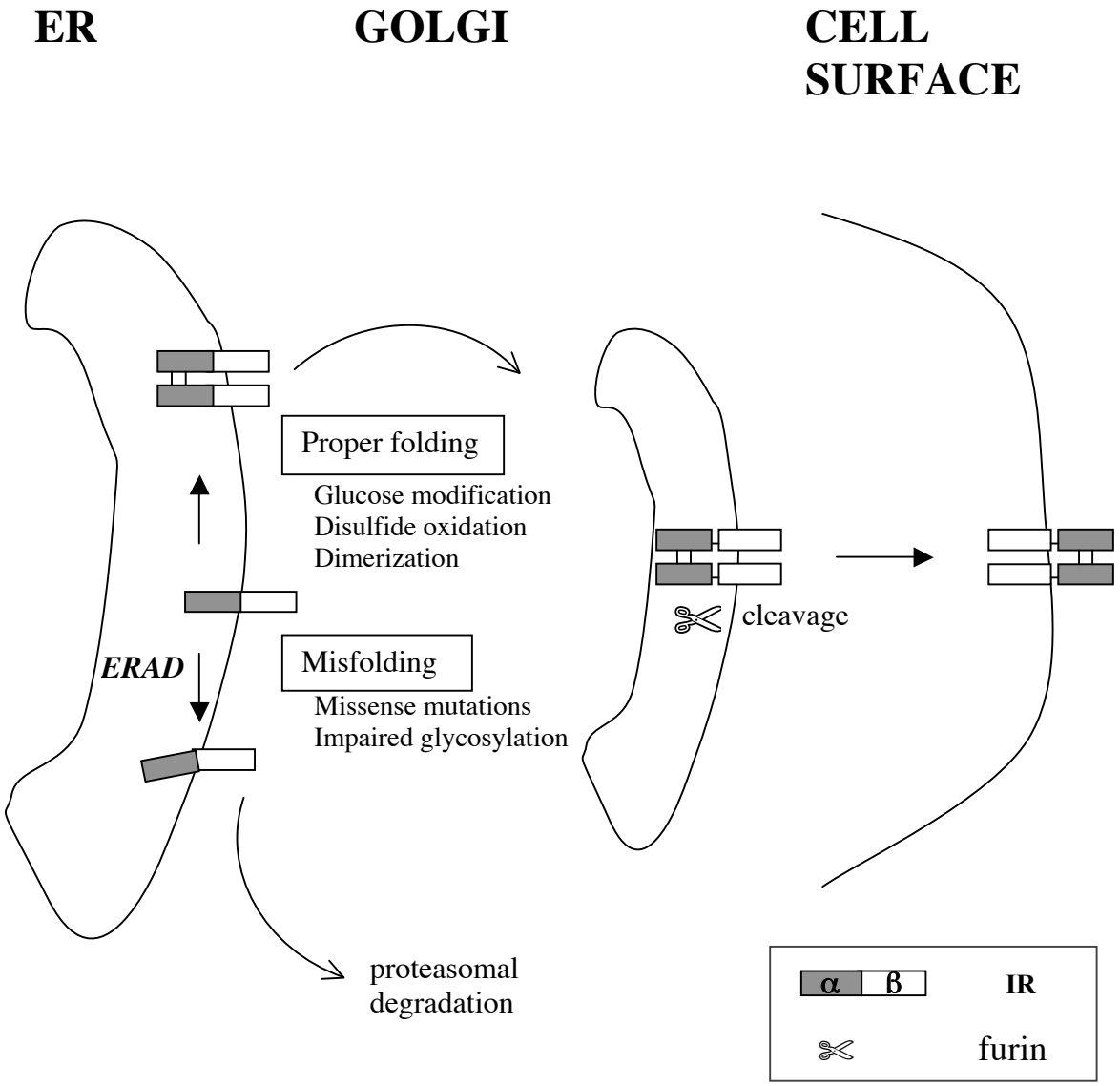

Figure 1.8. Insulin receptor maturation through the secretory pathway. The insulin receptor is a Type II transmembrane protein that is initially synthesized as a single polypeptide with co-translational insertion of its N-terminus in the ER lumen. N-linked glycosylation and glucose trimming influence chaperone binding facilitating folding and dimerization within the ER prior to export to the Golgi apparatus. The immature dimer then undergoes further glycan modification in the Golgi and is cleaved by furin into α and β subunits. The mature receptor is then expressed on the cell surface as a heterotetramer of stoichiometry $\alpha_2\beta_2$. Misfolding of the receptor due to missense mutations or perturbation of glycan maturation, diverts the receptor to the ER-associated degradation (ERAD) pathway where it is retrotranslocated out of the ER and into the cytosol to be degraded by the proteasome.

dimerization, the IR is transported to the Golgi apparatus where mannose trimming and addition of N-acetylglucosamine (GlcNAc), galactose, sialic acid, and fucose occurs (Helenius and Aebi, 2001). Glycosylation is important in receptor biosynthesis and function since either mutating the glycosylated asparagine residues in the IR (Caro et al., 1994; Elleman et al., 2000) or inhibiting glycosylation results in decreased cell surface expression and insulin signaling (Ronnett et al., 1984). Immediately prior to delivery to the cell surface, the proIR dimer undergoes proteolytic cleavage at a subtilisin-cleavage site (RKRR↓) essential for high-affinity insulin binding. The mature receptor is then delivered to the surface as a heterotetramer with a stoichiometry of 2 α and 2 β subunits (Ullrich et al., 1985).

1.8.1. Transcriptional regulation

Many of the metabolic effects mediated by insulin depend on the winged helix transcription factor, FOXO (*F*orkhead b*OX*-containing protein, *O* subfamily). FOXO was first identified in the worm *C. elegans* as *Daf-16*, a mutation that can suppress the increased lifespan caused by loss of *Daf-2*, the worm ortholog of the insulin receptor (Lin et al., 1997; Ogg et al., 1997). *Daf-16* (FOXO) also mediates the *Daf4/Daf7* (TGFβ) pathways in the worm and the same may be true in mammalian cells (Seoane et al., 2004). There is a single FOXO ortholog found in *Drosophila* (Puig et al., 2003) and three mammalian FOXO genes (*FOXO1, FOXO3a, FOXO4).* FOXO1 is the most ubiquitous isoform and most likely to mediate insulin action since haploinsufficiency leads to increased Glut4 mRNA and reduced hepatic expression of gluconeogenic genes.

AKT inhibits FOXO activity by phosphorylating the transcription factor, leading to its nuclear exclusion. However, inhibition of FOXO-dependent transcription can also be achieved

with constitutively nuclear FOXO mutants, suggesting that multiple mechanisms regulate FOXO activity in addition to nuclear localization. Recent studies show that deacetylation of FOXO by Sirt1 (Silent Infromation Regulator 2.1) in *C. elegans* modifies transcription of target genes and serves as another mode of regulating FOXO activity to promote cellular survival (Brunet et al., 2004; Motta et al., 2004).

Earlier studies investigating FOXO function showed that this transcription factor upregulates cellular glucose production by promoting expression of glucose-6-phosphatase and PEPCK to mediate glycogenolysis and gluconeogenesis, respectively, resulting in increased cellular glucose. However, recent studies in *Drosophila* have also implicated FOXO1 in transcription of the insulin receptor itself (Puig et al., 2003). Drosophila FOXO (dFOXO) activity resulted in upregulation of the translational regulator d4EBP and the dInR. These results established dFOXO as a key transcriptional regulator of the insulin pathway that modulates growth and proliferation.

I.8.2. Translational regulation

Translation of mRNAs containing a 5' cap structure (m^7GpppN, where N is any nucleotide) represents the standard mode of translation used by most cellular mRNAs. Cap-dependent translation is initiated by recruitment of ribosomes to the 5' end of mRNAs via the eukaryotic translation initiation factor 4F (eIF4F), a heterotrimeric complex comprised of a cap-binding subunit (eIF4E) and an RNA helicase (eIF4A) bridged by a scaffolding molecule (eIF4G). The translation factor eIF4E is held inactive by the hypophosphorylated translational repressor 4EBP1 to inhibit cap-dependent translation. Hyperphosphorylation of 4EBP1 by

mTOR disengages it from eIF4E, allowing the translation factor to initiate cap-dependent translation.

A second mechanism for translation initiation bypasses the need for the cap and eIF4E and instead relies on the presence of internal ribosome entry sites (IRES) in the untranslated regions (UTRs) found on specific mRNAs. IRESs form a well-defined and highly conserved RNA structure adjacent to AUG start sites which allow direct recruitment of ribosomes to mediate cap-independent translation (Baird et al., 2006). This mechanism of translation was first discovered in retroviruses as a way to boost production of viral proteins when regular cap-dependent translation of the infected host cell is shut down. However, recent studies have uncovered the existence of IRES sequences in cellular mRNAs (Baird et al., 2006; Fernandez et al., 2002; Fernandez et al., 2001; Svitkin et al., 2005) and provide an alternate translational pathway that is possibly utilized when the machinery for cap-dependent translation is compromised.

Studies in *Drosophila* IR (dIR) mRNA constructs uncovered three unusually long 5' UTRs in transcripts produced by dIR promoters that contained multiple AUG initiator codons upstream of the legitimate IR initiator codon (Marr et al., 2007). More importantly, these 5' UTR regions were demonstrated to contain IRES activity. Marr *et al* hypothesized that these 4EBP1-resistant IRES containing mRNAs serve to functionally couple transcription and translation and function to amplify insulin receptor production under conditions of nutrient depletion.

I.8.3. Post-translational regulation

The early maturation of transmembrane signaling receptor tyrosine kinases (RTKs) occurs in the endoplasmic reticulum (ER) under the surveillance of an intricate quality control (QC) system that redirects mutant or improperly processed proteins to ER-associated degradation (ERAD) (reviewed in (Hampton, 2002; McCracken and Brodsky, 2005; Sayeed and Ng, 2005; Werner et al., 1996). Like all integral membrane proteins, the level of expression and activity of RTKs are determined by the action of the QC network comprised of protein folding enzymes, molecular chaperones and proteases on both sides of the ER membrane. How these individual ER and cytosolic factors collaborate to redirect mutant or misprocessed proteins to degradation is still not completely clear. Genetic variants of the IR, and wild-type IR that are improperly glycosylated, are targeted for endoplasmic reticulum associated degradation (ERAD). Although substrates of ERAD ultimately undergo complete proteolysis, initial mechanisms for recognition, retention and targeting of such substrates to the ERAD pathway have not been fully elucidated.

Previous studies have provided detailed information on at least three major families of molecular chaperones that participate in membrane glycoprotein QC and ERAD. These include the ER lumenal Hsp70 family member BiP (binding protein) (Chillaron and Haas, 2000; Nishikawa et al., 2001; Plemper et al., 1997), calnexin (CNX) and calreticulin (CRT), homologous calcium-binding proteins localized to the ER (Molinari et al., 2004) and Grp94, the only known ER lumenal Hsp90 (Argon and Simen, 1999; Tatu and Helenius, 1997; Zhang et al., 1997). BiP associates with the broadest range of nascent proteins within the ER lumen and it has also been implicated in protein import into the ER (Hamman et al., 1998). Studies of exogenous viral membrane protein folding led to the prevailing model for glycan-mediated QC involving CNX and CRT. The model holds that glucose attachment and removal from high-mannose N-

linked glycans results in continuous cycles of release and recapture by CNX/CRT (Ellgaard et al., 1999; Helenius et al., 1997; Parodi, 2000b; Schrag et al., 2001). This model is based on *in vitro* evidence that glucosyltransferase is selective for N-linked groups in regions containing hydrophobic residues that become buried in the mature protein. Cycles of glucosylation and de-glucosylation are thus coupled to maturation and CNX/CRT binding. Interestingly, CNX has been shown to interact with multiple folding intermediates, whereas CRT associates with earlier conformers (Hebert et al., 1996; Hebert et al., 1997). Furthermore, several laboratories have uncovered a glycan-independent mode of CNX/CRT binding to client proteins (Danilczyk and Williams, 2001; Saito et al., 1999; Swanton et al., 2003).

In addition to ER lumenal chaperones, ERAD has been observed with pharmacological blockade of Hsp90, one of the most abundant eukaryotic chaperones in the cytosol. Interestingly, the ansamycin class of inhibitors of the heat shock protein of 90 kDa (Hsp90), herbimcyin A (HA) and geldanamycin (GA), which interfere with nucelotide binding to Hsp90, have been shown to induce rapid degradation of a number of integral membrane proteins including CFTR and members of the receptor tyrosine kinase superfamily including the EGFR, IGF1-R and IR (Loo et al., 1998; Saitoh et al., 2002; Sakagami et al., 1999; Sepp-Lorenzino et al., 1995; Supino-Rosin et al., 2000). Moreover, Hsp90 binding to the RTK epidermal growth factor type 2 (ErbB2) has been shown to involve interactions between residues in the receptor kinase domain and the cytosolic molecular chaperone (Tikhomirov and Carpenter, 2000; Xu et al., 2001).

Despite topological separation between the ER lumen and cytosol, ERAD is thought to involve integration of activities within both compartments. New studies using live cell imaging have shown that client proteins targeted to degradation retain a high degree of fluidity within the ER membrane (Haggie et al., 2002; Nehls et al., 2000). Yet, it remains unclear as to whether

substrates targeted to ERAD are first immobilized, or instead retain mobility within the plane of the ER.

1.8.4. Effect of altered IR structure on receptor function

Decreased cell surface expression of the IR has been implicated in both genetic and acquired forms of insulin resistance and may contribute to the pathogenesis of diabetes mellitus (Pessin and Saltiel, 2000; Shulman, 2000; Taylor, 2002). Most mutations in the insulin receptor gene that lead to impaired intracellular transport to the plasma membrane, or decreased insulin binding affinity, map to the extracellular domain of the receptor (Figure 1.9A). Previous work in our laboratory involved two missense mutations that was previously identified in a woman with classic congenital type A insulin resistance (Rouard et al., 1999; Rouard et al., 1997). The maternally inherited allele, D59G is located in a right angle bend between two β sheets while the paternally inherited allele, L62P, lies within the adjacent β strand, both within the L1 domain of the receptor. Substituting the aspartic acid at the amino acid residue 59 for a glycine did not substantially perturb maturation of the receptor. However, this substitution was shown to diminish affinity of the receptor for insulin. The leucine to proline substitution at amino acid residue 62, on the other hand, resulted in a significant structural change and served to destabilize the conformation of the receptor and diverted it to a degradative pathway from the ER, preventing it from reaching the cell surface. Specific residues involved in insulin binding have recently been elucidated in the crystal structure of the human IR (Lou et al., 2006; McKern et al., 2006). The residues essential for ligand specificity were found to cluster in the L1 domain of the human insulin receptor (Figure 1.9B) supporting our previous studies on D59 and L62 and highlighting the importance of these residues in insulin binding affinity and receptor stability.

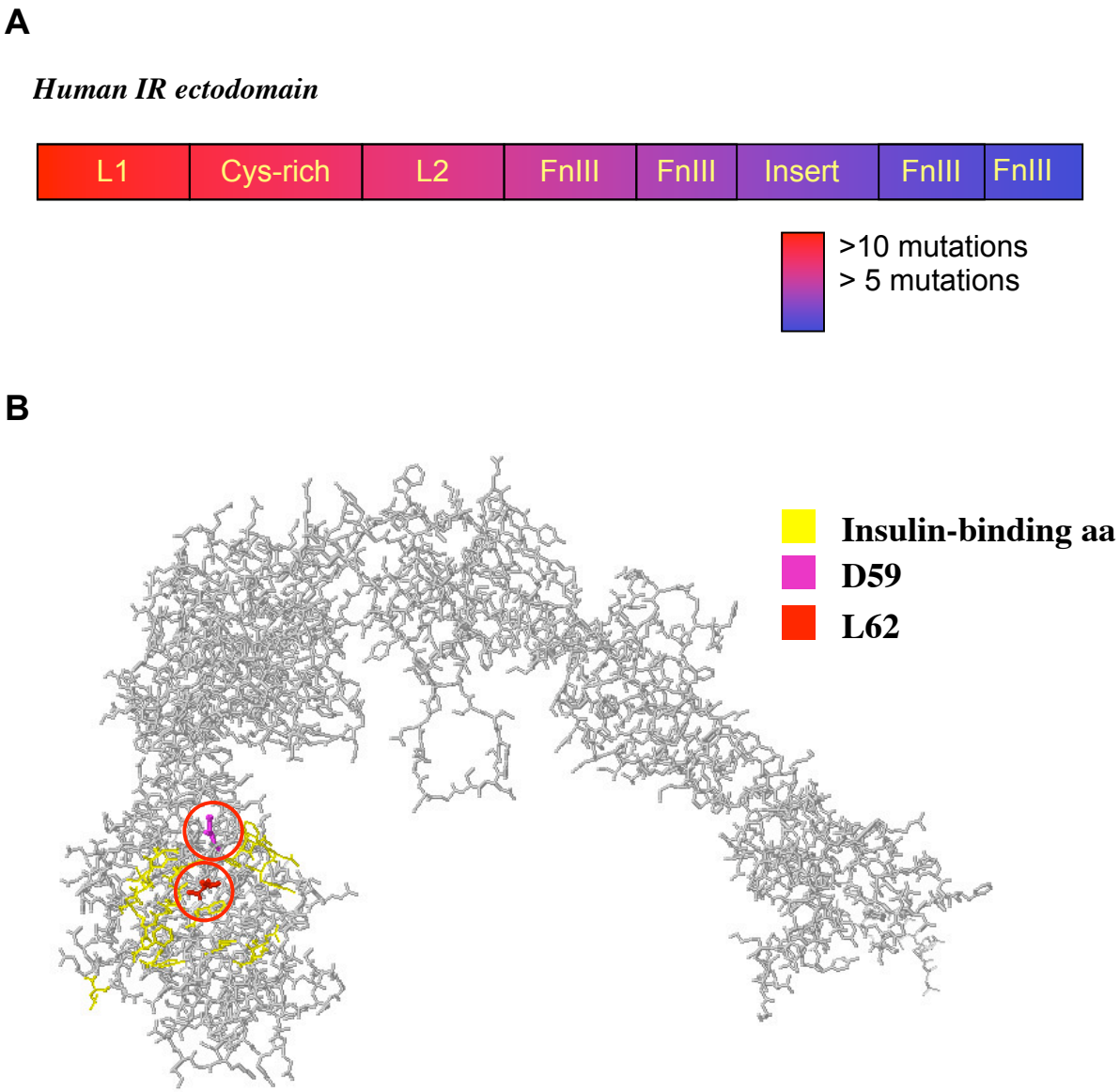

Figure 1.9. Human insulin receptor ectodomain. *A*. Inherited human mutations that have been discovered in patients exhibiting metabolic disorders have predominantly mapped to the extracellular region of the IR, with a great majority occurring within the L1 binding domain of the receptor. *B*. "Stick" model of the human IR ectodomain I generated using the structural software application, Jmol, based on recently published crystal structures (Lou et al., 2006; McKern et al., 2006). Insulin-binding residues are highlighted in yellow and the D59 and L62P residues found to be mutated in a young woman suffering from Type A insulin resistance are colored in magenta and red, respectively.

1.9. Goals of this thesis

Given the important roles that receptor tyrosine kinases play in cellular growth and development, aberrant regulation of RTK expression and degradation have been implicated in a variety of neoplastic and metabolic diseases. The insulin receptor is a prototypical RTK involved in glucose metabolism and organismal growth and much research has been performed to elucidate its signaling pathways and its biogenesis. Recently, altered expression of the IR has also been discovered in various forms of cancer. These properties make the insulin receptor an excellent tool to study RTK expression and degradation and serve as a model protein to investigate the complex mechanisms that regulate RTK homeostasis in mammalian systems. More specifically, I will address three different aspects that impact insulin receptor homeostasis.

First, I will investigate how signaling activity through the receptor serves as a negative feedback mechanism to regulate receptor function. Much research has been focused on investigating how insulin signaling is attenuated via inhibitory mechanisms directed at components of the pathway distal to the receptor. However, with the discovery and expansion of recent studies on the role of AKT in transcriptional and translational regulation of insulin receptor expression in *Drosophila*, I will perturb AKT expression or function in mouse or human models to determine the effect of this kinase on expression and stability of the insulin receptor in mammalian systems. We hypothesize that attenuating insulin signaling downstream of the receptor will lead to upregulation of insulin receptor expression.

Second, given the important role of the insulin receptor in glucose uptake and cell metabolism, I will explore how changes in glucose or ATP availability impacts IR expression and stability. Furthermore, as various steps in the PI3K/AKT/mTOR pathway have been shown

to be sensitive to ATP levels inside the cell, I will investigate whether there is cross-talk between insulin signaling and nutrient sensing to coordinately regulate IR stability.

Finally, I will examine the earliest steps involved in biogenesis of the membrane-spanning IR. Recent studies have shown that downregulation of the receptor from the cell surface is not the only mechanism by which RTK signaling is attenuated and that altered IR synthesis may also play a role. Thus, I will explore how IR biogenesis is modulated from the ER to the cell surface under varying conditions of cell stress and metabolic perturbations. In addition, as the RTKs are membrane-spanning proteins and interact with factors on both the lumenal side and the cytosolic side of the endoplasmic reticulum, I will investigate how chaperones located on both sides of the ER membrane affect IR stability when receptor misfolding is triggered by perturbing N-linked glycosylation or the introduction of missense mutations. Since protein maturation is a dynamic process involving movement through various compartments in the cell, I will also measure diffusional mobility of the IR using novel GFP chimeras. I hope to determine if movement through the ER is altered when the protein is misfolded and whether ER or cytosolic chaperones play a role in spatial and temporal regulation of the IR.

Insights from these studies will lead to a better understanding of insulin receptor homeostasis that can be extended to other members of the RTK superfamily and lead to development of more effective therapies for Type 2 diabetes, other metabolic diseases and neoplasia.

Chapter 2

In Vivo Regulation of Receptor Tyrosine Kinase Metabolism by PI3K/AKT/mTOR Signaling

2.1. Abstract

Receptor tyrosine kinases (RTKs) play an important role in eukaryote cell growth and metabolism by initiating an intracellular signal transduction cascade upon binding their growth factor ligands. However, the mechanisms by which signaling through these receptors regulate their own expression and degradation are not fully understood. Here we investigate how activity of kinases downstream of the insulin receptor (IR), a prototypical RTK which serves as the primary control node in cellular processes involving glucose metabolism and growth, affects its expression and degradation. Insulin signaling activity was altered through both genetic and pharmacologic methods and their effects on insulin receptor homeostasis was investigated. We discovered that eliminating AKT, a critical kinase downstream of the IR, either through transgenic knock-out mouse models or RNAi in human cells, increased IR expression and decreased its degradation. Similarly, inhibiting PI3K or mTOR activity, kinases upstream and downstream of AKT, respectively, also resulted in increased IR protein levels. In contrast, constitutively active PI3K decreased IR protein expression. Interestingly, expression of other RTKs such as EGFR and IGF1-R were similarly upregulated when PI3K/AKT/mTOR signaling was inhibited. Together, these data suggest that negative feedback mechanisms exist for the insulin signaling pathway at both transcriptional and post-translational levels to regulate RTK stability in mammalian systems.

2.2. Introduction

In eukaryotic cells, members of a large family of membrane-spanning receptors that possess intrinsic tyrosine kinase activity play a fundamental role in cell growth, metabolism and survival. Examples of these receptor tyrosine kinases (RTKs) include the epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor (IGF1-R). Altered cell surface expression of these receptor tyrosine kinases (RTKs) has profound effects on organismal physiology and is associated with both metabolic disease and neoplasia. The insulin receptor (IR) is a prototypical RTK that regulates metabolism and lifespan and both genetic and epigenetic changes in receptor expression are involved in the initiation and progression of human Type 2 diabetes. More recently, the IR and the highly related IGF1-R, have both been implicated in various forms of cancer (reviewed in (Denley et al., 2003) and (LeRoith and Roberts, 2003), respectively), making the IR an excellent tool to investigate the regulatory mechanisms that are involved in RTK homeostasis.

Signaling through the insulin receptor is the key step that initiates the intracellular signal transduction cascade needed for growth and glucose metabolism (Figure 2.1). Upon binding insulin, the IR undergoes a conformational change that enables *trans* autophosphorylation of key tyrosine residues in its catalytic domain (Eck et al., 1996; Hubbard et al., 1994). This leads to recruitment and phosphorylation of insulin-related substrates 1-4 (IRS 1-4), adaptor proteins which bind the IR via a phosphotyrosine binding domain (PTB) (Eck et al., 1996). IRS proteins then recruit and activate SH2-containing (Src-homology 2) effector proteins like PI3K, Grb-2 and SHP-2, which can then activate other kinases such as AKT and MAPK, among others. Activation of the MAPK pathway is associated with the proliferative effects of insulin while the PI3K/AKT/mTOR pathway is primarily responsible for the metabolic and anabolic effects of

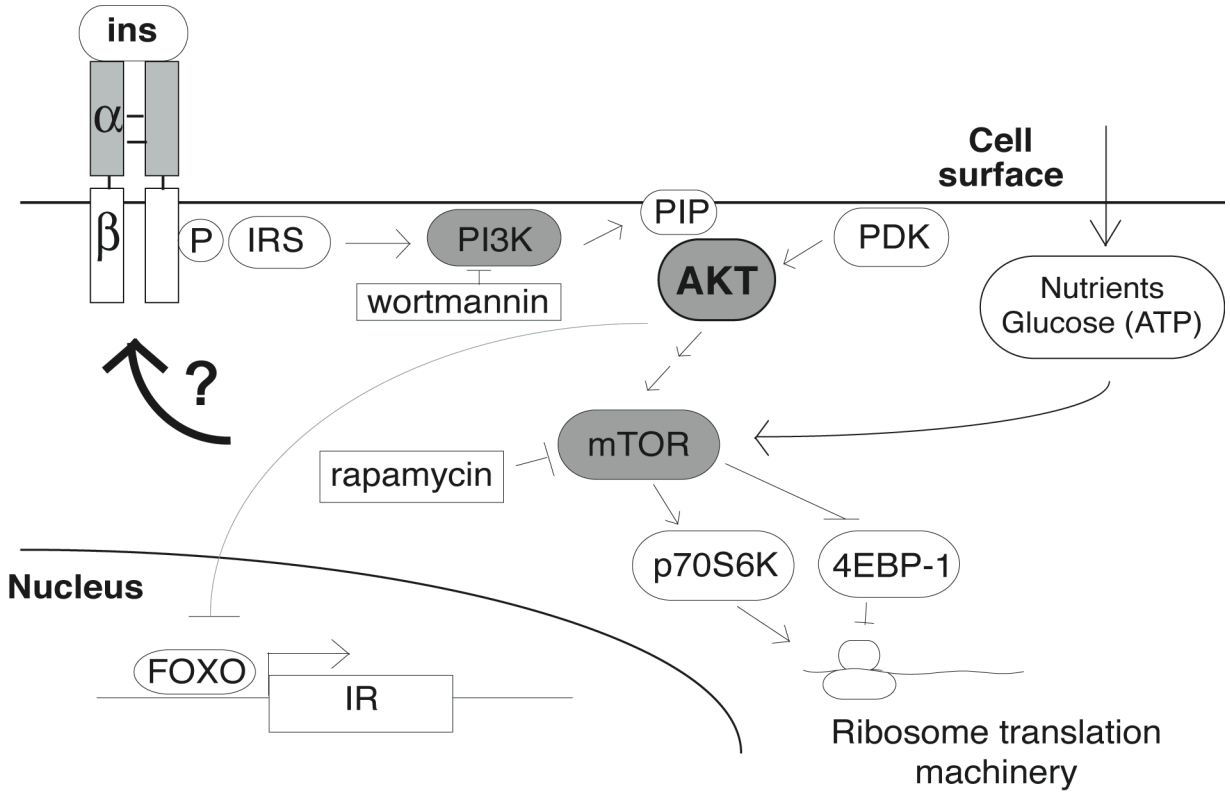

Figure 2.1. PI3K/AKT/mTOR signaling mediated by the insulin receptor. Upon binding insulin, the insulin receptor undergoes autophosphorylation of tyrosine residues in its catalytic domain, leading to recruitment and phosphorylation of insulin-related substrates 1-4 (IRS 1-4). IRS proteins then recruit and activate the lipid kinase PI3K. Activation of PI3K on the cell surface results in phosphorylation of phosphatidyl inositol 4,5-bisphosphate (PIP2) which in turn, recruits the serine-threonine kinase Akt beneath the cell membrane and facilitates its phosphorylation by phospholipid-associated PDK1 and PDK2. Akt then activates mTOR, an ATP sensor, which serves to initiate cap-dependent translation by activating p70S6K and inhibiting the 4EBP-1 repressor. AKT also phoshorylates the transcription factor, FOXO1, leading to its nuclear exclusion and preventing it from upregulating IR transcription. Pharmacologic inhibitors for particular steps in the insulin signaling pathway include wortmannin and rapamycin which block PI3K and mTOR activity, respectively. However, how the different steps in this pathway regulate insulin receptor expression and degradation remain unknown.

insulin. This signaling pathway can also be triggered by activation of other RTKs such as the highly-related IGF1-R and the EGFR. To further highlight its importance in growth and development, PI3K signaling is highly conserved in metazoans as it plays a crucial role in mediating cellular growth and metabolism that is coupled to lifespan. Recruitment and activation of PI3K to the cell surface results in phosphorylation of phosphatidyl inositol 4,5-bisphosphate (PIP2) and recruitment and phosphorylation of AKT by PDK1 (Alessi, 2001; Myers and White, 2002; Vanhaesebroeck and Alessi, 2000). AKT is a serine-threonine kinase which can directly activate mTOR by phosphorylating it at Ser2448 (Nave et al., 1999). AKT can also indirectly activate mTOR by phosphorylating TSC2, an mTOR inhibitor, and enabling mTOR activation by the small GTPase, Rheb (reviewed in (Hay and Sonenberg, 2004)). Recently, a third mechanism of action has been demonstrated which involves PRAS40 (proline-rich AKT substrate 40 kDa) phosphorylation by AKT, enabling the release of PRAS40 from mTOR resulting in mTOR activation (Vander Haar et al., 2007). Active mTOR then phosphorylates both p70S6K and the translational repressor 4EBP-1, enabling cap-dependent translation to occur.

Akt mediates many of the metabolic actions of insulin, including stimulation of glucose transport, glycogen synthesis, protein translation, inhibition of apoptosis and promotion of cell survival (Barber et al., 2001; Pap and Cooper, 1998; Summers and Birnbaum, 1997). AKT has also been shown to be involved in IR expression by phosphorylating the transcription factor FOXO1, leading to its cytoplasmic retention and preventing it from promoting IR transcription (Puig et al., 2003).

Like most signaling pathways, the insulin signaling cascade is regulated by both positive and negative factors resulting in either signal amplification or termination. For example,

serine/threonine phosphorylation of IRS 1-4 inhibits binding to the juxtamembrane region of the IR and negatively regulates insulin signaling (Paz et al., 1997; Pederson et al., 2001). Other important negative regulators include phosphatases such as protein tyrosine phosphatase 1B (PTP1B) and phosphoinositide 3-phosphatase (PTEN) which dephosphorylate key tyrosine residues in the insulin receptor(Nakashima et al., 2000). Pharmacological reagents, such as wortmannin and rapamycin, can also target particular steps in the pathway by inhibiting activity of PI3K an mTOR, respectively.

Studies investigating the PI3K/AKT/mTOR pathway have shown that chronic insulin signaling initiates a negative feedback mechanism through downregulation of proteins distal to the insulin receptor. However, research in obese mouse models show reduced insulin receptor expression (Kahn et al., 1973; Le Marchand-Brustel et al., 1978) and that insulin resistance in humans was also attributed to decreased insulin receptor expression (Caro et al., 1987; Kolterman et al., 1981)). In addition, recent evidence in *Drosophila* suggests that regulation at the level of insulin receptor biosynthesis may also have important implications in growth and development (Puig et al., 2003; Puig and Tjian, 2006). These suggest that understanding the mechanisms that regulate homeostasis of the insulin receptor, the initial point of contact for insulin, is crucial for a more comprehensive understanding of the complex signaling pathway it initiates and perhaps lead to better therapies to combat type 2 diabetes.

In order to understand how signaling through the receptor may regulate its own biosynthesis or downregulation, we investigated how specifically altering activity of AKT, a crucial kinase in the PI3K signaling pathway which mediates many of the metabolic actions of insulin, affects IR expression in both mouse and human models. AKT has three different isoforms that share greater than 85% sequence homology, all of which play important roles in

growth but vary in their tissue-specific expression. AKT1 and AKT2 are both ubiquitously expressed throughout the organism, with the latter having a higher level of expression in insulin-responsive tissues, such as skeletal muscle, liver and fat (Altomare et al., 1998). The third isoform, AKT3, is expressed at the lowest level and is found solely in the testes and the brain and is not essential for glucose homeostasis (Easton et al., 2005). To further dissect how different steps in the insulin signaling pathway affect IR expression, we utilized both pharmacological and genetic methods to investigate the role of PI3K and mTOR, kinases upstream and downstream of AKT, in regulating IR homeostasis.

Here we provide novel evidence that clearly shows that inhibiting AKT function, in both mouse and human models, increased steady-state protein levels of the insulin receptor through increased transcription and decreased degradation. Altering activity of PI3K or mTOR had a similar but more modest effect on IR expression and highlights the central role that AKT plays in regulating expression of the insulin receptor. Interestingly, similar effects were observed on other RTKs such as EGFR and IGF1-R suggesting that common regulatory mechanisms exist to regulate homeostasis of RTKs. Together, these results suggest that negative feedback mechanisms exist for the insulin signaling pathway at both transcriptional and post-translational levels to regulate RTK stability in mammalian systems.

2.3. Materials and Methods

Miscellaneous

Cell culture reagents were from Mediatech (Manassas, VA). Antibodies were from Cell Signaling Technology (Beverly, MA) except for AKT2 (Stressgen), FOXO1 (Abcam), transferrin receptor (Zymed) and tubulin (Calbiochem). Secondary antibodies were from GE Healthcare. Chemiluminescence reagents were from Pierce and Millipore. Autoradiography film was from MidSci (St. Louis, MO). Wortmannin and rapamycin were from Calbiochem. All other reagents were purchased from Sigma or Fisher Scientific (Fairlawn, NJ).

Protein analysis of mouse embryonic fibroblasts and livers from AKT1$^{-/-}$ mice

Generation of Akt1$^{-/-}$ mice has been previously described (Chen et al., 2001). Primary mouse embryonic fibroblasts (MEFs) were isolated from 14.5-day-old embryos of wild-type C57BL/6J and *AKT1*$^{-/-}$ mice and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2mM glutamine, 0.1mM non-essential amino acids, 1mM sodium pyruvate and 100U/mL penicillin-streptomycin at 37°C in 5% CO_2. MEFs were grown in poly-lysine coated 6-well plates (Falcon) for 24 hours prior to lysis in 100 uL of lysis buffer with thaw-freeze-cycle extraction, as previously described (Peng et al., 2003). Liver samples from wild-type and AKT1 knock-out mice were lysed via sonication in lysis buffer containing protease and phosphatase inhibitors (Sigma). Protein concentration was measured using the Bradford protein quantitation protocol and equivalent amounts of lysate per sample was resolved by SDS-PAGE and then transferred to polyvinylidene difluoride membrane and incubated with a blocking buffer (5% nonfat milk in 0.2% Tween-20 in PBS) for 1 hour at room temperature. The membranes were incubated with primary antibodies overnight at 4°C, washed thrice with 0.2% Tween-

20/PBS, incubated with horseradish peroxidase-conjugated mouse or rabbit secondary antibodies (1:3000-1:5000 dilution) for 2 hours at room temperature, washed five times and detected with enhanced chemiluminescence using West Pico Chemiluminescence (Pierce Chemical Co., Rockford, IL) or Millipore (Billerica, MA). Blots were exposed to autoradiography film and developed using Futura 3000 SV (Fisher Industries, Inc). Bands were quantitated by densitometry using LabWorks software on the Epi Chemi II darkroom system from UVP (Upland, CA).

For multiple blotting, PVDF membranes were stripped by soaking in 2% SDS, 100mM β-mercaptoethanol and 62.5 mM Tris-Cl, pH 6.8 and incubating at 65°C for 30 minutes before re-probing with another primary antibody.

RNA analysis

Total RNA was extracted from frozen tissue or fibroblasts with TRI Reagent (Molecular Research Center, Inc.), and real-time PCR was performed and analyzed using an Applied Biosystems 7900HT Fast Real-Time PCR System with 1X SYBR green master mix (Applied Biosystems) and gene-specific primers (Appendix). PCR conditions were 10 min at 95°C followed by 35 cycles of 10 s at 95°C, 15 s at 60°C. Relative expression levels were determined using the comparative C_T method to normalize target gene mRNA to *GAPDH*.

Transfection and RNAi of 293T HEK cells

Pooled siRNA oligomers targeting AKT or mTOR were obtained from Dharmacon and conditions were optimized for maximum knockdown of the target gene after 72 hours of transfection in 293T human embryonal kidney (HEK) cells. Transient transfections were

performed using Lipofectamine 2000 (Invitrogen) and grown in minimum essential medium (MEM) with Eagle's salts containing 10% heat-inactivated fetal bovine serum, 2mM glutamine, 1mM sodium pyruvate, 0.1mM non-essential amino acids and 100 U/mL of penicillin/streptomycin at 37°C in 5% CO_2. After transfection, cells were washed twice with PBS before lysis in Triton X-100 lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.55, 150mM mM NaCl) and freshly prepared protease inhibitors from Sigma including PMSF (20ug/mL), leupeptin (0.5 ug/mL), aprotinin (0.1 ug/mL), pepstatin (1ug/mL), sodium fluoride (100mM), tetrasodium pyrophosphate (4mM) and sodium orthovanadate (2mM). Separate samples were used for protein and RNA extraction. Protein lysates were processed as described above for immunoblotting. Total RNA was extracted from cells with TRI Reagent (Molecular Research Center, Inc.) and processed for real-time PCR as described above.

Cycloheximide chase

MEFs were washed twice with PBS before incubation with media supplemented with 50 ug/mL of cycloheximide (Calbiochem) at 37°C and 5% CO_2 for increasing time periods. After each time point, cells were washed with PBS and lysed and processed as described above for immunoblotting.

2.4. Results

Transgenic AKT1 knock-out mice have increased IR and EGFR expression and decreased degradation

To investigate the role of AKT in insulin receptor homeostasis, protein and mRNA levels of the IR were compared in embryonic fibroblasts from both wild-type mice and mice where the *akt1* gene ($AKT1^{-/-}$), the most highly expressed AKT isoform in MEFs, was deleted. Regulation of the IR during its biogenesis can be monitored through a series of characteristic gel shifts that correspond with movement of its pro-form (220 kD) from ER to *trans* Golgi, where it is cleaved into α and β subunits of 135 and 90 kD, respectively. Figure 2.2A and B shows that steady-state protein expression of both the uncleaved, immature proIR and the cleaved, mature IR β is increased in MEFs from the $AKT1^{-/-}$ mouse. This increase was similarly observed for the epidermal growth factor receptor (EGFR), another RTK, but not for the transferrin receptor, an unrelated transmembrane protein with no intrinsic signaling activity. When mRNA expression was examined through real-time PCR analysis, a slight increase in IR transcripts was observed in the $AKT1^{-/-}$ mice. Interestingly, EGFR transcripts, but not that of the transferrin receptor, was significantly increased in the AKT1 knockout MEFs, similar to the results obtained by analyzing steady-state protein expression.

A similar analysis of protein and mRNA expression was performed in liver samples from the wild-type and $AKT1^{-/-}$ mice. Figure 2.3 shows that indeed, IR and EGFR protein (Figure 2.3A) and mRNA expression (Figure 2.3B) are also increased in the livers from $AKT1^{-/-}$ mice, similar to the observations seen with the MEFs. In addition, protein levels of downstream insulin signaling components such as mTOR, p70S6K and 4EBP1 were not significantly

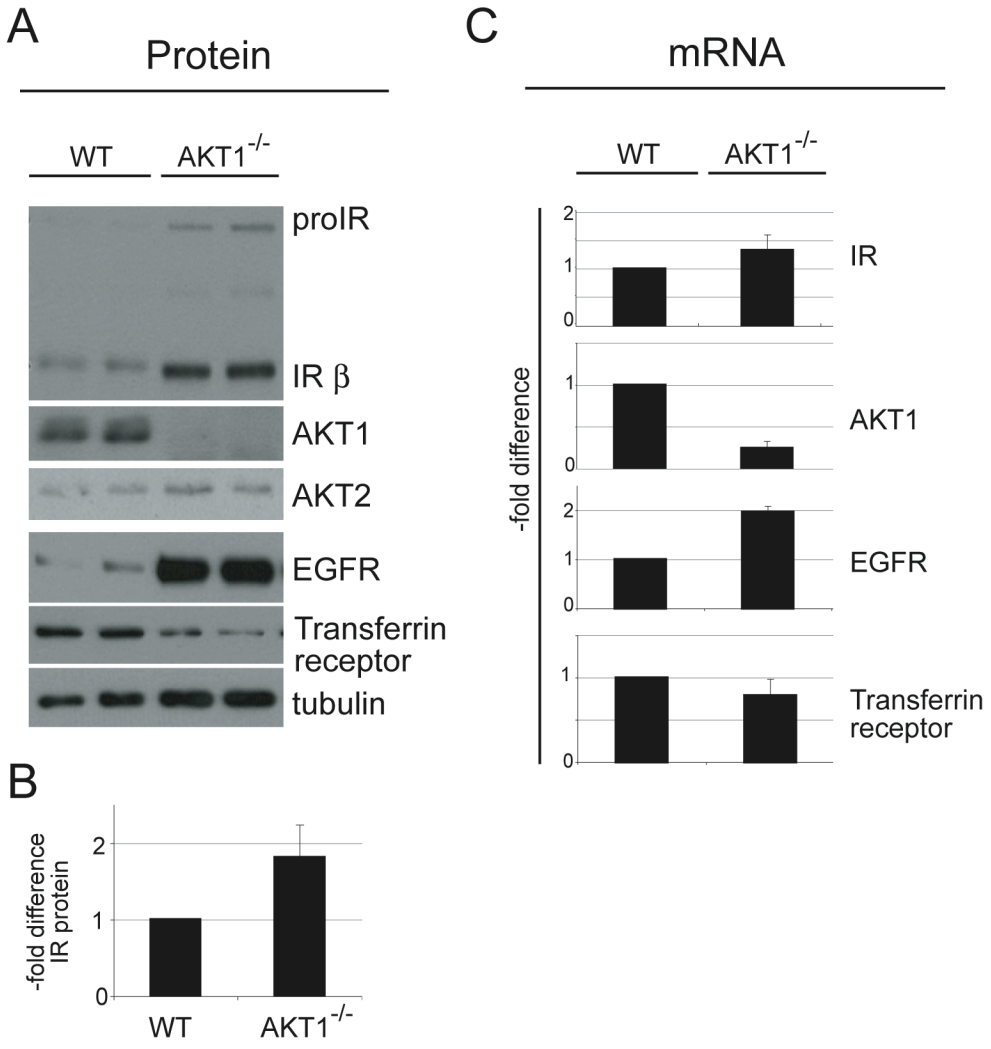

Figure 2.2. Deletion of AKT1 increases IR and EGFR expression in mouse embryonic fibroblasts. *A.* Fibroblasts from 14.5 dpc embryos of wild-type and AKT1$^{-/-}$ mice were grown overnight in 6-well plates, lysed and processed for western blotting, as described in the methods. *B*. Band intensities of IR proteins were measured using densitometric analysis and normalized to tubulin expression (n=4). *C*. Total RNA was extracted from MEFs grown overnight in culture and converted to cDNA prior to real-time PCR analysis using gene-specific primers as described in the methods (n=4).

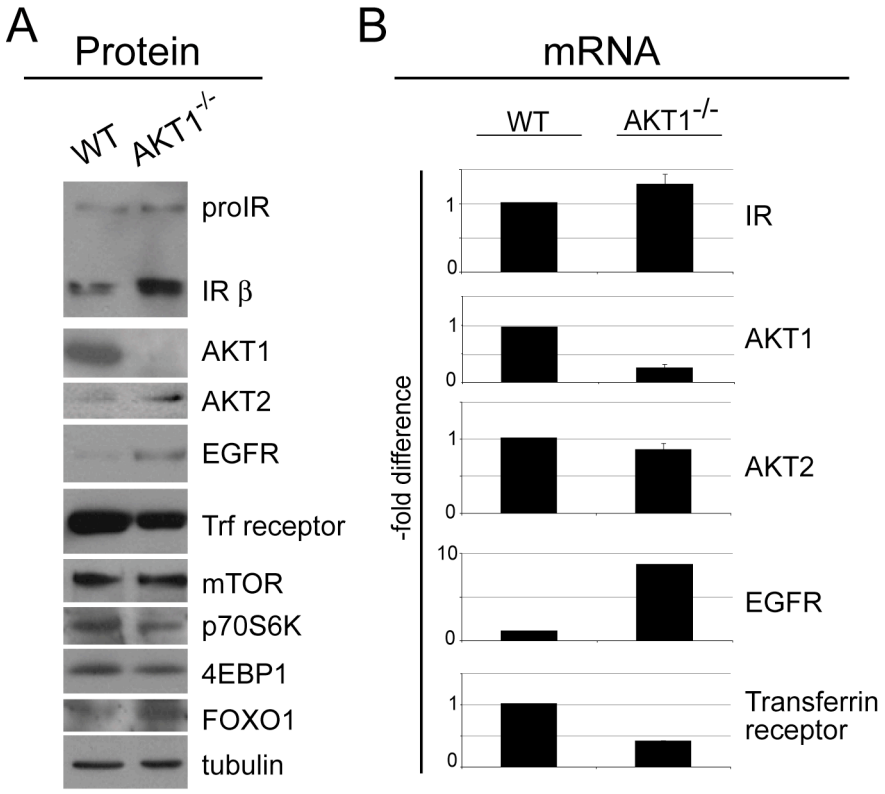

Figure 2.3. RTK expression is increased in liver samples from AKT1$^{-/-}$ mice. *A*. Livers from wild-type and AKT1$^{-/-}$ mice were sonicated and processed for Western blotting, as described in the methods. *B*. RNA from a portion of the same livers in *A* was extracted and converted to cDNA prior to real-time PCR analysis, as described in the methods.

changed. However, basal expression of FOXO1, a transcription factor found to upregulate IR transcription in *Drosophila* (Puig et al., 2003), was increased in AKT1$^{-/-}$ mice. This confirms previous studies which showed that AKT activity is necessary for ubiquitin-mediated proteasomal degradation of FOXO (Plas and Thompson, 2003).

The increase in IR mRNA and immature proIR protein in Figures 2.2 and 2.3 suggests that increased protein synthesis may contribute to the increase in steady-state levels of the IR. However, to test whether a change in rate of degradation also plays a role, cycloheximide (CHX) chase analysis was performed. Comparing lane 4 to lane 1 in Figure 2.4A confirms the increase in immature proIR and mature IR β found in AKT1$^{-/-}$ MEFs, as previously shown in Figure 2.2A. Furthermore, when new protein synthesis is inhibited with CHX, the amount of remaining IR is increased in the absence of AKT1 by approximately 25% (Figure 2.4A and B). Interestingly, a similar effect was observed for EGFR, but not the unrelated transferrin receptor (Figure 2.4C). Furthermore, basal expression of p70S6K, a kinase involved in cap-dependent translation, was increased in the AKT1$^{-/-}$ MEFs and its degradation was similarly attenuated in the absence of AKT1 (Figure 2.4C).

Eliminating AKT in human cells increases RTK expression and decreases degradation

To test whether the loss of AKT would have a similar effect on IR expression in human cells, siRNA oligonucleotides that targeted AKT1 and AKT2, the isoforms most highly expressed in insulin-responsive tissues, were transfected into 293T human embryonal kidney (HEK) cells. AKT1 has been implicated in organismal growth (Chen et al., 2001) while the pivotal role AKT2 plays in the insulin signaling pathway is further demonstrated by the fact that

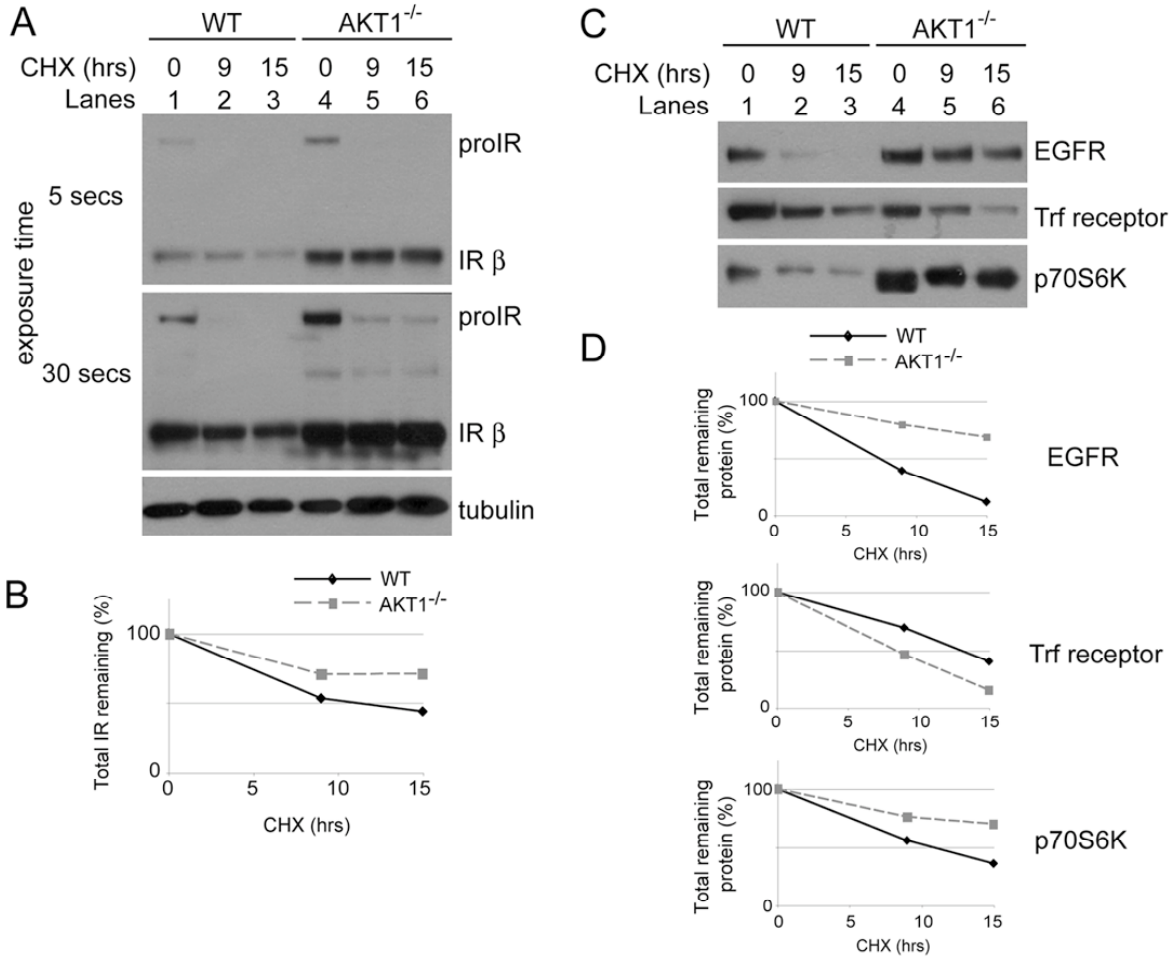

Figure 2.4. Deletion of AKT1 in MEFs attenuates degradation of RTKs. *A.* MEFs from wild-type or AKT1-/- mice were treated with 50 ug/mL of cycloheximide for 9 and 15 hours before cell lysis and processing for Western blotting, as previously described. *B.* IR band intensity from *A* was quantitated and graphed as a percentage of total IR observed before CHX treatment. *C.* The same membrane in *A* was stripped and reprobed using the indicated antibodies. *D.* Band intensities from *C* were quantitated and graphed as a percentage of total protein before CHX treatment.

inactivating mutations in this AKT isoform have been associated with severe insulin resistance and type 2 diabetes (George et al., 2004). Using 293T cells transfected with a scrambled siRNA sequence as a control, a 75% decrease in both AKT1 and AKT2 protein was observed after a 72-hour transfection period (Figure 2.5A). Similar to the results observed in the MEFs and liver samples from wild-type and AKT knock-out mice in Figures 2 and 3, IR protein and mRNA, as well as EGFR protein, was also increased when AKT expression was reduced in human cells (Figure 2.5A and B). Interestingly, p70S6K, a positive regulator of cap-dependent translation was also increased, while 4EBP1, a repressor of cap-dependent translation was decreased (Figure 2.5A). In addition, inactive phosphorylated FOXO1 protein was decreased despite higher protein levels of this transcription factor (Figure 2.5A), suggesting that increased mRNA transcription mediated by active FOXO1 may contribute to the observed increase in steady-state IR levels observed in this human cell line.

To determine if IR degradation in human cells was also decreased when AKT expression was knocked-down, protein turnover was measured when new protein synthesis was inhibited by cycloheximide. Knocking-down AKT expression decreases the rate of degradation of the insulin receptor in 293T cells compared to cells transfected with the control siRNA (Figure 2.5C and D), an effect similar to that observed with MEFs from AKT1 knock-out mice in Figure 2.4. In addition, degradation was also attenuated for the EGFR and IGF-1 receptor, two other members of the receptor tyrosine kinase superfamily, but not for the unrelated transferrin receptor (Figure 2.5E and F).

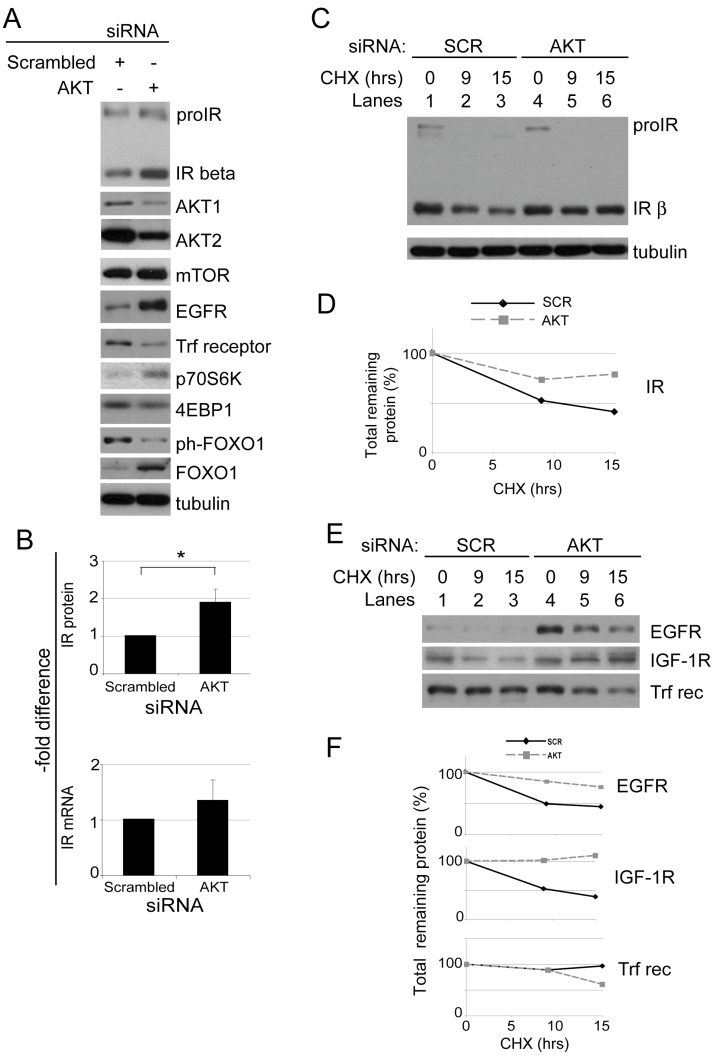

Figure 2.5. AKT knockdown in human 293T HEK cells leads to increased IR expression and decreased RTK degradation. *A*. 293T HEK cells were transfected with pooled siRNA oligomers containing either a scrambled sequence or human AKT. Lysates were extracted after 72 hours and processed for Western blotting, as described previously. *B*. *(Top)* IR band intensities from the immunoblots were quantitated and graphed as a percentage of total IR found in cells transfected with the scrambled siRNA oligomer. (n=4) *(Bottom)* 293T cells transfected with scrambled or AKT siRNA were lysed and mRNA was extracted and processed for real-time PCR analysis, using primers specific to human IR, as described in the methods. (n=4) *C*. 293T cells were transfected with siRNA containing a scrambled sequence or AKT for 72 hours before incubation with 50 ug/mL of cycloheximide (CHX) for 9 and 15 hours. After CHX treatment, cells were lysed and processed for western blotting, as previously described. *D*. IR band intensities from *C* were quantitated and graphed as a percentage of total IR observed before CHX treatment. *E*. The same membrane in *E* was stripped and reprobed using the indicated antibodies. *F*. Band intensities from *E* were quantitated and graphed as a percentage of total protein before CHX treatment.

Regulation of IR expression upstream and downstream of AKT in human cells

To test whether the activity of other kinases within the insulin signaling pathway also affects IR homeostasis, the activity or expression of PI3K and mTOR, kinases upstream and downstream, respectively, of AKT, was altered in 293T cells. Figure 2.6A and B show that inhibiting PI3K function with wortmannin increased both immature proIR and mature IR β. To test whether increased PI3K activity would have the opposite effect on IR protein levels, overexpression of the p110 catalytic subunit of PI3K or another variant, p110-CAAX, was performed. p110-CAAX is a modified p110 that has a COOH-terminal CAAX motif. This motif signals post-translation prenylation leading to membrane localization and activation. In Figure 2.6C and D, we observed that transfection of cDNA with either the catalytic subunit of PI3K or a constitutively active form of PI3K decreased both immature proIR found in the ER and mature IR β.

Inhibiting mTOR function with rapamycin (Figure 2.7A and B) resulted in a similar increase in immature proIR and mature IR β as observed with wortmannin treatment (Figure 2.6A and B). Similarly, when mTOR expression was knocked down using RNAi, steady state expression of IR protein and mRNA (Figure 2.7C and D) was increased. Protein levels of EGFR, another RTK was increased while the unrelated transferrin receptor was unchaged, similar to the results observed in the $AKT1^{-/-}$ mouse (Figures 2.2-2.4) and when AKT was knocked down in human cells (Figure 2.6). Decreased mTOR expression also resulted in an increase in IRS-1, which supports previous results implicating mTOR in facilitating IRS-1 degradation (Berg et al., 2002; Haruta et al., 2000; Pederson et al., 2001; Takano et al., 2001). p70S6K and FOXO1 protein were also increased, similar to results obtained with RNAi of AKT

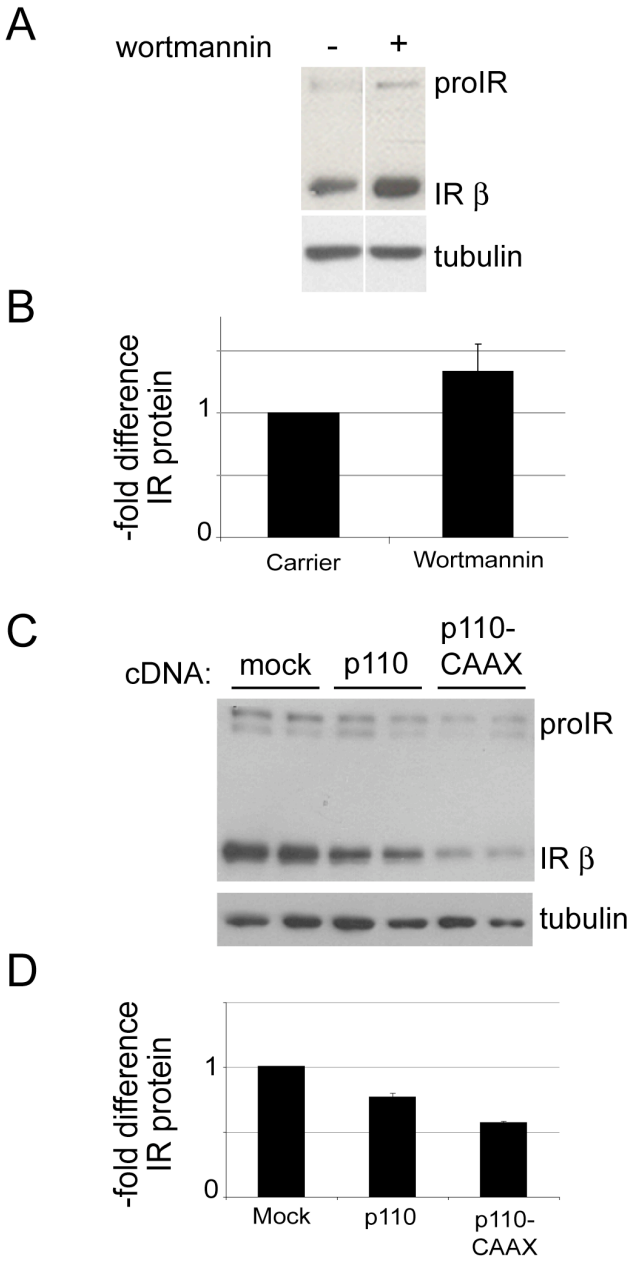

Figure 2.6. PI3K activity negatively regulates IR expression. *A*. 293T HEK cells endogenously expressing human IR were treated with carrier (0.5% DMSO) or wortmannin (1uM) for 20 hours and cell lysates were processed for immunoblotting, as described previously. *B*. IR protein expression in blots from independent experiments were quantitated and graphed (n=4). *C*. 293T HEK cells were transfected with cDNA containing the wild-type p110 catalytic subunit of PI3K or with a constitutively active form of p110 (p110-CAAX). Lysates were processed for immunoblotting, as described previously. *D*. IR protein expression in blots from independent experiments were quantitated and graphed (n=2).

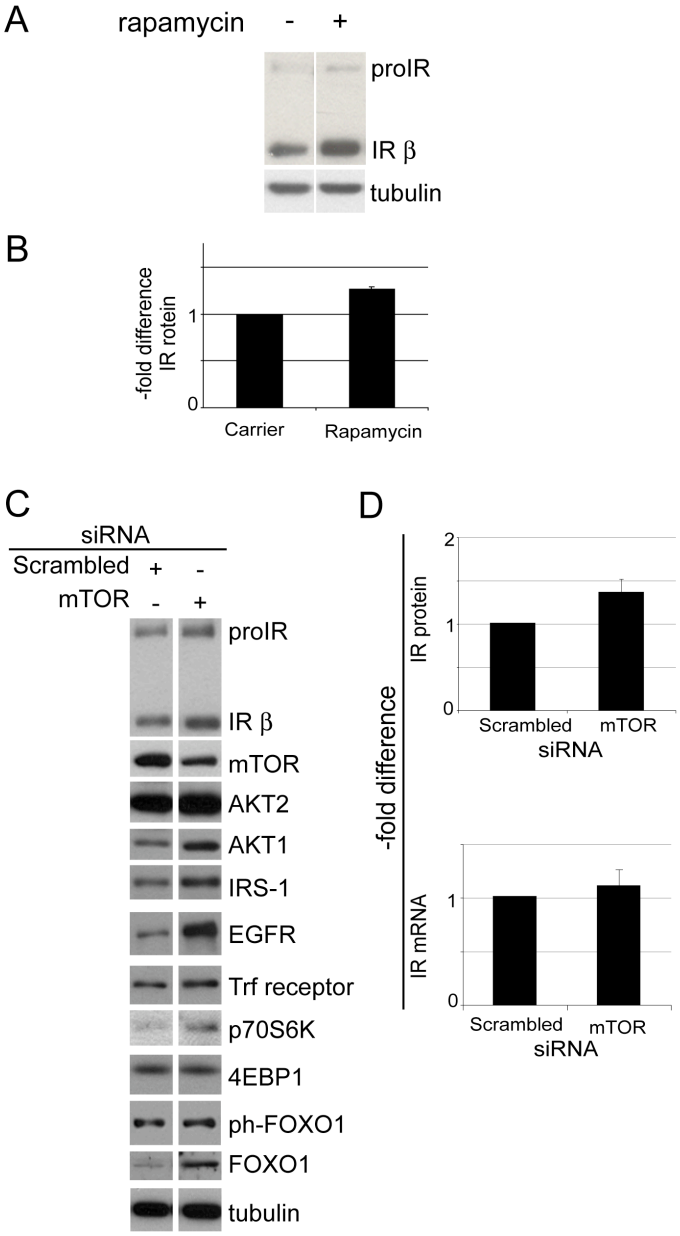

Figure 2.7. Inhibiting mTOR increases RTK levels. *A* . 293T HEK cells endogenously expressing human IR were treated with carrier (0.5% DMSO) or rapamycin (300 nM) for 20 hours and cell lysates were processed for immunoblotting, as described previously. *B.* IR protein expression in blots from independent experiments were quantitated and graphed (n=4). *C.* 293T HEK cells were transfected with scrambled or mTOR siRNA oligomers for 72 hours and then cell lysates were processed for immunoblotting, as described in the methods. *D. Top:* IR band intensities from the immunoblots were quantitated and graphed as a percentage of total IR found in cells transfected with the scrambled siRNA oligomer. (n=4) *Bottom:* 293T cells transfected with scrambled or mTOR siRNA were lysed and mRNA was extracted and processed for real-time PCR analysis, using primers specific to human IR, as described in the methods. (n=4).

in Figure 2.5A but in contrast, 4EBP1 expression and levels of inactive phosphorylated FOXO were unchanged with RNAi of mTOR (Figure 2.7C).

2.5. Discussion

Type 2 diabetes is a growing worldwide epidemic that is also believed to trigger a rise in other associated complications such as neuropathy and cardiovascular disease. Although much work has been done to help understand the complex signaling pathways that are triggered by binding of insulin to its receptor, much remains unknown about the regulatory mechanisms that are involved in maintaining the dynamic balance needed to fine-tune the appropriate physiological response. One of the defining features of type 2 diabetes is the prevalence of diminished sensitivity to insulin, despite upregulated expression of this metabolic hormone. Chronic insulin signaling has been shown to lead to an insulin-resistant state, even in healthy subjects (Del Prato et al., 1994; Iozzo et al., 2001). This feedback inhibition of insulin signaling has been previously attributed to receptor internalization (Marshall, 1985; Trischitta et al., 1989) and negative regulation of kinases downstream of the receptor. However, *in vitro* studies have also shown that this insulin-induced decrease in signaling activity can be attributed to increased degradation of the receptor itself (Cruz et al., 2004; Huang et al., 2002; Okabayashi et al., 1989). Extending this analysis to humans, it has also been shown that obese human patients have reduced insulin receptor expression and that the observed insulin resistance was due to decreased IR expression (Arner et al., 1983; Caro et al., 1987; Kolterman et al., 1981). These studies suggest that increased insulin signaling activity can decrease IR expression. However, the converse hypothesis of whether inhibiting insulin signaling can upregulate insulin receptor expression has not been fully investigated. Earlier studies on tyrosine-kinase deficient IR provided the first clues that inhibiting insulin signaling could prevent downregulation of the receptor (Grako et al., 1992) and increase its mRNA expression (Nishiyama et al., 1994) but the intermediate steps between insulin binding and altered IR expression remained unclear. Here we

provide a more specific approach by targeting kinases downstream of the IR in its primary signaling pathway to provide a better understanding of how insulin signaling regulates insulin receptor expression and degradation.

We show that inhibiting activity of AKT, a critical kinase that mediates the many metabolic and anabolic effects of insulin, leads to an increase in steady-state IR protein levels. The observed increase included higher levels of immature proIR, suggesting that the increased protein expression observed is not solely due to decreased downregulation of the receptor from the cell surface. We propose several mechanisms that contribute to increased IR levels when AKT activity is altered. First, increased IR transcription was observed when AKT activity was decreased in mouse (Figures 2.2C and 2.3B) and human models (Figure 2.5C). This supports a recent study which showed that depriving cells of serum, and thus inhibiting growth factor signaling through receptors on the cell surface, resulted in an increase in IR expression (Puig and Tjian, 2005). The increase in IR mRNA we observe here may be a direct result of upregulated transcription mediated by the transcription factor FOXO1 since an increase in FOXO1 protein expression was similarly detected, along with a decrease of its inactive, phosphorylated form (Figure 2.5A). This correlates well with a converse experiment wherein knocking-down FOXO1 expression in primary hepatocytes showed a decrease in IR expression (Matsumoto et al., 2007). It has been shown that insulin signaling leads to degradation of FOXO itself (Guo et al., 2006). Thus, the increase in steady-state FOXO protein expression may be due to decreased degradation of this transcription factor in the absence of AKT activity. This supports previous studies that showed decreased FOXO1 expression when chicken embryo fibroblasts were transformed with AKT (Aoki et al., 2004) since AKT activity facilitates ubiquitination and proteasomal degradation of FOXO (Plas and Thompson, 2003). Together, these results show a similar role

for AKT in regulating FOXO activity in mouse and human models and highlights its importance in regulating FOXO-mediated IR transcription in mammalian systems as previously observed in *Drosophila* (Puig et al., 2003; Puig and Tjian, 2005).

Putative FOXO1 binding sites were found in the promoter region of the highly-related IGF1-R (Xuan and Zhang, 2005) and we observed increased expression of this RTK when AKT activity was deleted. Further studies are needed to confirm if IGF1R is a target gene downstream of FOXO1. Interestingly, we also observed a significant increase in transcription levels of EGFR (Figures 2.2C and 2.3B), another important RTK, raising the intriguing possibility that FOXO1 may also be involved in regulating transcription of other members of the RTK family.

Previous studies have shown that although AKT2 is overexpressed in 12% of ovarian carcinomas and 3% in breast carcinomas, no significant association between AKT2 and amplification of ErbB2, an epidermal growth factor receptor isoform, has been found (Bellacosa et al., 1995). Our results here may help explain this phenomenon since we see that increased AKT expression actually negatively regulates expression of RTKs such as the IR, EGFR and IGF1R. However, increasing insulin receptor expression through upregulation of FOXO may not be enough to overcome insulin resistance, since increased FOXO activity may also lead to increased cellular glucose production (Nakae et al., 2002) by upregulating G-6-Pase and PEPCK, rate-limiting enzymes involved in glycogenolysis and gluconeogensis (reviewed by (Greer and Brunet, 2005)).

Second, the increase in steady-state IR protein levels, and that of other RTKs such as EGFR and IGF1-R, can also be attributed to a decrease in the rate of degradation, as analyzed by cycloheximide chase analysis (Figures 2.4 and 2.5). This observation is in agreement with previous studies which showed increased proteasomal degradation of the insulin receptor after

both acute and prolonged insulin signaling (Cruz et al., 2004; Huang et al., 2002; Okabayashi et al., 1989) and suggests that AKT activity may play an important role in regulating insulin receptor turnover. In addition, AKT may also play a previously unrecognized role in regulating turnover of other important RTKs such as EGFR and IGF1-R.

Increased steady-state levels of p70S6K in the AKT knock-out MEFs (Figure 2.4C) and in 293T cells wherein AKT was knocked-down (Figure 2.5A) suggest that upregulation of cap-dependent protein translation may also play a role in the observed increase in IR expression upon AKT inhibition. However, since AKT activity has been shown to be required for p70S6K activation and repression of 4EBP-1 (reviewed in (Whiteman et al., 2002)), events necessary for cap-dependent translation, increased cap-dependent protein translation may probably have a modest contribution to the observed increase in IR protein levels. A possible alternative may be internal ribosome entry site (IRES)-mediated translation. AKT has been shown to play a role in IRES-mdiated translation of cellular mRNAs including that of cyclin D1 (Shi et al., 2005a). This, combined with the recent discovery of internal ribosome entry sites in *Drosophila* IR mRNA (Marr et al., 2007) and in rat IGF1-R (Giraud et al., 2001), suggests that cap-independent, IRES-mediated translation may play a role in increased IR protein levels when AKT activity is attenuated. IRES sequences have also been found in mRNA for fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) (reviewed in (Baird et al., 2006)), ligands for other RTKs and it would be interesting to see if similar sequences are found in the untranslated regions in the corresponding RTK transcripts.

Investigating other components of the primary insulin signaling pathway, we observe similar effects on upregulation of IR expression when activity of kinases upstream and downstream of AKT was inhibited. PI3K lies upstream of AKT as it phosphorylates lipids on

the cell membrane upon insulin stimulation, which then recruit AKT and enable its activation. When PI3K activity was inhibited with wortmannin, insulin receptor protein expression was increased (Figure 2.6A and B). Conversely, we observe that increasing PI3K activity by introduction of a constitutively active PI3K lead to a decrease in steady-state insulin receptor levels (Figure 2.6C and D). Previous studies have shown that inhibiting PI3K activity with wortmannin (Burgering and Coffer, 1995) or ablating the catalytic subunit p110α (Zhao et al., 2006), decreased AKT activation. Recently, it was shown that overexpression of a phosphatase that dephosphorylates PI3K phospholipids also reduced AKT activity and increased nuclear localization of FOXO1 (Cho et al., 2006). Thus, the effects we observe on IR expression with altered PI3K activity may be due to negative feedback mechanisms mediated by AKT and FOXO 1 as described above.

A critical downstream effector of AKT is the serine-threonine kinase mTOR. When mTOR activity was inhibited either by treatment with a specific inhibitor rapamycin (Figure 2.7A) or through RNAi (Figure 2.7B), insulin receptor expression was increased. This IR upregulation we observe after inhibiting mTOR activity supports previous results which showed that constitutively elevated mTOR signaling in the absence of its inhibitor, tuberous sclerosis complex (TSC), results in almost undetectable levels of insulin receptor protein (Shah et al., 2004). Similarly, TSC-null cell lines have reduced expression of the PDGF receptor, another RTK (Zhang et al., 2003). However, although an increase in p70S6K was observed with knock-down of mTOR, protein levels of the translational repressor 4EBP1 was not significantly changed. This raises the possibility that instead of cap-dependent translation, the increase in IR protein levels with mTOR inhibition in mammalian systems may instead be attributed to IRES-mediated translation. In addition, we also observed a more subtle increase in IR levels when

mTOR expression was knocked down compared to when AKT expression was reduced (compare Figure 2.7C with Figure 2.5B). This blunted effect may be due to a compensating increase in AKT1 expression upon mTOR inhibition. This observation is consistent with a previous study which saw an increase in AKT levels when mTOR shRNA was used to knock-down mTOR expression in *Drosophila* (Sarbassov dos et al., 2005). It has also been previously shown that blocking mTOR function or expression with rapamycin or RNAi, respectively, results in increased AKT signaling possibly due to attenuation of mTOR-mediated IRS-1 degradation (Khamzina et al., 2005; Shi et al., 2005b; Tremblay et al., 2005; Wan et al., 2006). In addition, the increased FOXO1 expression observed with mTOR siRNA may bind to TSC2 (Cao et al., 2006) and lead to degradation of this indirect mTOR inhibitor, attenuating the reduction in mTOR activity. Thus, the increase in AKT activity that accompanies inhibition of mTOR activity may dampen the negative feedback that mTOR activity may have on IR homeostasis.

Even though disrupting AKT in *Drosophila* impaired normal cell survival during embryogenesis and decreased cell size (Scanga et al., 2000; Verdu et al., 1999), reduced AKT1 activity was well tolerated in a mammalian mouse model and it was suggested that this kinase can be used as a target for therapy in cancers where the PI3K/AKT pathway is constitutively activated (Chen et al., 2001). However, our results here suggest that caution must be taken with this strategy as inhibiting AKT activity can serve to upregulate receptor tyrosine kinase expression, which may lead to increased signaling of other growth-related pathways and may not effectively diminish tumor growth and development. Furthermore, regulation of IR expression by insulin signaling is by no means a linear pathway and various factors come together to control receptor expression and degradation. For example, deleting PTP1B, which dephosphorylates

activated IR, resulted in increased insulin signaling but had no observable effect on IR levels (Elchebly et al., 1999).

Taken together, these results suggest that insulin signaling downstream of the receptor leads to a negative feedback mechanism to regulate insulin receptor expression and stability and that AKT plays a central role in maintaining IR homeostasis. As the PI3K/AKT signaling cascade can be triggered by signaling through receptors for many growth factors and cytokines, including epidermal growth factor (EGF) family members, insulin, and IGF-I, it was not surprising to see that inhibiting AKT expression also increased protein levels of IGF1-R and EGFR. Recent studies have suggested a possible connection between insulin receptor expression and cancer progression (reviewed in (Denley et al., 2003)). This suggests that common mechanisms mediated by AKT signaling exist to regulate RTK expression and degradation. Observations from these studies may thus provide greater insight in both metabolic disease and neoplasia and help in the development of better therapies for diabetes and cancer.

Chapter 3

Role of Redox Flux and PI3K Signaling in IR Homeostasis

3.1. Abstract

Receptor tyrosine kinases (RTKs) comprise a large group of transmembrane receptors with intrinsic tyrosine kinase activity that play an essential role in eukaryotic cell growth, metabolism and survival. Given their crucial role in mediating signals from extracellular cues, cell surface expression of these receptors is a dynamic process that is highly sensitive to metabolic flux. Studies in our lab and others have revealed that the insulin receptor (IR), a class II RTK, is subject to intracellular degradation upon nutrient deprivation. However, the molecular mechanisms linking metabolic homeostasis and growth factor signaling are not completely understood understood. Here we show that both inhibition of glycolysis and mitochondrial oxidative phosphorylation result in reduction of IR protein levels, suggesting that glucose metabolism is linked to IR biogenesis. Interestingly, we found that the decrease in steady-state IR protein expression due to ATP depletion is attenuated when PI3K signaling through AKT and mTOR is blocked by either pharmacological or genetic manipulation. Together, these results indicate that new pathways interconnecting insulin signaling with metabolic flux may reflect a coordinated and reciprocal regulation of IR homeostasis.

3.2. Introduction

Receptor tyrosine kinases (RTKs) represent a class of integral membrane proteins that are involved in numerous signaling pathways within the cell, including cell proliferation, differentiation, migration and metabolism. RTK signaling and expression is tightly controlled and is highly sensitive to extracellular cues. However, the detailed mechanisms that coordinate RTK homeostasis with metabolism remain poorly understood. The insulin receptor (IR) is a prototypical RTK that facilitates uptake of glucose to regulate cell metabolism and gene expression, making it an excellent tool to investigate how nutrient availability and receptor signaling coordinately regulate IR homeostasis.

IR levels are highly dynamic and inappropriate expression and activity of these receptors may have profound consequences. Decreased cell surface expression of the IR has been implicated in acquired forms of insulin resistance and may contribute to the pathogenesis of Type 2 diabetes (Pessin and Saltiel, 2000; Shulman, 2000; Taylor, 2002). The insulin resistance found in patients exhibiting impaired glucose tolerance was also attributed to decreased IR expression (Kolterman et al., 1981). Interestingly, other studies have shown that glucose withdrawal from adipocyte cells *in vitro* induces insulin receptor proteolysis, possibly due to depletion of uridine diphosphate-N-acetyl-glucosamine and impaired N-linked glycosylation and/or activation of the cell stress response (Hwang and Frost, 1999; Rossetti, 2000) suggesting that nutrient availability may impact receptor expression and stability.

Recent studies have highlighted the ability of the cell to sense the nutrient status of in its environment via activation of key proteins sensitive to amino acid availability and intracellular levels of ATP. Two important proteins that have been underscored in these studies are AKT and mTOR, serine-threonine kinases that play crucial roles in mediating insulin-induced signaling to

facilitate glucose metabolism. The serine/threonine kinase AKT is activated by PI3K and is an upstream positive regulator of the mammalian target of rapamycin (mTOR). Activated AKT maintains intracellular ATP levels and inhibits AMP-activated protein kinase (AMPK), an enzyme activated by ATP depletion (Hahn-Windgassen et al., 2005). Inhibition of AMPK prevents phosphorylation of tuberous sclerosis complex 2 (TSC2), an inhibitor of the Rheb GTPase that activates mTOR (Inoki et al., 2003). In addition to its sensitivity to availability of amino acids, mTOR has been shown to be inhibited by low intracellular ATP levels, independent of the abundance of amino acids (Dennis et al., 2001).

We have recently discovered that inhibiting PI3K/AKT/mTOR signaling upregulates IR expression in mammalian systems and leads to increased receptor levels in the cell. Thus, we hypothesized that inhibiting insulin signaling may help attenuate the degradation induced by metabolic inhibition. Here we demonstrate that inhibiting glycolysis and oxidative phsophorylation in the cell specifically decreased total insulin receptor protein levels and diminished its cell surface expression. However, inhibiting PI3K/AKT/mTOR signaling with either pharmacological reagents or through RNAi attenuated this decrease. Together, these results show that ATP depletion and insulin signaling can coordinately regulate insulin receptor homeostasis and help provide insights for development of better therapies for Type 2 diabetes.

3.3. Materials and Methods

Miscellaneous

Cell culture reagents were from Mediatech (Manassas, VA). Antibodies were from Cell Signaling Technology (Beverly, MA) except for AKT2 (Stressgen), FOXO1 (Abcam), transferrin receptor (Zymed) and tubulin (Calbiochem). Secondary antibodies were from GE Healthcare. Chemiluminescence reagents were from Pierce and Millipore. Autoradiography film was from MidSci (St. Louis, MO). Wortmannin and rapamycin were from Calbiochem. 2-deoxyglucose (2DG) and sodium azide were purchased from Sigma Aldrich (St. Louis, MO).

Cell lines

293T HEK and HepG2 cells were grown in minimum essential medium (MEM) with Eagle's salts containing 10% heat-inactivated fetal bovine serum, 2mM glutamine, 1mM sodium pyruvate, 0.1mM non-essential amino acids and 100 U/mL of penicillin/streptomycin at 37°C in 5% CO_2.

$AKT1^{-/-}$ mouse embryonic fibroblasts

Generation of $Akt1^{-/-}$ mice has been previously described (Chen et al., 2001). Primary mouse embryonic fibroblasts were isolated from 14.5-day-old embryos of wild-type C57BL/6J and $AKT1^{-/-}$ mice and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2mM glutamine, 0.1mM non-essential amino acids, 1mM sodium pyruvate and 100U/mL penicillin-streptomycin at 37°C in 5% CO2. MEFs were grown in poly-lysine coated 6-well plates (Falcon) for 24 hours prior to lysis in 100 uL of lysis buffer with thaw-freeze-cycle extraction, as previously described (Peng et al., 2003).

Transfection and RNAi of 293T HEK cells

Short interfering RNA (siRNA) oligomers targeting AKT or mTOR were obtained from Dharmacon and conditions were optimized for maximum knockdown of the target gene after 72 hours of transfection in 293T human embryonal kidney (HEK) cells. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) and grown in minimum essential medium (MEM) with Eagle's salts containing 10% heat-inactivated fetal bovine serum, 2mM glutamine, 1mM sodium pyruvate, 0.1mM non-essential amino acids and 100 U/mL of penicillin/streptomycin at 37°C in 5% CO_2. After transfection, cells were washed twice with PBS before lysis in Triton X-100 lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.55, 150mM mM NaCl) and freshly prepared protease inhibitors from Sigma including PMSF (20ug/mL), leupeptin (0.5 ug/mL), aprotinin (0.1 ug/mL), pepstatin (1ug/mL), sodium fluoride (100mM), tetrasodium pyrophosphate (4mM) and sodium orthovanadate (2mM). Separate samples were used for protein and RNA extraction. Protein lysates were processed as described above for immunoblotting. Total RNA was extracted from cells with TRI Reagent (Molecular Research Center, Inc.) and converted to cDNA using a high-capacity cDNA archive kit from Applied Biosystems (Foster City, CA). Real-time PCR was performed with primers specific for human insulin signaling genes (Appendix A.1) and 1X SYBR green master mix and analyzed using an Applied Biosystems 7900HT Fast Real-Time PCR System using *GAPDH* expression as a control.

Immunoprecipitation, gel electrophoresis and immunoblotting

Cell were lysed in Triton X-100 lysis buffer (1% Triton X-100, 50 mM HEPES, pH 7.55, 150mM mM NaCl) and freshly prepared protease inhibitors from Sigma including PMSF (20ug/mL), leupeptin (0.5 ug/mL), aprotinin (0.1 ug/mL), pepstatin (1ug/mL), sodium fluoride (100mM), tetrasodium pyrophosphate (4mM) and sodium orthovanadate (2mM). Equivalent amounts of lysate protein from the different samples, as determined using the Bradford protein quantitation protocol, were immunoprecipitated by incubating with 0.5 uL of 8314, an antibody against the α subunit of the human hIR, overnight at 4°C. After overnight incubation, protein A-agarose (Pierce) was added and allowed to mix with the sample for 1-3 hours at 4°C. Samples were briefly microfuged and 10 uL of the supernatant was set aside to use for blotting with tubulin for comparision of total protein concentration in each immunoprecipitated sample. The immune complexes in the pellet were washed with lysis buffer twice then diluted in concentrated Laemmli sample buffer to yield a final 1X concentration (2% SDS, 50 mM Tris, 2mM EDTA, 0.01% bromophenol blue, 10% glycerol and 144 mM β-mercaptoethanol). Redissolved samples were boiled in for 10 minutes prior to electrophoresis through 5% stacking and 8% running (5/8%) gel in a Bio-Rad Mini-Protean III Electrophoresis tank at 120 V for two hours. Laemmli sample buffer was added to the supernatant that was initially set aside as a protein loading control and these samples were also processed for electrophoresis and blotted with anti-tubulin (1:5000).

Immunoblotting was performed after electrophoresis by transferring the proteins from SDS-PAGE to Immobilon P (membrane presoaked for ~1 min in methanol; Millipore Corp., Waters Chromatography, Milford, MA), in buffer containing 25 mM Tris-Hcl, pH 7.4, 192 mM glycine and 20% methanol at 4°C and either 85 V for 2 hrs or 30 V overnight. The membranes

were soaked in blocking buffer (5% wt/vol nonfat dry milk and 0.2% Tween 20 vol/vol in PBS) for 1 hour and then incubated overnight at 4°C overnight with the primary antibody (or at room temperature for 1 h) while mixing. The membranes were rinsed again in 0.2 % Tween 20/PBS, incubated with HRP-linked secondary antibodies, and then rinsed and developed using Pierce West Pico ECL reagents.

Biotinylation

To compare insulin receptor expression levels, cells endogenously expressing the receptor were washed with PBS (with no Mg^{++} or Ca^{++}) and then incubated with 0.5 mg/mL of sulfo-N-hydroxysulfosuccinimide ester-biotin (Pierce Chemical Co.) for 30 min. on ice. Monolayers were washed in PBS containing 15 mM glycine and then lysed in Triton X-100 lysis buffer. Immunoprecipitation and electrophoresis and membrane transfer was as described above. Membranes were blocked with 1% BSA (Sigma, Fraction V) overnight and probed with streptavidin-HRP (1:3000 dilution; Amersham Pharmacia Biotech, Inc.). ECL was used to visualize the biotinylated receptors (Pierce Chemical Company). Blots were subsequently stripped and re-probed with anti-hIR β subunit antibodies (1:10,000).

3.4. Results

Insulin receptor protein expression is specifically decreased by the metabolic inhibitors 2DG and sodium azide

The mature IR is initially synthesized as a single chain preproreceptor (proIR) containing a signal peptide that directs it co-translationally to the endoplasmic reticulum (ER). The proreceptor is composed of an α-subunit, containing the insulin binding site, and the β- subunit, containing a single transmembrane domain and a tyrosine kinase domain. Upon translocation into the lumen of the ER and signal peptide cleavage, the proIR undergoes extensive asparagine-linked glycosylation at 17 sites within the α-subunit (Ullrich et al., 1985). Glycosylation is important in receptor biosynthesis and function since either mutating the glycosylated asparagine residues in the IR (Caro et al., 1994; Elleman et al., 2000) or inhibiting glycosylation results in decreased cell surface expression and insulin signaling (Ronnett et al., 1984). Following dimerization in the ER, the IR is transported to the Golgi apparatus where the proIR dimer undergoes proteolytic cleavage at a subtilisin-cleavage site (RKRR↓) essential for high-affinity insulin binding. After cleavage, the mature receptor is delivered to the surface as a heterotetramer with a stoichiometry of 2α and 2β subunits (Ullrich et al., 1985). Thus, regulation of the IR during its biogenesis can be monitored by a series of characteristic gel shifts which correspond with movement of its pro-form (220 kDa) from the ER to the *trans* Golgi, where it is cleaved into α and β subunits of 135 and 90 kDa, respectively (Olson et al., 1988).

In models of non-insulin dependent Type 2 diabetes, such as the Goto-Kakisaki (GK) rat, it has been shown that the significant decrease in glucose uptake in the liver corresponds with a reduction in the number of insulin receptors (Bisbis et al., 1993). Furthermore, we and others have previously demonstrated that glucose deprivation and insulin resistance lead to reduced

expression of the insulin receptor in human models (Bass et al., 1998; Hwang et al., 2000). More recently, we demonstrated that following exposure of cells to 2-deoxyglucose (2DG), a non-metabolizable glucose analogue that blocks the production of UDP-glucose, we observed a reduction in the amount of immature proIR and mature IR β and abnormalities in the gel migration of the proIR, consistent with impaired glycan processing (Ramos et al., 2007). To further investigate the effect of depleting cellular ATP on IR protein expression, sodium azide, an inhibitor of the cytochrome C oxidase-complex IV of the mitochondrial transport chain (Gasparini et al., 1997), was combined with 2DG. Treatment with 2DG, in combination with other metabolic inhibitors of ATP synthesis, for up to 8 hours, has previously been shown to reduce cellular ATP by up to 80% (Gunn et al., 1985) with varying effects on cell viability. To determine optimum incubation times using 2DG and sodium azide, a preliminary time-course experiment in 293T human embryonic kidney fibroblasts was performed. Figure 3.1 demonstrates that treatment with metabolic inhibitors effectively reduced cell surface expression of the IR, but not that of an unrelated transmembrane protein, the transferrin receptor. The decrease in total IR was observed starting at one hour of treatment and was sustained with up to four hours of incubation.

To further investigate if the effects of metabolic inhibitors were specific to the insulin receptor in human cells, both 293T HEK cells and HepG2, a human hepatocellular liver carcinoma cell line, were used for 2DG and sodium azide treatment. Treatment with 2DG and sodium azide reduced total IR proteins, including those found on the cell surface, in both cell lines (Figure 3.2). None of the downstream insulin signaling proteins such as AKT, mTOR, p70S6K, 4EBP1 or FOXO1, were affected, nor were protein expression levels of the unrelated transferrin receptor altered.

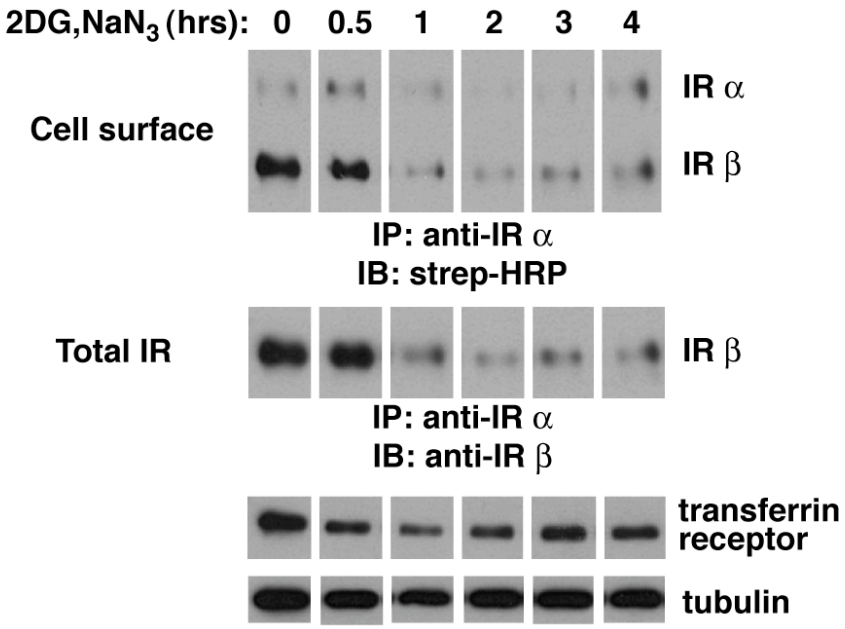

Figure 3.1. Metabolic inhibition decreases cell surface IR expression and total protein steady-state levels. 293T HEK cells endogenously expressing insulin receptors were treated with 2-deoxyglucose (50mM) and sodium azide, NaN_3 (0.05%) for increasing time periods. After treatment, cells were biotinylated, lysed and processed for Western blotting (as described in the methods).

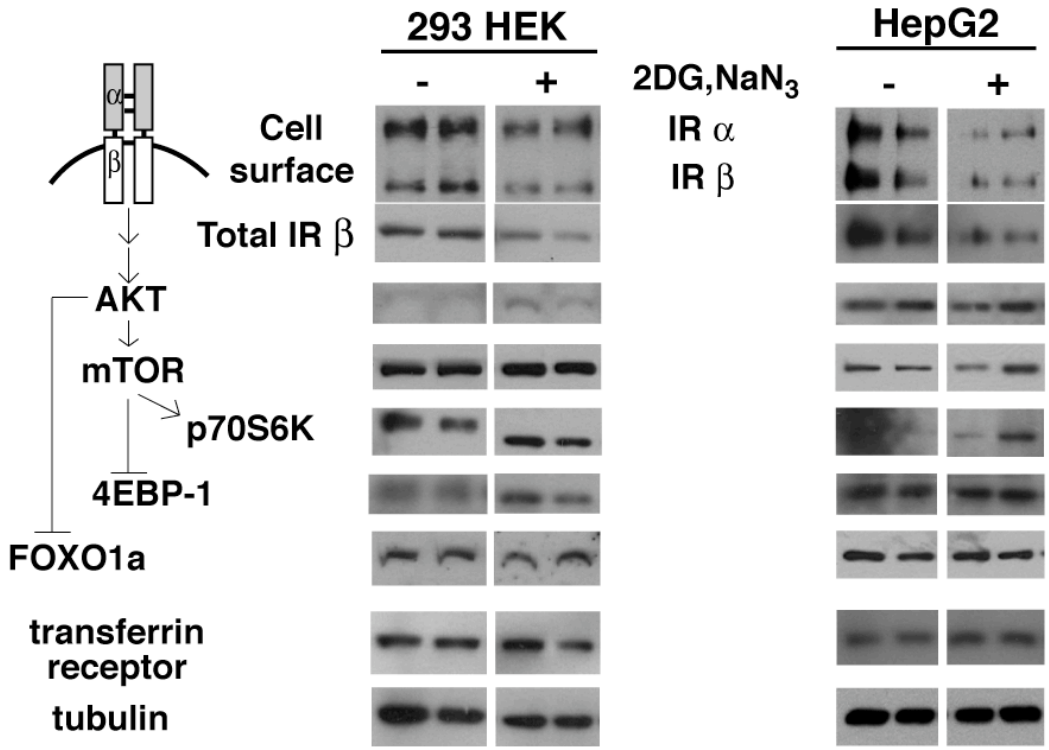

Figure 3.2. Metabolic inhibition specifically decreases cell surface IR expression and total IR protein in both human kidney and liver cell lines. 293T HEK (*left)* and HepG2 cells (*right*) endogenously expressing insulin receptors were treated with 2-deoxyglucose (50mM) and sodium azide, NaN_3 (0.05%) for four hours. After treatment, cells were biotinylated, lysed and processed for Western blotting (as described in the methods).

Inhibiting PI3K or mTOR activity increases IR expression and attenuates reduction induced by 2DG and sodium azide

Previous studies have shown that chronic insulin treatment leads to a reduction in insulin receptors, facilitating development of insulin resistance. In particular, studies showing that persistent activation of PI3K leads to insulin resistance (Egawa et al., 2000; Egawa et al., 1999), combined with observations that inhibiting mTOR leads to increased insulin receptor expression in partially hepatectomized rats (Escribano et al., 2002), suggested to us that inhibiting the PI3K signaling pathway may affect insulin receptor expression. Indeed, inhibiting PI3K or mTOR with wortmannin or rapamycin, respectively, increased IR protein levels in 293T cells (Figure 3.3, lanes 9-12). Furthermore, PI3K or mTOR inhibition served to attenuate the degradation induced by the metabolic inhibitors 2DG and sodium azide (Figure 3.3, lanes 3-8).

Genetic manipulation of AKT and mTOR attenuate degradation induced by metabolic inhibitors

To confirm whether the observed upregulation in IR protein levels with wortmannin and rapamycin treatment was due to specific inhibition of the PI3K signaling pathway, genetic manipulation was also performed. RNAi of AKT and mTOR was performed by transfecting siRNA oligomers containing AKT or mTOR-specific sequences into 293T HEK cells endogenously expressing the insulin receptor. After a 72-hr transfection period, cells were lysed and protein expression of the targeted gene, as well as the insulin receptor, were measured using Western blotting. RNAi of either mTOR or AKT lead to increased insulin receptor expression but did not affect protein levels of the unrelated transferrin receptor (Figure 3.4A), similar to results previously shown in Chapter 2.

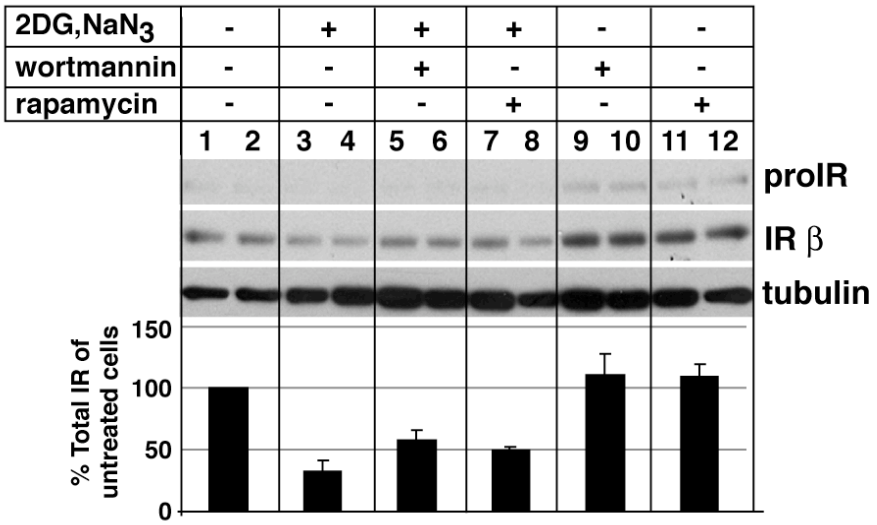

Figure 3.3. Inhibiting PI3K or mTOR increases IR expression and attenuates the decrease in receptor levels induced by metabolic inhibition. 293T HEK endogenously expressing insulin receptors were treated with 2-deoxyglucose (50mM) and sodium azide, NaN_3 (0.05%), wortmannin (1uM), rapamycin (20 nM) or a combination of these treatments, for hours. After treatment, cells were lysed and processed for Western blotting (as described in the methods). Band intensities were measured using densitometric analysis and IR expression normalized to tubulin was quantitated.

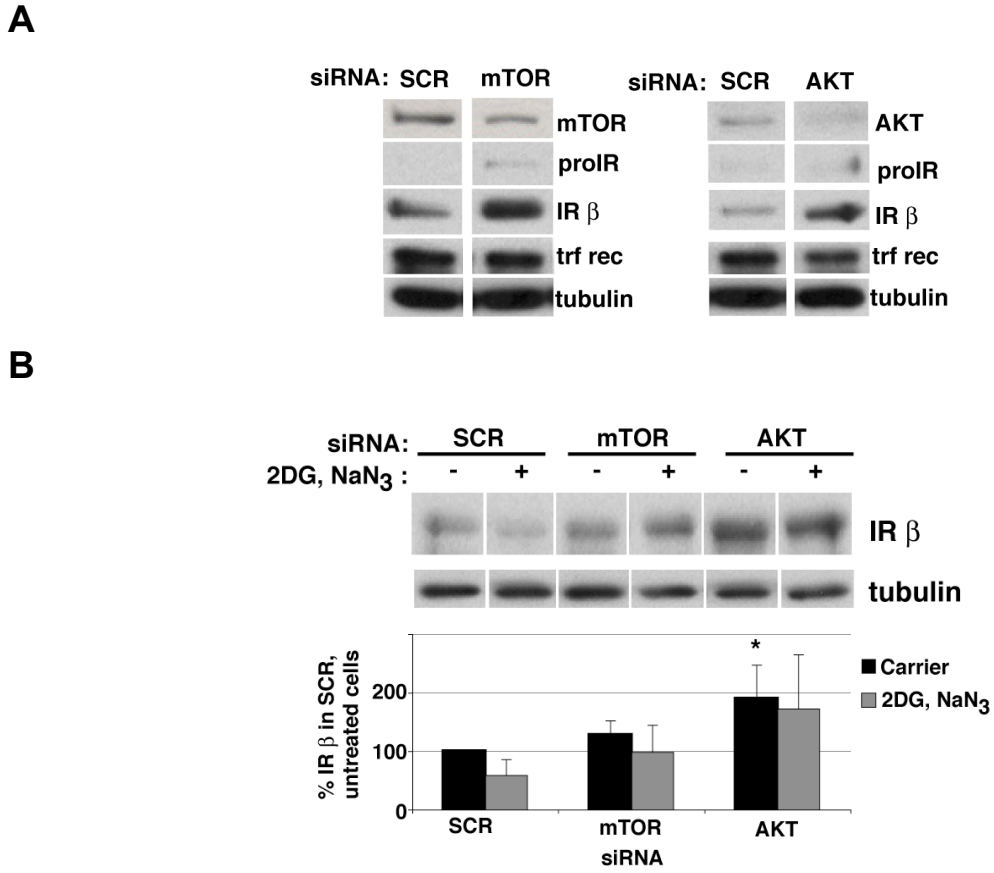

Figure 3.4. RNAi of mTOR or AKT2 increases IR levels and attenuates diminished receptor expression induced by metabolic inhibitors. *A*. 293T HEK endogenously expressing insulin receptors were transfected with siRNA oligomers containing a scrambled sequence (SCR), mTOR or AKT. After 72 hours, transfected cells were lysed and processed for Western blotting, as described in the methods. *B*. 293T HEK cells were transfected with siRNA, as described for A. After transfection, cells were treated with carrier or 2-deoxyglucose (50mM) and sodium azide, NaN_3 (0.05%), for four hours. After treatment, cells were lysed and processed for Western blotting, as described avove. Band intensities were measured using densitometric analysis and IR expression normalized to tubulin was quantitated. * $p < 0.05$ compared to untreated cells transfected with SCR siRNA oligomers.

Furthermore, 2DG and sodium azide treatment of cells transfected with mTOR and AKT siRNA showed attenuated reduction in insulin receptor protein levels (Figure 3.4B), similar to the effect observed with pharmacological inhibition of PI3K and mTOR with wortmannin and rapamycin, respectively.

To further test the hypothesis that inhibiting AKT signaling can upregulate IR expression, we measured insulin receptor content in mouse embryonic fibroblasts (MEFs) from mice where AKT1, the most ubiquitous isoform in MEFS, was deleted (Chen et al., 2001). Figure 3.5A confirms results previously shown in Chapter 2 that demonstrates increased insulin receptor protein levels in $AKT1^{-/-}$ MEFs. In addition, treating the wild-type and $AKT1^{-/-}$ MEFs with 2DG and sodium azide showed a blunted effect on insulin receptor proteins induced by the metabolic inhibitors, confirming the results observed with RNAi of AKT in human fibroblasts.

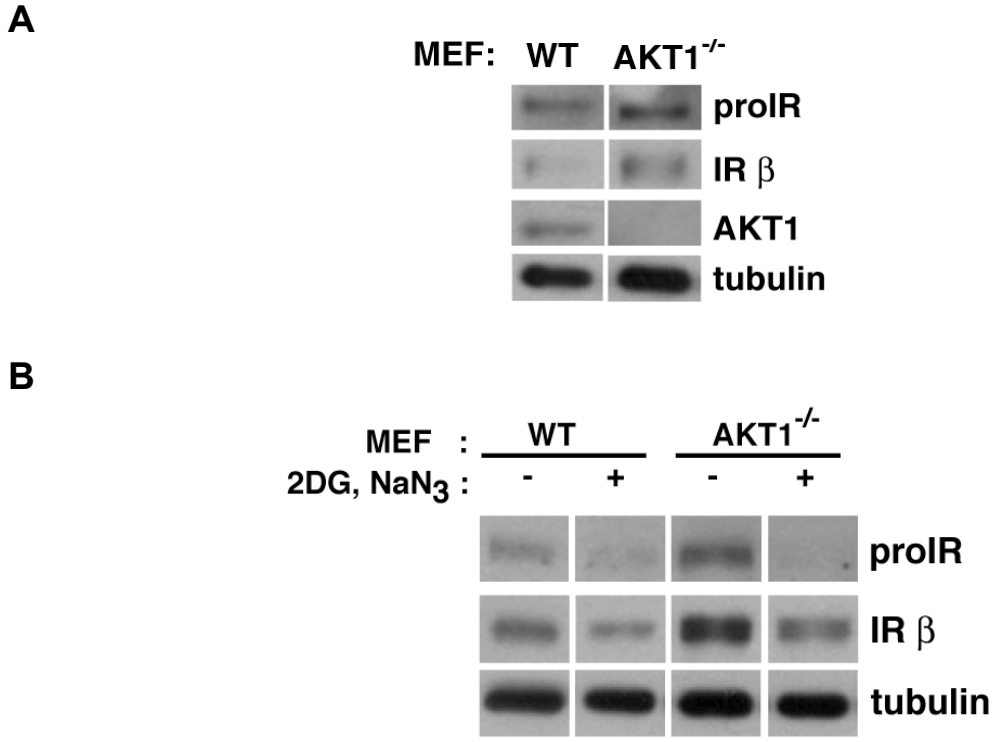

Figure 3.5. Deletion of AKT1 in mouse embryonic fibroblasts increased IR levels and attenuated diminished receptor expression induced by metabolic inhibitors. *A*. 293T HEK endogenously expressing insulin receptors were transfected with siRNA oligomers containing a scrambled sequence (SCR), mTOR or AKT2. After 72 hours, transfected cells were lysed and processed for Western blotting, as described in the methods. *B*. 293T HEK cells were transfected with siRNA, as described for A. After transfection, cells were treated with carrier or 2-deoxyglucose (50mM) and sodium azide, NaN_3 (0.05%), for four hours. After treatment, cells were lysed and processed for Western blotting, as described avove.

3.5. Discussion

Insulin receptor signaling plays a vital role in cell growth and metabolism and it is important to understand how nutrient and energy availability affect insulin receptor expression. Here we showed that decreasing intracellular ATP via the metabolic inhibitors, 2DG and sodium azide, specifically decreased insulin receptor protein levels, without altering protein expression of other insulin signaling proteins or an unrelated transmembrane protein. Mitochondrial dysfunction has been implicated in the development of insulin resistance (Petersen et al., 2003) and type 2 diabetes (Lowell and Shulman, 2005) and our observations here support the role of mitochondrial function in ATP production to mediate insulin receptor expression. Furthermore, RTK overexpression has been observed in a variety of cancers and the decreased IR expression observed after metabolic inhibition may provide a possible explanation for the increased sensitivity of tumor cells to glycolytic inhibitors when oxidative phosphorylation was inhibited (Liu et al., 2001).

The decrease in IR protein levels with metabolic inhibition may have been mediated by a decrease in insulin receptor mRNA expression, as suggested by previous studies involving glucose starvation (Briata et al., 1990). However, decreased cellular ATP mediated by 2DG and sodium azide elicits effects distinct from those shown by Puig *et al* wherein they demonstrated that serum-deprivation of insect cells leads to an increase in IR expression (Puig and Tjian, 2005; Puig and Tjian, 2006). These results suggest that availability of glucose or ATP may trigger mechanisms that regulate IR expression that are distinct from those mediated by growth factor signaling.

The increased IR expression we observe after inhibiting PI3K/AKT/mTOR signaling on IR expression is in agreement with previous observations from serum deprivation experiments

demonstrating negative feedback on IR levels. Inhibition of insulin signaling reflects a similar situation in which cells are starved of growth factors and thus both conditions lead to increased insulin receptor expression. In addition, it has been shown that inhibition of PI3K activity phenocopies starvation in *Drosophila* (Britton et al., 2002) and our observations here further strengthens the similarity in effects on IR expression mediated by inhibiting insulin signaling activity with growth factor deprivation (Puig and Tjian, 2005; Puig and Tjian, 2006).

Furthermore, glycosylation plays an important role in protein processing of the insulin receptor (Hwang and Frost, 1999; Leconte et al., 1994) and the effect of metabolic inhibitors on post-translational maturation of the IR protein would need to be addressed, in addition to its role in IR mRNA transcription. Moreover, effects on protein synthesis itself will also need to be considered as mammalian translation factors have been shown to be affected by nutrient availability (Proud, 2002). The decrease in IR protein levels we observe with metabolic inhibitors may be due to decreased protein translation, induced by depletion of intracellular ATP.

Together, our observations from this study show that depleting intracellular ATP with both glycolytic and mitochondrial inhibitors specifically reduced IR protein expression and that this decrease was attenuated by inhibiting signaling through the receptor through either pharmacological or genetic manipulation of PI3K, AKT or mTOR. Thus, redox flux and insulin signaling through PI3K both coordinately regulate IR expression.

3.6. Acknowledgments

We thank Veronique Nogueira for her help with growth and maintenance of the mouse ebryoinc fibroblasts from the *AKT1*$^{-/-}$ mice. This work was supported by the National Institutes of Health (1RO3DK62056-01), Astellas and an International Fellowship from the American Association of University Women (AAUW) to R. R. R. We also acknowledge the generous support of the Lazarus Family Foundation and the Charles Walgreen Jr. Trust.

Chapter 4

Calreticulin and Hsp90 Stabilize the Human Insulin Receptor and Promote Its Mobility in the Endoplasmic Reticulum

The work presented in Chapter 4 has been published with the following citation:
Ramos, R.R., Swanson, A.J., Bass, J. (2007). Calreticulin and Hsp90 stabilize the human insulin receptor and promote its mobility in the ER. PNAS *104*, 10470-10475.

4.1. Abstract

Elimination of misfolded membrane proteins in the ER affects cell survival and growth and can be triggered by either local physiologic events or disease-associated mutations. Regulation of signaling receptor degradation involves both cytosolic and ER lumenal molecular chaperones but the mechanisms and timing of this process remain uncertain. Here we report that calreticulin (CRT) and Hsp90 exert distinct effects on the stability and cell surface levels of native and misfolded forms of the human insulin receptor (hIR) and a human variant found in type A insulin resistance. CRT was unique in stabilizing the disease variant and in augmenting hIR expression when glycolysis was abrogated. Effects of Hsp90 were independent of receptor tyrosine phosphorylation and did not change levels of downstream signaling kinases. Live cell imaging revealed that movement of the hIR through the ER was accelerated by misfolding or by overexpression of either CRT or Hsp90. Together, our results indicate that both CRT and Hsp90 control expression of hIR at its earliest maturation stages and modulate its movement within the ER prior to either degradation or cell surface expression.

4.2. Introduction

The early maturation of transmembrane signaling receptor tyrosine kinases (RTKs) occurs in the endoplasmic reticulum (ER) under the surveillance of an intricate quality control (QC) system that redirects mutant or improperly processed proteins to ER-associated degradation (ERAD) (reviewed in (McCracken and Brodsky, 2005; Meusser et al., 2005)). The level of expression and activity of RTKs affects cell metabolism and growth and is thought to involve a network of protein folding enzymes, molecular chaperones and proteases on both sides of the ER membrane. However, how these individual ER and cytosolic factors collaborate to redirect mutant or misprocessed receptors to degradation and the factors that modulate cell surface levels of wild-type RTKs under basal conditions are not fully known.

Studies of exogenous viral membrane protein folding have led to a model implicating glycan maturation as a key step in signaling receptor quality control (Hammond et al., 1994). This process begins with the binding of calnexin (CNX) and calreticulin (CRT) to N-linked carbohydrates, and proceeds with cycles of glucosylation and deglucosylation that are coupled to maturation and CNX/CRT dissociation (Molinari et al., 2004; Parodi, 2000a). Thus, CNX/CRT are candidates for control of RTK expression. In addition to ER lumenal chaperones, RTK expression is also influenced by Hsp90, one of the most abundant cytosolic proteins. Inhibiting Hsp90 leads to degradation of RTKs through effects that involve autophosphorylation (Cabrera et al., 1996). However, Hsp90 also participates in ERAD of non-phosphorylated ER proteins including the cystic fibrosis transmembrane conductance regulator (CFTR) (Loo et al., 1998).

An additional important mechanism involved in control of RTK expression involves the movement of these proteins through the ER, a process dependent upon the rate of folding, oligomerization, and binding and dissociation from both ER lumenal and cytosolic chaperones.

Here we have analyzed the spatiotemporal dynamics in the maturation and quality control of the human insulin receptor (hIR), a prototypical RTK, to better understand stages in receptor expression that affect its function and delivery to the cell surface. Previous studies have established distinct stages in the maturation of the hIR, a major RTK involved in metabolism and energy balance. Receptor folding, glycosylation and dimerization are post-translational steps involved in the acquisition of the active binding site for insulin (Elleman et al., 2000; Olson et al., 1988). Previously, we exploited chemical crosslinking to identify the major molecular components associated with the nascent insulin receptor and the impact of N-linked glycosylation on IR biogenesis (Bass et al., 1998). We found that the immature receptor monomer is present in complexes comprised of the glycan-specific chaperones CNX and CRT in addition to BiP, and that CRT was the predominant ER lumenal chaperone associated with the newly translated receptor. Previous studies have also shown that Hsp90 interacts with the hIR on its cytosolic surface. However, the effect of Hsp90 and the possible involvement of kinase phosphorylation in receptor degradation remain controversial. Thus, to better define the roles of CRT and Hsp90 in ERAD of the hIR, we exploited novel bioluminescent forms of both the native protein and a misfolded disease variant and investigated how interactions with lumenal and cytosolic chaperones influence the steady state expression of functional receptors.

4.3. Materials and Methods

Miscellaneous

Molecular biology reagents were purchased from Pierce, Cell Signaling and Stressgen. The mAb 83-14 was a gift from K. Siddle (Cambridge, UK). Purified bovine insulin was from Novo Nordisk. The Hsp90 cDNA was a gift of Dr. R. Morimoto (Northwestern University) and subcloned into pBK-CMV. The calreticulin cDNA was a gift of Dr. M. Michalak (University of Alberta).

Mutagenesis of hIR tyrosine kinase domain

Site-directed mutagenesis of the hIR was conducted using QuikChange® (Stratagene). Forward/reverse strand primers for hIR^{Y3F} were 5'–GACCAGAGACATCTTTGAAA CGGATTTCTTCCGGAAAGG-3'/5'CCTTTCCGGAAGAAATCCGTTTCAAAGATCTCTCG CTC-3' for mutagenesis of tyrosines in the catalytic triad of the hIR kinase domain at amino acid position 1158, 1162 and 1163 to phenylalanine (the mutated nucleotides are underlined).

Cloning of hIR-GFP

The terminal region of the 3'end of wild-type hIR from pSel-hIR was amplified with *Pfu* Turbo polymerase using forward and reverse primers (5'-GACGGTACCAGATCTTGCG CATGTGCTGGCAATTC-3'/5'-CGAGGGGCCCGGAAGGATTGGACCGAGGCAAG-3'), to facilitate cloning hIR in-frame into the pEGFP-N2 vector (Clontech). The 355 bp PCR product was blunt ligated into pCR-BluntII-TOPO (Invitrogen). An EcoRI/ApaI fragment was then subcloned into the MCS of pEGFP-N2. An XbaI/EcoRI fragment of pSel-HIR was then

subcloned into the NheI/EcoRI sites of pEGFP-N2. The entire hIR-GFP cDNA was sequenced at restriction junctions and across the entire coding region of the hIR.

Biotinylation, immunoprecipitation, gel electrophoresis and immunoblotting

Transient transfections were performed in 293T HEK cells using Lipofectamine 2000 (Invitrogen) and grown in conditions as described previously. Biotinylation, cell lysis, immunoprecipitation, gel electrophoresis and immunoblotting were performed as previously described (Bass et al., 1998). For studies of IR expression, total protein was measured (Bradford assay) and equivalent protein amounts were immunoprecipitated with anti-IR α antibody (8314) or loaded directly onto an SDS gel. Tubulin blotting was performed on all Western blots to confirm equivalent amounts of total loaded protein.

Metabolic labeling

293T cells were starved for 1 hour by preincubation in met/cys-free DME containing 5% dialyzed FBS. Pulse labeling was then performed by addition of met/cys-free medium containing 200 μCi/mL of [^{35}S] methionine and [^{35}S] cysteine for 1 hr and chased with cold media for up to 18 hours. After each time point, cells were washed and equivalent amounts of lysates were immunoprecipitated with anti-IR α (8314) and processed as described previously.

Photobleaching and live cell imaging

293T HEK cells were seeded on 35-mm dishes with coverslip bottoms obtained from MatTek (Ashland, MA) and transfected with the appropriate cDNA. After transfection, cells were either incubated with 5 ug/mL of brefeldin A (Sigma) for a minimum of 6 hours, or dishes were

directly mounted onto a Zeiss 510 confocal laser scanning microscope and maintained at 37ºC for the duration of the experiment. The 488-nm and 405-nm lasers and 100x plan apo objective were used in imaging. An ER-specific dye, ER-Tracker-Blue-White (Invitrogen), was used to specifically label the ER prior to targeted photobleaching. A laser power of 2.5-5% transmission was used in image acquisition and 100% transmission was used for photobleaching. For FRAP analysis, a single image was taken at 4^{th} zoom power and an area of ~1μm^2 was bleached for 3 seconds (100 iterations), after which an image was collected every 2 seconds until fluorescence recovery had plateaued. Relative fluorescence intensity (RFI) and mobile fraction were determined as described previously (Kim et al., 2002). Since the photobleached region was a circular area, the diffusion coefficient was determined for two-dimensional recovery in spot photobleaching (Axelrod et al., 1976). Briefly, an RFI versus time curve was fit to the equation $F_t=F_{final}[1-\exp(-\pi Dt/\omega^2)]$, where ω^2 is the bleached area and D is the effective diffusion coefficient. Prism software 4.0 (GraphPad Software) was used to determine the D value that allowed best-fit with earlier time points and to perform one-way ANOVA to test for statistical significance.

4.4. Results

Calreticulin, an ER chaperone, buffers degradation of the insulin receptor

Expression of unliganded RTK proteins in mammalian cells varies under different physiologic conditions and is also linked to protein structural maturation. Regulation of hIR during its biogenesis can be monitored through a series of characteristic gel shifts that correspond with movement of its pro-form (220 kD) from ER to *trans* Golgi, where it is cleaved into α and β subunits of 135 and 90 kD, respectively (Olson et al., 1988). We and others have previously demonstrated that glucose deprivation and insulin resistance lead to reduced expression of the insulin receptor (Bass et al., 1998; Hwang et al., 2000). For example, following exposure of cells to 2-deoxyglucose (2DG), a non-metabolizable glucose analogue that blocks the production of UDP-glucose, we observed a reduction in the amount of immature proIR and mature IR β (Figure 4.1A, left panel, lane 2)), and abnormalities in the gel migration of the proIR, consistent with impaired glycan processing. The magnitude of receptor degradation caused by 2DG was similar to the effects of inhibition of glycan modification with castanospermine (CST), an inhibitor of glucosidases I and II in the ER (Figure 4.1A, right panel, lane 2). The decrease in steady-state levels of the receptor was due to proteasomal degradation since treatment with lactacystin, a potent proteasomal inhibitor, attenuated the reduction in hIR induced by 2DG. We also used pulse-chase labeling to determine whether the reduction in receptor levels was due to increased degradation. Figure 4.1B shows that pretreatment with 2DG and CST decreased the stability and increased degradation of the receptor. Together, these observations indicated that impaired glycan maturation and/or reduced availability of intracellular UDP-glucose trigger receptor downregulation.

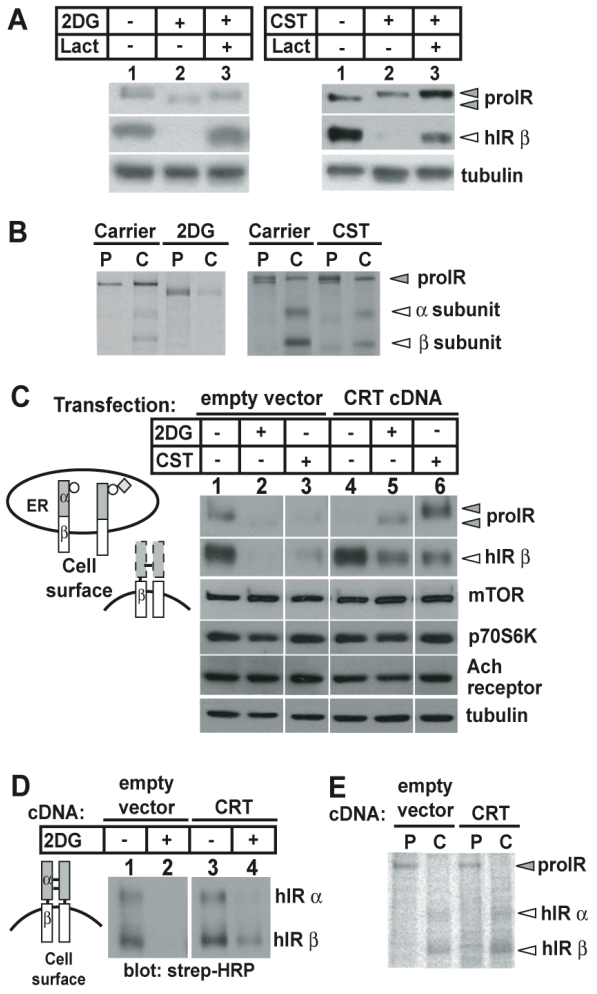

Figure 4.1. ER chaperone CRT selectively attenuates ER-associated degradation of IR induced by altered N-linked glycosylation. *A*. 293T cells stably expressing hIR were treated with 50mM 2DG for 20 hrs (left panel) or 1mM CST for 72 hrs (right panel) with or without 4.5 μM lactacystin. Cell lysates were processed as described previously and immunoblotted with anti-IR β and anti-tubulin. Closed and open triangles refer to proIR and hIR β subunits, respectively. *B*. Cells were pre-treated with 2DG or CST prior to performing pulse-chase analysis. Lysates from the pulse (P) and 6-hr chase (C) were processed as described previously. *C*. Cells were transfected with an empty vector or with CRT cDNA and treated with 2DG or CST and processed as above. Circles and diamonds indicate N-linked glycans and terminal glucose, respectively. *D*. Cells stably expressing hIR were transfected with an empty vector or with CRT cDNA and treated with 2DG as above. Cell surface receptors were biotinylated and analyzed as described previously. *E*. Pulse-chase was performed on cells transfected with an empty vector or with CRT cDNA. Lysates were harvested immediately after the pulse (P) and 6-hr chase (C) and processed as described previously.

In previous studies we identified hIR-CRT interactions as an important step linking glucose metabolism to receptor maturation (Bass et al., 1998). We therefore sought to determine whether changes in CRT levels affect expression of the hIR in cells grown under normal concentrations of glucose or following inhibition of glycolysis. We found that CRT overexpression overcame degradation of the hIR induced by either 2DG or CST (Figure 4.1C, lanes 5-6). Interestingly, CRT did not alter basal expression of two signaling proteins downstream of the IR, mTOR and p70S6K (reviewed in (Wullschleger et al., 2006)), consistent with its restricted role in maturation of proteins in the secretory pathway. Moreover, the effect of CRT on hIR was selective since expression of another transmembrane glycoprotein, the muscarinic acetylcholine (Ach) receptor, was unchanged under identical conditions. More importantly, under both basal conditions and following glycan misprocessing, CRT increased not only steady-state levels of the nascent hIR proreceptor and total processed β-subunit, but also increased the cell surface expression of the mature receptor (Figure 4.1D). In addition, pulse-chase experiments also showed that CRT overexpression increased the stability of mature α and β subunits of the hIR (Figure 4.1E). These results indicate that CRT may function independently of glycan binding to stabilize the hIR. Interestingly, glycan-independent interactions of CRT have also been observed with IgY, citrate synthase and malate dehydrogenase (Leach et al., 2002; Saito et al., 1999).

Second, we directly tested the effects of CRT on expression of a missense mutant variant of the receptor (hIRmut) (Figure 4.2A) that we discovered in a young woman with classical type A insulin resistance (Bass et al., 2000). We previously reported that the hIR^{mut} protein is present primarily as an immature precursor (proIR) that is converted to an 80 kD species that is rapidly

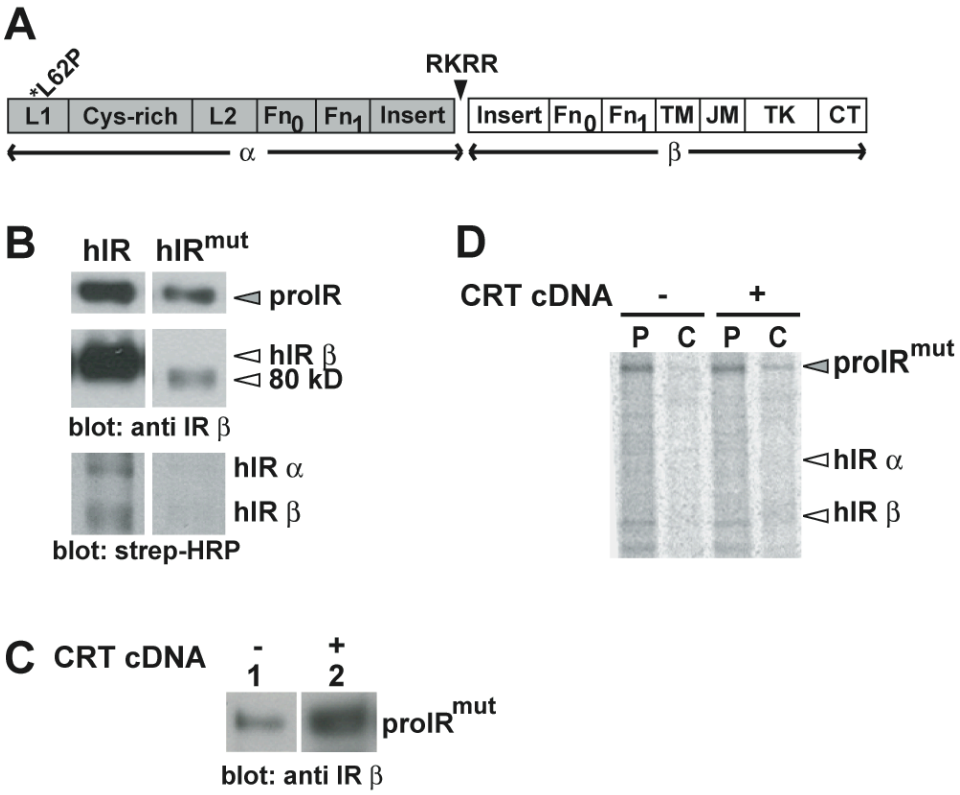

Figure 4.2. CRT abrogates ER-associated degradation of IR from subject with congenital type A insulin resistance. *A*. The Leu→Pro mutation at aa62 (*L62P) is in the first leucine-rich repeat domain (L1) of the IR. Protein domains in the IR include: leucine-rich repeat domains (L1 and L2), cys-rich region, fibronectin type III domains (Fn_0, Fn_1, Fn_2) and Fn_1 insert, transmembrane (TM), juxtamembrane (JM) and tyrosine kinase (TK) domains, and carboxy-terminal tail (CT). Furin recognition at the RKRR site cleaves the polypeptide into α and β subunits. *B*. 293T cells were transfected with wild-type hIR or hIR^{mut} cDNA. Cell surface receptors were biotinylated and processed as described previously. *C*. Cells were transfected with hIR^{mut} cDNA alone or with CRT cDNA and processed for Western blotting or for pulse-chase analysis (D), as described previously.

degraded and unable to reach the cell surface (Figure 4.2B). When we examined processing of the hIR^{mut} variant, we found that glycan maturation was altered and interactions with calreticulin were prolonged (Bass et al., 1998). However, the effect of CRT on maturation and homeostasis of the mutant receptor was not previously investigated. Here, we show that overexpression of CRT increased the steady-state levels of hIR^{mut} (Figure 4.2C) and stabilized the $proIR^{mut}$ (Figure 4.2D). Nonetheless, CRT had no effect on cell surface expression of hIR^{mut} (data not shown). Taken together, our results suggest that CRT buffers the expression of the native wild-type protein and has the capacity to attenuate degradation when glucose metabolism is abrogated. However, severe structural disruption caused by human mutations in the hIR gene could not be overcome by CRT overexpression.

Cytoplasmic Hsp90 stabilizes the nascent insulin receptor

In addition to binding to CRT within the lumenal surface of the ER, the transmembrane IR kinase is positioned in the cytoplasm where it interacts with cytosolic Hsp90. Since the effects of Hsp90 and its mechanism of action in controlling RTK expression have not yet been fully elucidated, we next examined how the cytosolic chaperone Hsp90 might affect levels of wild-type receptors. We took advantage of the ansamycin geldanamycin (GA), an Hsp90 inhibitor, to delineate the impact of cytoplasmic Hsp90 on receptor maturation, as GA has been previously shown to exhibit 3-fold higher affinity for cytoplasmic Hsp90 than for ER lumenal Grp94 (Xu et al., 2001), and association of hIR with Grp94 was not observed by co-immunoprecipitation (Bass et al., 1998). In contrast to our studies with glycan inhibition, we found that inhibitors of Hsp90 led to complete degradation of the fully processed cell surface

receptor and an increase in immature proIR (Figs. 4.3A and B, lanes 1 and 2). Receptor degradation following inhibition of Hsp90 was blocked by lactacystin, a potent proteasomal inhibitor (Figs. 4.3A and B, lane 3). Furthermore, lysosomal function was not required for ansamycin-mediated hIR degradation since addition of a lysosomal inhibitor, ammonium chloride, did not abrogate degradation induced by GA (Figure 4.3C, lane 4). Since previous studies had demonstrated a role for the kinase domain in degradation of the homologous RTK, ErbB2 (Xu et al., 2001), we tested the effect of GA on a kinase-dead insulin receptor variant (hIR^{Y3F}) (Figure 4.3D). Surprisingly, the kinase-dead insulin receptor variant was still degraded in the presence of GA (Figure 4.3E), indicating that Hsp90 interactions involve the non-phosphorylated receptor. We also found that overexpression of Hsp90 led to increased levels of the mature β subunits of hIR (Figure 4.3F, lane 3) whereas inhibition of Hsp90 with GA caused the accumulation of the immature species (Figure 4.3F, lanes 2 and 4). We conclude that Hsp90 function is important in the conversion of immature proIR to its mature α/β subunits. These studies also reveal that Hsp90 selectively affects hIR levels since the downstream levels of mTOR, and the unrelated transferrin receptor, were not altered under identical conditions (Figure 4.3F).

Non-overlapping function of CRT and Hsp90 in hIR quality control

To test whether the effects of CRT and Hsp90 on hIR expression were functionally overlapping or distinct, we tested the reciprocal effects of each chaperone in the presence of either glycolytic inhibition (2DG) or Hsp90 blockade (GA). Figure 4.4A, lane 3 shows that CRT increased the basal level of hIR expression in cells grown in standard medium and following

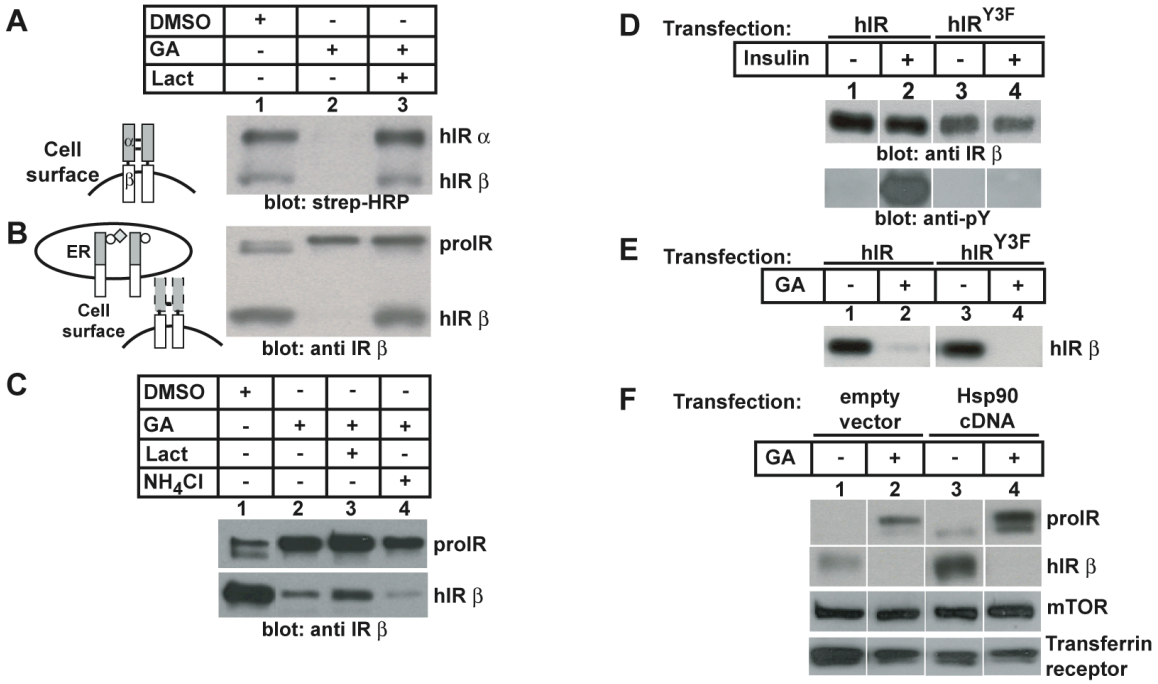

Figure 4.3. Kinase-independent ansamycin degradation of the hIR is attenuated by Hsp90. *A*. 293T cells expressing wild-type hIR were treated with either carrier, 4.5 μM GA, or both GA and 9.5μM lactacystin, for 20 hrs. Cell surface receptors were biotinylated and processed as described previously. *B*. Blot in *A* was stripped and re-probed with anti-IR β. Circles and diamonds indicate N-linked glycans and terminal glucose, respectively. *C*. 293T cells were treated with GA and either lactacystin or ammonium chloride (25mM) for 20 hrs. and lysates were processed as described previously. *D*. 293T cells were transfected with either wild-type hIR or hIR^{Y3F} cDNA, serum-deprived for 24 hours before treatment with 10^{-7} M insulin for 5 mins. Cell lysates were immunoprecipitated with anti-IR α (8314) and processed for Western blotting with anti-IR β (top) and anti-phosphotyrosine antibodies (bottom). *E*. Cells were transfected with either wild-type hIR or hIR^{Y3F} cDNA and treated with either carrier (0.5%DMSO) or 4.5μM GA for 20 hours. Cell lysates were processed as described previously. *F*. 293T cells stably expressing wild-type hIR were transfected with an empty expression vector or Hsp90 cDNA and treated with either DMSO or GA and processed as described previously.

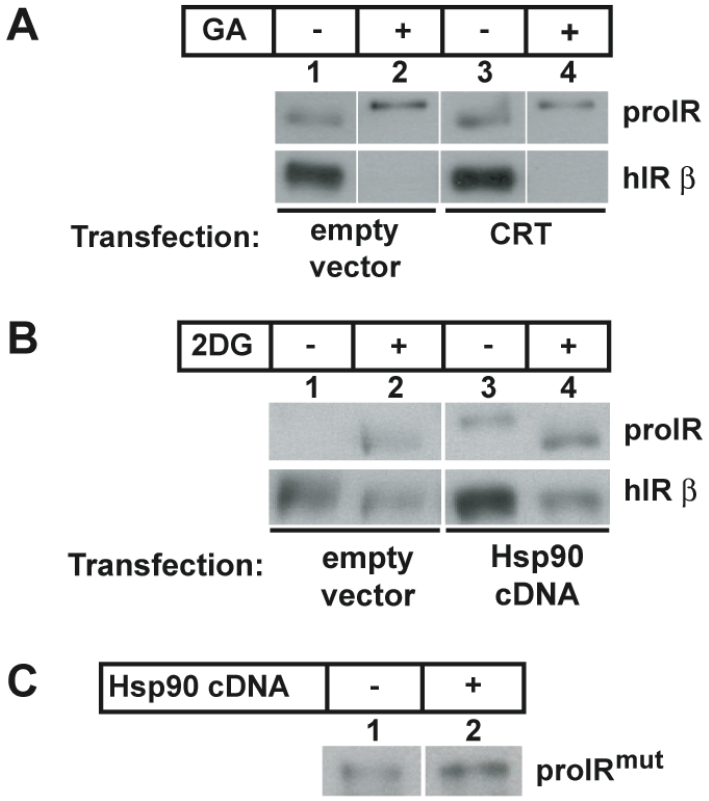

Figure 4.4. CRT and Hsp90 have independent roles in ERAD of IR. *A*. 293T cells expressing wild-type hIR were transfected with an empty vector or with CRT cDNA and treated with carrier or 4.5μM GA for 20 hrs. Cell lysates were processed as described previously. *B*. Cells stably expressing wild-type hIR were transfected with an empty vector or with Hsp90 cDNA and treated with either carrier or 50mM 2DG and ysates were processed as described previously. *C*. Cells were transfected with hIR^{mut} cDNA alone or with Hsp90 cDNA and lysates were processed as described previously.

2DG treatment (Figure 4.1C), but did not stabilize degradation induced by GA (Figure 4.4A, lane 4). Conversely, overexpression of cytosolic Hsp90 enhanced basal levels of wild-type hIR expression (Figure 4.4B, lane 3, and in Figure 4.3F), but did not attenuate degradation of hIR induced by glycolytic inhibition with 2DG (Figure 4.4B, lane 4). Interestingly, we found that CRT had a more potent effect on stabilizing the disease-causing mutant than Hsp90 (compare Figure 4.4C with Figure 4.2C). Together, these results indicate that the capacity of ER and cytoplasmic chaperones to attenuate degradation of a membrane-spanning protein are non-overlapping and may be dependent upon the location of the misfolded domain within the ER lumen or cytosol.

Effect of mutation in lumenal domain on diffusional mobility within the ER

To extend our biochemical analyses of folding of wild-type and disease-associated IR, we generated novel wild-type and mutant IR-GFP chimeras and established live cell imaging techniques to monitor the dynamics of this process. Fusion to GFP did not impair biogenesis (Figure 4.5A, C-E), nor signaling of the chimeric protein compared to wild-type receptors (Figure 4.5B). Two strategies enabled us to restrict our analysis of hIR movement within the ER: first, we used brefeldin A (BFA) to block ER-Golgi transport, and second, we analyzed movement of misfolded hIR^{mut}-GFP chimeras that remain trapped in the ER (Figure 4.6A). We used fluorescence recovery after photobleaching (FRAP) to compare the kinetics of movement of the wild-type with those of the ER-trapped hIR^{mut}-GFP chimera. A region of the ER was rapidly bleached at 100% laser power to reduce fluorescence to 10-20% of its initial value and subsequent fluorescence recovery was monitored using an attenuated laser.

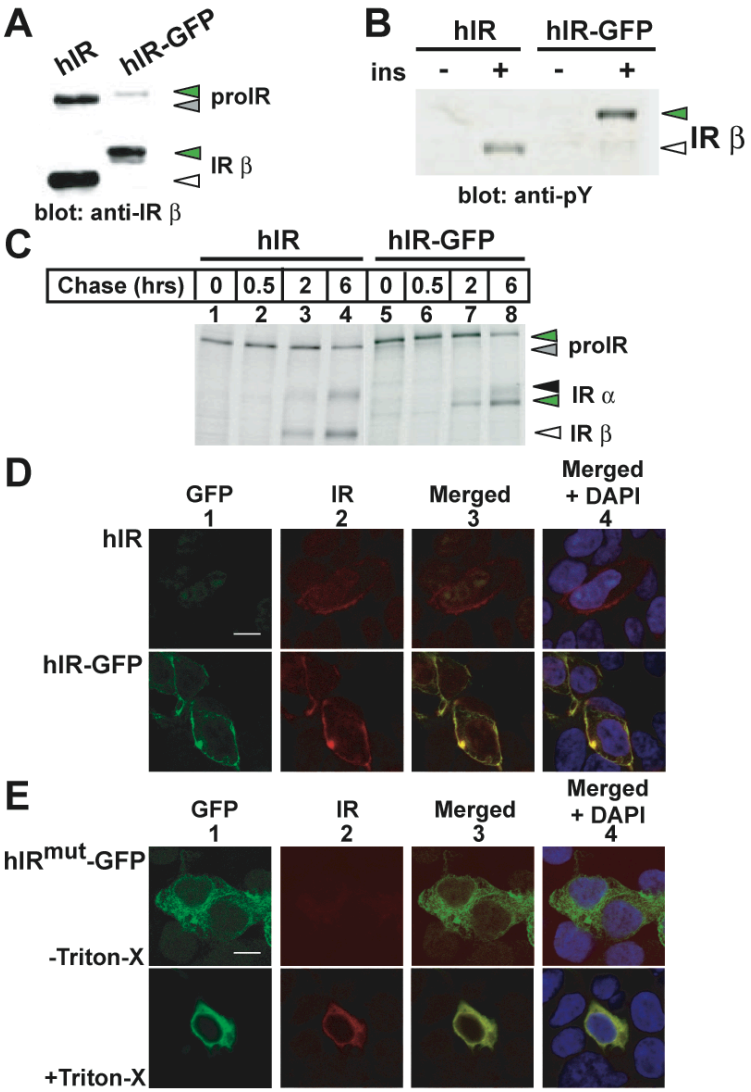

Figure 4.5. Characterization of bioluminescent hIR-GFP chimeras. *A*. Green fluorescent protein (GFP) was fused in-frame to the carboxy terminus of the insulin receptor (hIR-GFP) as described in the Methods. 293T cells were transfected with either hIR or hIR-GFP cDNA and processed as described previously. *B*. 293T cells transfected with either hIR or hIR-GFP cDNA were serum-starved 24 hours prior to incubation with 10^{-7} M insulin for 5 minutes and processed as described previously. Green triangles refer to IR-GFP fusion proteins. *C*. 293T cells were transfected with either hIR or hIR-GFP cDNA and processed for pulse-chase analysis as described previously. *D*. Cells expressing hIR or hIR-GFP were fixed, permeabilized, incubated with mouse anti-IR α (8314) followed by Texas Red donkey anti-mouse as the secondary antibody. GFP (green, Panel 1) and Texas Red (red, Panel 2) fluorescence were visualized using epifluorescence. Nuclei were stained with DAPI (blue). Bar = 10 microns. *E*. Cells expressing hIR^{mut}-GFP were fixed and kept intact (top) or permeabilized with Triton-X (bottom) and then processed as above.

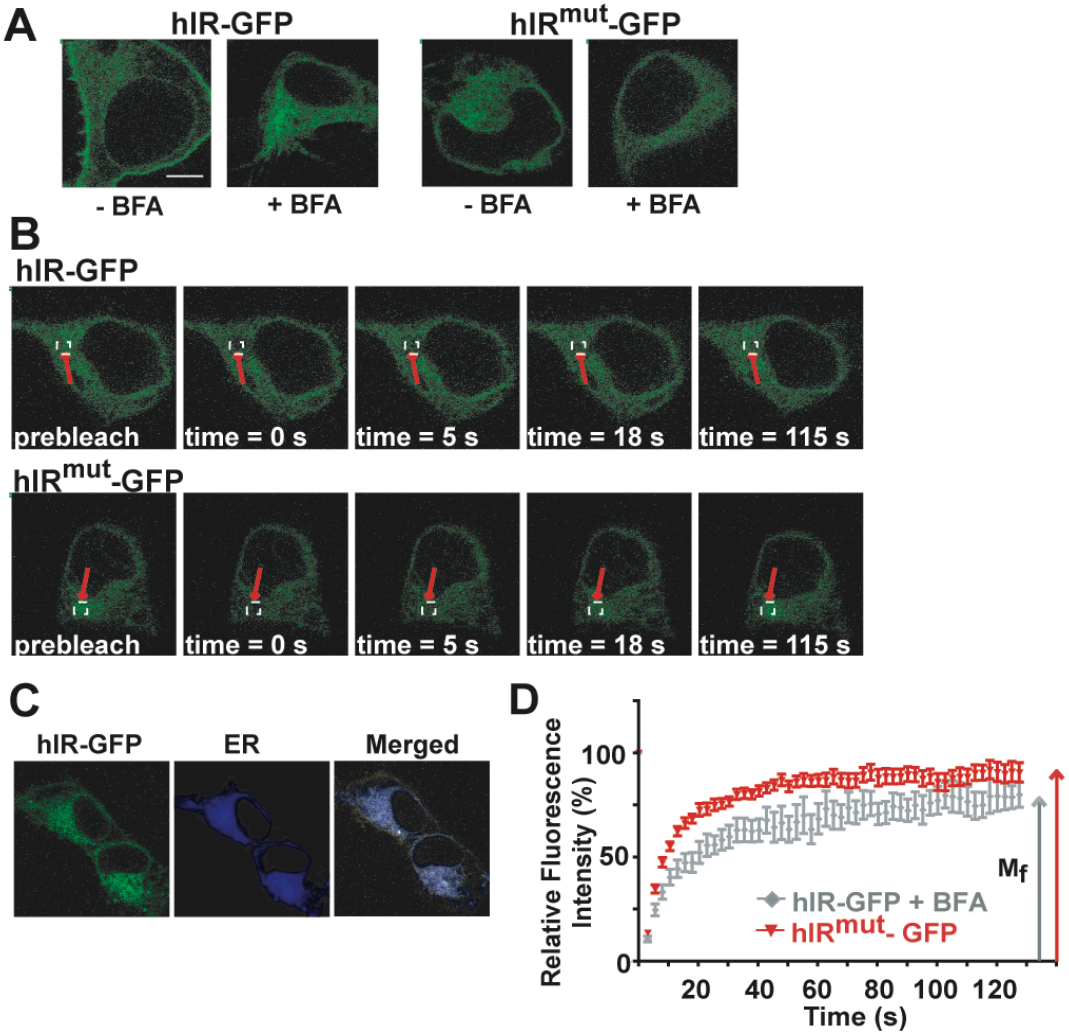

Figure 4.6. Visualization of ER mobility of wild-type and mutant hIR by live-cell imaging. *A.* Live 293T cells expressing hIR-GFP or hIR^{mut}-GFP were incubated in media alone, or in the presence of 5ug/mL BFA, at 37°C. Images were obtained at 100X magnification and 4X zoom. Bar = 10 microns. *B.* Live 293T cells expressing hIR-GFP in the presence of BFA (top) or hIR^{mut}-GFP (bottom) were kept at 37°C on a temperature-controlled stage using a Zeiss LSM 510 laser confocal microscope. FRAP was performed on these cells as described in the Methods. *C.* The live cells were incubated with an ER-specific dye (ER Tracker Blue-White) for 15 mins at 37°C prior to FRAP for targeted bleaching of the hIR-GFP within the ER. *D.* The ratio between mean fluorescent intensity in the photobleached ER region and in a non-photobleached region of the cell (relative fluorescent intensity) was plotted as a function of time to generate a fluorescence recovery curve, as described in the Methods.

Fluorescence recovery curves were generated as described (Axelrod et al., 1976; Ellenberg et al., 1997; Kim et al., 2002; Lippincott-Schwartz et al., 2001), and the diffusion coefficient (D) and mobile fraction (M_f) were then calculated. Figure 4.6B shows a comparison of time-lapse images captured by FRAP of wild-type hIR-GFP in the presence of BFA and hIR^{mut}-GFP. Studies of hIR-GFP monitored in BFA-treated cells, and untreated cells expressing hIR^{mut}-GFP, revealed a typical ER distribution that was confirmed by colocalization using an ER tracker dye (Figure 4.6C). Quantitation of fluorescence recovery in the photobleached area showed that both wild-type and mutant hIR-GFP had similar mobile fractions within the ER and diffusion coefficients of 2.3 and 3.4 x 10^{-10} cm^2/s, respectively (Figure 4.6D and Table 4.1). Our observations with FRAP analysis were further confirmed when we also performed studies using fluorescence loss in photobleaching (FLIP) (Figure 4.7). Specifically, by continuously bleaching a focal region within the ER and then monitoring loss of fluorescence in adjacent, but distinct, regions of the cell, we detected equivalent disappearance of fluorescent signal in cells expressing either wild-type or the mutant ER-trapped variant. Interestingly, the misfolded receptor exhibited a significantly higher diffusion rate compared to that of the wild-type hIR (Table 4.1). However, in cells expressing the wild-type receptor, we did not observe a difference in diffusion rate following incubation with BFA. These results suggest that ER retention of the receptor is not sufficient to influence its rate of diffusion but rather, alterations of structure, folding and oligomerization are necessary to impact diffusion. Interestingly, we also observed that augmenting chaperone expression significantly increased the diffusional mobility of wild-type receptor, but only moderately increased the diffusional mobility of the misfolded variant. We conclude that CRT and Hsp90 influence the mobility of the native wild-type receptor to a greater extent than the non-native misfolded mutant variant.

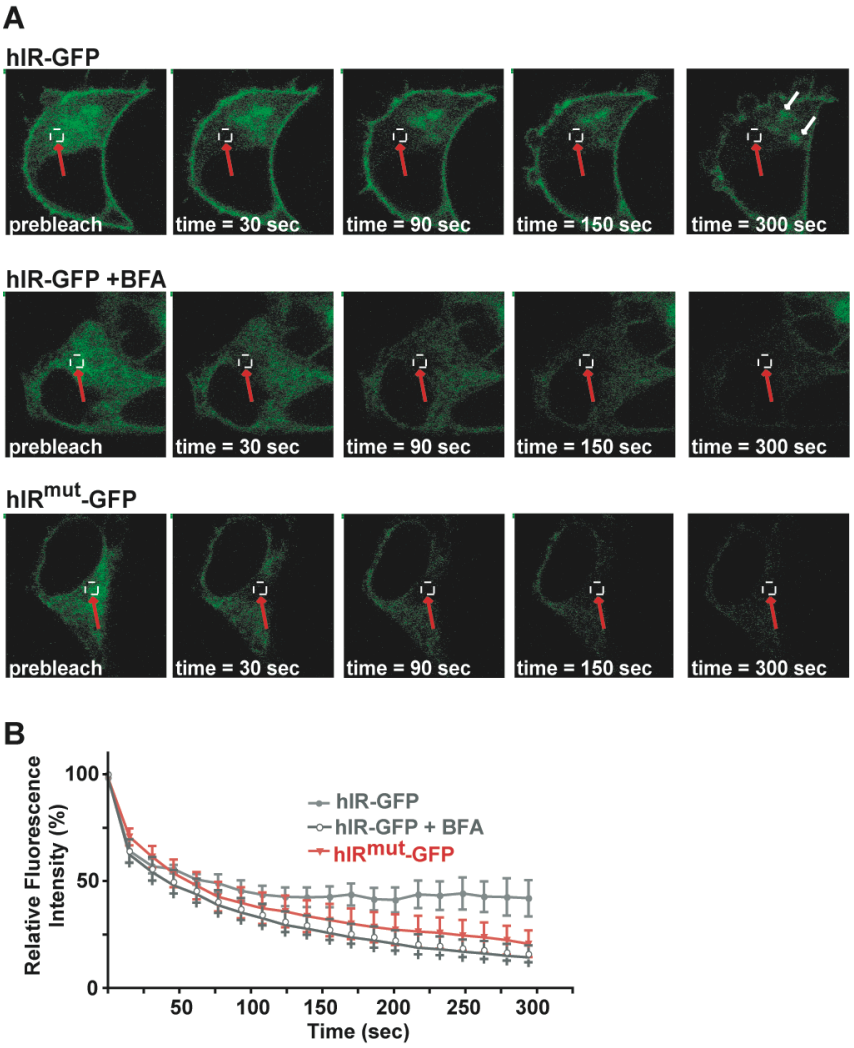

Figure 4.7. ER-retained wild-type or mutant hIR remains mobile within a continuous ER compartment. *A.* Live 293T cells expressing hIR-GFP in the absence or presence of BFA (top and middle panels, respectively) or hIR^{mut}-GFP (bottom) were maintained at 37°C on a temperature-controlled stage using a Zeiss LSM 510 laser confocal microscope. A ~1 μm^2 area (white square) was continuously bleached at 100% laser power and continuous loss of fluorescence was observed in images collected every 15 seconds (600 iterations) for 5 minutes. Remaining intracellular pockets of hIR-GFP fluorescence in the top panel are indicated by the white arrows. *B.* The ratio between mean fluorescent intensity in the photobleached ER region and mean total fluorescence intensity of the intracellular region of the cell (relative fluorescent intensity) was plotted as a function of time to generate a fluorescence loss curve, as described in the methods, with n=12-14 different cells each for wild-type and mutant hIR-GFP.

Table 4.1. Diffusion coefficients (D) and mobile fractions (M_f) of hIR-GFP and hIR^{mut}-GFP

Treatment	D ($x10^{-10}$ cm^2/s) ± S. E.		M_f (%) ± S. E.	
	hIR-GFP	hIR^{mut}-GFP	hIR-GFP	hIR^{mut}-GFP
- BFA	2.35 ± 0.12	3.32 ± 0.17	69.7 ± 6.3	81.0 ± 2.9
+ BFA	2.29 ± 0.20	3.38 ± 0.30*	76.8 ± 4.1	82.3 ± 4.4
+ CRT	3.57 ± 0.26$^{+,**}$	3.79 ± 0.23***	84.0 ± 2.2^{+}	81.2 ± 3.9
+ Hps90	3.55 ± 0.29$^{+,**}$	3.60 ± 0.37**	86.0 ± 3.6^{+}	78.0 ± 4.9

$^{+}$ in the presence of BFA n = 11-22 individual cells per set of experiments

$^{*, **, ***}$: $p<0.05$, 0.01, 0.001, respectively, compared to hIR-GFP + BFA

4.5. Discussion

While cytosolic and lumenal molecular chaperones have been implicated in ER quality control, the present study provides several novel findings that support a function for CRT and Hsp90 in the dynamics of QC during biogenesis of a transmembrane signaling receptor kinase in living cells. First, we demonstrate that CRT, a major ER lumenal chaperone, augments cell surface expression of native wild-type proteins under basal metabolic conditions, and attenuates degradation of a human mutant variant.

Second, our experiments clarify the mechanisms whereby Hsp90 influences cell surface expression of one subtype of receptor tyrosine kinases. Although previous studies with EGFR and GA indicated a role for receptor autophosphorylation in Hsp90-mediated degradation (Xu et al., 2001), our analysis revealed that kinase-dead hIR remained susceptible to degradation (Figure 4.3E). A related observation concerns the stage in hIR biogenesis during which cytosolic Hsp90 affects expression. Specifically, our results showed that Hsp90 promoted the conversion of immature hIR to mature proteolytically processed native protein (Figure 4.3A and F) which indicate that Hsp90 affects not only the cell surface population of hIR but it may also facilitate movement of the immature receptor to post-ER compartments and promote the progression of the receptor through earlier stages of maturation. Indeed, previous studies with CFTR have shown that Hsp90 participates in the ER degradation of the ΔF508 CFTR mutant variant (Fuller and Cuthbert, 2000; Wang et al., 2006), indicating an important and broad role for cytosolic Hsp90 in basal membrane protein biogenesis and quality control.

A third aspect of our studies addressed temporal dynamics of hIR movement within the ER. Calculations from our fluorescence recovery studies showed a diffusion coefficient of approximately 2.3-3.4 x 10^{-10} cm^2/sec for the hIR-GFP chimeras. While previous studies have

established similar diffusion coefficients for various forms of aquaporin2 (AQP2), a multi-spanning transmembrane protein (Levin et al., 2001), our results provide new evidence to show comparable diffusion rates for native and misfolded proteins with single-pass topology. Interestingly, we found that hIR^{mut} had increased movement compared to wild-type hIR-GFP. In contrast, previous experiments with AQP2 and CFTR showed that missense mutant variants had mobilities equivalent to native proteins within the ER (Haggie et al., 2002; Levin et al., 2001). More recently, mutant Gas3/PMP22, a misfolded tetraspan component of myelin protein, was found to be less mobile than its wild-type counterpart (Fontanini et al., 2005), an effect attributed to differences in the Stokes' radius of the mutant protein oligomers. Additional studies support a relationship between diffusional mobility and the physical size of the transmembrane domain (Hughes et al., 1982; Lippincott-Schwartz et al., 2001). Given these observations, it is noteworthy that our previous biochemical studies have shown that hIR^{mut} does not dimerize in the ER (Bass et al., 2000). We speculate that a reduced membrane-spanning radius and/or failure to engage in the proper maturation steps enhances the movement of the mutant monomer through the ER.

Fourth, our data further indicate a role for both CRT and Hsp90 in the temporal dynamics of hIR movement. CRT significantly increased the diffusion coefficient of the wild-type hIR-GFP, resulting in a rate of mobility similar to that of the misfolded hIR^{mut}-GFP (Table 4.1). Recent analyses have shown that CRT itself is highly mobile within the ER (Snapp et al., 2006) and may contribute to movement of its client proteins. Moreover, overexpression of BiP has been shown to increase the solubility of pαF and decrease its degradation (Kabani et al., 2003). Thus, ER chaperones appear to generally promote protein mobility even following misfolding. However, it should be noted that effects of each chaperone may depend upon the specific

substrate since BiP overexpression was also shown to immobilize misfolded VSVG (Nehls et al., 2000).

Cytosolic chaperones have been shown to play a role in ER substrate mobility and our FRAP experiments showed that Hsp90 was also able to significantly increase mobility of the wild-type receptor (Table 4.1). Our genetic transfection experiments extend previous observations that inhibiting Hsp90 decreased CFTR mobility (Haggie et al., 2002) and that Hsp90 maintains solubility of an aggregation-prone domain of CFTR (Youker et al., 2004). Overall, we propose that Hsp90 also plays a facilitative role in transmembrane protein movement within the ER.

Together, our studies reveal that CRT and Hsp90, chaperones found on opposite sides of the ER membrane, exert distinct roles on the stability and mobility of the hIR, and ultimately its cell surface expression. Our observations raise the intriguing possibility that regulation of movement of transmembrane proteins within the ER may be substrate-specific and result from distinct interactions with ER lumenal and cytosolic chaperones. Future studies elucidating the spatiotemporal steps in RTK biogenesis may improve our understanding of disease conditions, including diabetes and obesity, both of which are associated with dysregulation of IR homeostasis.

4.6. Acknowledgments

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Chapter 5

Conclusions and Future Directions

5.1. Conclusions

Receptor tyrosine kinases enable the cell to maintain constant communication with the extracellular environment and facilitate the appropriate physiological response to promote cell growth and development. Altered expression and activity of RTKs has been implicated in a variety of diseases including metabolic disorders and a wide range of cancers. Thus, it is important to understand the mechanisms that regulate RTK expression and degradation in order to enable development of better therapies for metabolic disease and neoplasia.

Here, I used the insulin receptor and its signaling and biogenesis pathways as a model system to investigate three different aspects of RTK homeostasis.

5.1.1. PI3K/AKT/mTOR signaling negatively regulates RTK expression

Previous studies have shown that downregulation of the IR and other RTKs occurs at the cell surface via internalization upon phosphorylation of the kinase domain or via inhibitory serine phosphorylation of the receptor or other proteins involved in the signal transduction cascade. However, in chapter 2, I showed that activity of the PI3K/AKT/mTOR pathway, a signal transduction cascade shared by many RTKS, directly affected IR, EGFR and IGF1-R expression at both the protein and mRNA levels.

Deleting AKT1 in mice or knocking down AKT expression in human cells via RNAi both showed similar increases in IR and EGFR protein expression. A corresponding increase in mRNA transcripts was also observed. Active AKT phosphorylates FOXO leading to its sequestration in the cytoplasm, preventing it from promoting transcription of its target genes. Thus, deletion of AKT would increase active FOXO in the nucleus. The observed increase in IR mRNA expression in mouse and human models when AKT was deleted confirms previous

studies in the *Drosophila* insulin receptor that showed FOXO-mediated transcription of this gene (Puig and Tjian, 2005). However, the observed increase in EGFR was unexpected. Even though signaling through EGFR has been shown to affect FOXO activity on target genes including *dx1, Foxa2,* and *Insulin2* (Buteau et al., 2006) and TNFα-related apoptosis inducing ligand (Morris et al., 2005), there are no reports that implicate FOXO in transcription of another RTK aside from the IR and IGF1R (Xuan and Zhang, 2005).

Deleting AKT in mice and human cells also showed an attenuated rate of degradation for RTKs when new protein synthesis was inhibited, suggesting a role for AKT in the turnover of RTKs. This decrease in degradation is independent of receptor kinase activity and internalization via ubiquitination of phosphorylated receptors since the cells were endogenously expressing wild-type receptors and inhibition of insulin signaling was performed downstream of the receptor. However, the exact mechanism by which AKT activity is involved in degradation of RTKS remains unclear.

Altering activity of PI3K confirmed initial observations that signaling through the PI3K/AKT/mTOR pathway negatively regulated RTK expression. These effects were probably more directly mediated by AKT which is downstream of PI3K. Inhibiting mTOR function resulted in a more blunted increase in RTK expression. This may be due to the different signaling loops that AKT and mTOR are involved with that regulate each other's activity, such that inhibiting mTOR leads to increased AKT activity, highlighting the non-linearity of these signaling pathways.

5.1.2. Inhibiting PI3K signaling attenuates the decrease in IR induced by metabolic inhibitors

Recent studies have highlighted the importance of intracellular glucose and availability in maintaining cellular homeostasis. Previous research has shown that glucose deprivation leads to aberrant IR production (Hwang and Frost, 1999). However, the use of more specific metabolic inhibitors such as 2-deoxyglucose, a non-metabolizable analogue of glucose, and sodium azide, an inhibitor of the cytochrome C oxidase, enables a more targeted approach to depleting intracellular ATP.

I showed in Chapter 3 that treatment with these metabolic inhibitors lead to a decrease in cell surface expression and total protein levels of the insulin receptor, but did not alter protein expression of other proteins in the insulin signaling pathway nor of unrelated transmembrane proteins. This result is different from that observed in *Drosophila* experiments wherein insect cells that were serum-deprived had increased IR production. This suggests that there may be distinct mechanisms mediated by cellular ATP and growth factor signaling that regulate IR expression.

To test whether the increased RTK production observed with inhibition of PI3K signaling shown in Chapter 2 can partially rescue or reverse the decrease in IR proteins induced by metabolic inhibitors, signaling was inhibited through both pharmacological and genetic manipulation in combination with 2DG and sodium azide treatment. Results from Chapter 3 show that indeed, inhibiting PI3K, AKT or mTOR activity or expression attenuated the reduction in IR levels induced by 2DG and sodium azide. These observations suggest that both redox flux and PI3K signaling can coordinately regulate IR homeostasis.

5.1.3. IR maturation is spatially and temporally regulated by ER luminal and cytosolic chaperones

The observations shown in Chapters 2 and 3 involved looking at how altering growth factor-mediated and energy-mediated signaling pathways affect the mature, functional receptor and hinted at possible regulatory mechanisms at the earliest points of IR synthesis. In chapter 4, I investigated the steps involved in the maturation of the IR, by specifically looking at how interactions with chaperones topologically separated on either side of the ER membrane influence IR homeostasis.

First, I found that perturbation of luminal domains of the IR by either perturbing N-linked glycosylation or the introduction of a missense mutation lead to proteasomal degradation of the receptor but was attenuated by overexpression of the cytosolic chaperone, calreticulin. Second, I discovered that Hsp90 overexpression increased IR protein levels and that IR degradation mediated by inhibiting Hsp90 function was independent of receptor kinase activity. Furthermore, CRT and Hsp90 had distinct, non-overlapping roles in facilitating IR maturation.

A third aspect I investigated was diffusional mobility of the IR. Previous research has shown that misfolded proteins can have equivalent or decreased mobilities in the ER compared to their wild-type counterparts. However, in Chapter 4, I made a novel observation which showed that the misfolded mutant IR had significantly increased mobility in the ER compared to the wild-type isoform and that this mobility could not be further increased by overexpression of CRT nor Hsp90. This demonstrated that misfolding of a luminal domain does not impede movement within the ER and suggested that distinct intermolecular interactions within the ER may be modulating its movement.

5.1.4. Significance of this thesis

The results presented in this thesis provide insights into how signaling activity, cellular metabolic status and polypeptide structural changes coordinately regulate expression and degradation of RTKs using the insulin receptor as a model protein. In this work, I uncovered an alternate strategy to control RTK signaling activity. I demonstrated that signaling through the PI3K/AKT/mTOR pathway negativelys regulate receptor activity by diminishing expression and increasing degradation of the RTK itself. Furthermore, inhibiting signaling activity partially reversed RTK degradation induced by metabolic inhibitors. Since similar effects were observed for IR and IGF1-R, class II RTKs, and EGFR, a class I RTK, my research findings establish a novel regulatory mechanism important in RTK constancy and function.

In addition, my live-cell imaging experiments, using novel IR-GFP chimeras, surprisingly revealed that misfolded receptors retained in the ER remained mobile within this subcellular compartment and possessed a higher diffusional mobility compared to natively folded proteins. This observation, combined with previous studies which showed equivalent or slower diffusion of other non-native proteins, demonstrates that diffusional mobility of misfolded proteins in the ER may be modulated by substrate-specific interactions with the quality control machinery.

Collectively, this thesis work provides a more comprehensive understanding of the different regulatory mechanisms involved in RTK homeostasis and may enable development of better strategies to regulate RTK expression as a possible therapy for metabolic disease and cancer.

5.2. Future directions

Although several interesting and novel observations have been made in this thesis while exploring the effects of signaling and nutrient availability on spatial and temporal regulation of RTK homeostasis, more questions arise from these findings and I propose possible experiments to explore these new hypotheses.

5.2.1. FOXO-mediated transcription of other RTKs

Bioinformatics methods to screen mammalian genes (Xuan and Zhang, 2005) and genetic screens in *Drosophila* for DAF-16 targets (Pinkston-Gosse and Kenyon, 2007) did not reveal new RTKs downstream of FOXO/DAF-16. Targets obtained from a chromatin immunoprecipitation (ChIP) assay in *Drosophila* also did not reveal RTKs as FOXO target genes and failed to detect a confirmed FOXO target gene, the dInR (Oh et al., 2006). The results in chapter 2 suggests that human EGFR may also be a downstream target of FOXO, however considering that previous general screens failed to pick up even confirmed RTKs as FOXO target genes, perhaps a more targeted approach will be needed to investigate FOXO's role in the transcription of RTKs other than the IR and IGF1-R.

Given FOXO's known roles in upregulating enzymes involved in cellular glucose production and growth, discovering additional RTK genes that involve FOXO-mediated transcription will provide a more comprehensive understanding of the pleiotropic effects of FOXO.

In addition, serine phosphorylation may not only play a role in downregulation of protein activity, but it may also facilitate additional downstream mechanisms to regulate RTK expression. Knockdown of the serine/threonine cyclin G-associated kinase (GAK) by RNAi lead

to a 50-fold increase in EGFR expression (Zhang et al., 2004). It will be important to learn whether this increase in EGFR expression was mediated by FOXO, or a different pathway.

5.2.2. Regulation of FOXO expression by RTKs

PI3K and AKT activity downstream of most RTK signaling pathways has been shown to be necessary for proteasomal degradation of FOXOs (Aoki et al., 2004; Guo et al., 2006). The E3 ubiquitin ligase, Skp2, is responsible for recognizing phosphorylated FOXO (Ser-256), facilitating ubiquitination and proteasomal degradation of the transcription factor. Confirming the role of FOXO in upregulating RTK expression can be further tested by performing various experiments that manipulate FOXO levels. First, RNAi of FOXO1 can be performed to test whether mRNA expression of IR and other RTKs is diminished. Second, a converse experiment would involve generating a non-phosphorylatable FOXO mutant (Ser256Ala), preventing its cytoplasmic sequestration and proteasomal degradation, and then examine if RTK levels increase. However, since FOXO is involved in upregulating expression of a wide variety of transcripts involved in cell growth and metabolism, it would be wise to monitor expression of other target genes downstream of FOXO such as those involved in metabolic activities of the cell like PEPCK, G-6-Pase to assess the more global impact of altered FOXO expression on the cell.

5.2.3. Translational regulation of RTK synthesis

The increase in the immature form of the IR when AKT signaling was inhibited shown in chapter 2 suggests that increased protein synthesis may also play a role in the observed upregulation of RTK expression. To address whether rates of translation were similarly increased when PI3K signaling was inhibited, polyribosomes containing actively translated mRNA can be

extracted from cells that have AKT deleted or have been treated with inhibitors of PI3K and mTOR and the attached mRNA can be reverse-transcribed, amplified and quantitated using primers specific for various RTKs.

However, this analysis will only show whether general protein translation rates are affected but it will not enable identification of the type of translation initiation that occurred on the attached mRNAs, whether it is cap-dependent or IRES-mediated translation. A way to discriminate between these two possibilities would be to mutate the IRES sequences found upstream of the insulin receptor promoter and see if this changes the rate of IR translation.

The very recent discovery of IRES sequences in cellular mRNA has opened up a whole new paradigm in understanding how protein translation is regulated in metazoans. It has been shown that serum deprivation increases IRES-mediated translation of 4EBP1, a repressor of cap-dependent translation, thereby downregulating the bulk of protein synthesis. This phenomenon, however, is accompanied by an increase in IR protein (Marr et al., 2007) and it has been hypothesized that this is the mechanism by which a cell tries to conserve its resources and primes itself for glucose uptake by increasing production of the IR. But we also know that there are other IRES-containing sequences whose protein products are involved in a variety of cell growth processes (Baird et al., 2006). What effect would serum-deprivation have on translation of these other proteins and how would it help the cell cope with the nutrition-deficient conditions? In addition to studies of IR translation, monitoring other IRES-containing mRNAs relevant to regulation of growth might provide a more comprehensive picture of the cell's response to such conditions. Candidate proteins to examine would include the pro-apoptotic factor, Apaf1, or growth hormones such as fibroblast growth factor, since these proteins have previously been shown to contain IRES sequences (reviewed in (Baird et al., 2006)).

Would depleting cells of ATP, instead of growth factors, have a similar effect on upregulation of IR protein synthesis? Results from chapter 3 suggested that the cell mechanisms to cope with ATP depletion were distinct from those mediated by serum deprivation. Since protein synthesis, whether via cap-dependent or IRES-dependent initiation requires energy, it would be interesting to see whether IRES-mediated translation is similarly affected or whether this pathway has developed alternative strategies to cope with nutrient depletion.

5.2.4. Effect of metabolic inhibitors on other aspects of RTK homeostasis

I showed in Chapters 3 and 4 that metabolic inhibition results in decreased IR protein levels but we did not investigate its effect on RTK transcription. Previous studies have shown that depletion of ATP can prevent phosphorylation and activation of proteins in the cell. It would be intriguing to see whether FOXO-mediated transcription is affected by depletion of ATP. Secondly, would movement of RTKs within the ER and through the secretory pathway be affecected by ATP availability?

In addition, recent studies have shown that ATP levels can be regulated by AKT by inhibiting AMP-activated protein kinase (AMPK), a kinase activated by ATP depletion (Hahn-Windgassen et al., 2005). In addition, mTOR is also responsive to intracellular ATP and may act downstream of AKT as inhibition of AMPK prevents phosphorylation of tuberous sclerosis complex 2 (TSC2), an inhibitor of the Rheb GTPase that activates mTOR (Inoki et al., 2003). Understanding how metabolic changes in the cell may similarly affect other important signaling pathways is crucial to obtaining a more comprehensive understanding of its cellular response.

5.2.5. Signal transactivation between other receptors

Crosstalk between different receptors resulting in multi-dimensional signaling between various receptors has been observed between different members of the RTK family. In addition, crosstalk between RTKs and other classes of receptors have also been reported. One example involves signal transactivation between progesterone receptors and IGF1 receptors (Cui et al., 2003; Lee et al., 2003). Given the importance of nuclear hormone receptors in the progression of diseases such as breast cancer, where overexpression of RTKs like ErbB2 and EGFR have also been implicated, it would be worth investigating how the signaling pathways from these two different receptor classes may affect each other's expression and homeostasis.

5.3. Final comments

Receptor tyrosine kinases play important roles in normal cell growth and development and aberrant expression and function of these proteins are implicated in both metabolic disease and tumor progression. The results presented in this thesis provide insight into how signaling activity, cellular metabolic status, and polypeptide structural changes coordinately regulate expression and degradation of RTKs using the insulin receptor as a model protein. Here we provide evidence that activity of the PI3K/AKT/mTOR signaling pathway shared by various RTKs negatively regulates RTK homeostasis, and that inhibiting the function of the crucial kinases involved in signal transduction upregulates RTK expression. Furthermore, inhibiting signaling activity is able to partially protect against metabolic inhibitors by attenuating the degradation of RTKs.

In addition, I have established new insight into chaperone-mediated maturation of IR biogenesis, revealing the surprising observation that misfolded receptors remained mobile within the ER and possessed a higher diffusional mobility compared to natively folded proteins.

Elucidating the molecular components of RTK homeostasis pathways may ultimately enable development of better strategies in therapeutic efforts to treat metabolic disease and cancer.

References

Alessi, D. R. (2001). Discovery of PDK1, one of the missing links in insulin signal transduction. Colworth Medal Lecture. Biochem Soc Trans *29*, 1-14.

Altomare, D. A., Lyons, G. E., Mitsuuchi, Y., Cheng, J. Q., and Testa, J. R. (1998). Akt2 mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin. Oncogene *16*, 2407-2411.

Aoki, M., Jiang, H., and Vogt, P. K. (2004). Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. Proc Natl Acad Sci U S A *101*, 13613-13617.

Argon, Y., and Simen, B. B. (1999). GRP94, an ER chaperone with protein and peptide binding properties. Semin Cell Dev Biol *10*, 495-505.

Arner, P., Einarsson, K., Backman, L., Nilsell, K., Lerea, K. M., and Livingston, J. N. (1983). Studies of liver insulin receptors in non-obese and obese human subjects. J Clin Invest *72*, 1729-1736.

Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E., and Webb, W. W. (1976). Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. Biophys J *16*, 1055-1069.

Baird, S. D., Turcotte, M., Korneluk, R. G., and Holcik, M. (2006). Searching for IRES. Rna *12*, 1755-1785.

Barber, A. J., Nakamura, M., Wolpert, E. B., Reiter, C. E., Seigel, G. M., Antonetti, D. A., and Gardner, T. W. (2001). Insulin rescues retinal neurons from apoptosis by a phosphatidylinositol 3-kinase/Akt-mediated mechanism that reduces the activation of caspase-3. J Biol Chem *276*, 32814-32821.

Bass, J., Chiu, G., Argon, Y., and Steiner, D. F. (1998). Folding of insulin receptor monomers is facilitated by the molecular chaperones calnexin and calreticulin and impaired by rapid dimerization. J Cell Biol *141*, 637-646.

Bass, J., Turck, C., Rouard, M., and Steiner, D. F. (2000). Furin-mediated processing in the early secretory pathway: sequential cleavage and degradation of misfolded insulin receptors. Proc Natl Acad Sci USA *97*, 11905-11909.

Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V.*, et al.* (1995). Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int J Cancer *64*, 280-285.

Bellacosa, A., Testa, J. R., Staal, S. P., and Tsichlis, P. N. (1991). A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science *254*, 274-277.

Benecke, H., Flier, J. S., and Moller, D. E. (1992). Alternatively spliced variants of the insulin receptor protein. Expression in normal and diabetic human tissues. J Clin Invest *89*, 2066-2070.

Berg, C. E., Lavan, B. E., and Rondinone, C. M. (2002). Rapamycin partially prevents insulin resistance induced by chronic insulin treatment. Biochem Biophys Res Commun *293*, 1021-1027.

Bisbis, S., Bailbe, D., Tormo, M. A., Picarel-Blanchot, F., Derouet, M., Simon, J., and Portha, B. (1993). Insulin resistance in the GK rat: decreased receptor number but normal kinase activity in liver. Am J Physiol *265*, E807-813.

Blume-Jensen, P., and Hunter, T. (2001). Oncogenic kinase signalling. Nature *411*, 355-365.

Briata, P., Briata, L., and Gherzi, R. (1990). Glucose starvation and glycosylation inhibitors reduce insulin receptor gene expression: characterization and potential mechanism in human cells. Biochem Biophys Res Commun *169*, 397-405.

Britton, J. S., Lockwood, W. K., Li, L., Cohen, S. M., and Edgar, B. A. (2002). Drosophila's insulin/PI3-kinase pathway coordinates cellular metabolism with nutritional conditions. Dev Cell *2*, 239-249.

Brunet, A., Sweeney, L. B., Sturgill, J. F., Chua, K. F., Greer, P. L., Lin, Y., Tran, H., Ross, S. E., Mostoslavsky, R., Cohen, H. Y.*, et al.* (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.

Burgering, B. M., and Coffer, P. J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature *376*, 599-602.

Buteau, J., Spatz, M. L., and Accili, D. (2006). Transcription factor FoxO1 mediates glucagon-like peptide-1 effects on pancreatic beta-cell mass. Diabetes *55*, 1190-1196.

Cabrera, N., Diaz-Rodriguez, E., Becker, E., Martin-Zanca, D., and Pandiella, A. (1996). TrkA receptor ectodomain cleavage generates a tyrosine-phosphorylated cell-associated fragment. J Cell Biol *132*, 427-436.

Cao, Y., Kamioka, Y., Yokoi, N., Kobayashi, T., Hino, O., Onodera, M., Mochizuki, N., and Nakae, J. (2006). Interaction of FoxO1 and TSC2 induces insulin resistance through activation of the mammalian target of rapamycin/p70 S6K pathway. J Biol Chem *281*, 40242-40251.

Caro, J. F., Sinha, M. K., Raju, S. M., Ittoop, O., Pories, W. J., Flickinger, E. G., Meelheim, D., and Dohm, G. L. (1987). Insulin receptor kinase in human skeletal muscle from obese subjects with and without noninsulin dependent diabetes. J Clin Invest *79*, 1330-1337.

Caro, L. H., Ohali, A., Gorden, P., and Collier, E. (1994). Mutational analysis of the NH2-terminal glycosylation sites of the insulin receptor alpha-subunit. Diabetes *43*, 240-246.

Chen, H., Ma, J., Li, W., Eliseenkova, A. V., Xu, C., Neubert, T. A., Miller, W. T., and Mohammadi, M. (2007). A molecular brake in the kinase hinge region regulates the activity of receptor tyrosine kinases. Mol Cell *27*, 717-730.

Chen, W. S., Xu, P. Z., Gottlob, K., Chen, M. L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K.*, et al.* (2001). Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev *15*, 2203-2208.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res *31*, 3497-3500.

Chillaron, J., and Haas, I. G. (2000). Dissociation from BiP and retrotranslocation of unassembled immunoglobulin light chains are tightly coupled to proteasome activity. Mol Biol Cell *11*, 217-226.

Cho, C. Y., Koo, S. H., Wang, Y., Callaway, S., Hedrick, S., Mak, P. A., Orth, A. P., Peters, E. C., Saez, E., Montminy, M.*, et al.* (2006). Identification of the tyrosine phosphatase PTP-MEG2 as an antagonist of hepatic insulin signaling. Cell Metab *3*, 367-378.

Cho, H., Thorvaldsen, J. L., Chu, Q., Feng, F., and Birnbaum, M. J. (2001). Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J Biol Chem *276*, 38349-38352.

Countaway, J. L., McQuilkin, P., Girones, N., and Davis, R. J. (1990). Multisite phosphorylation of the epidermal growth factor receptor. Use of site-directed mutagenesis to examine the role of serine/threonine phosphorylation. J Biol Chem *265*, 3407-3416.

Countaway, J. L., Nairn, A. C., and Davis, R. J. (1992). Mechanism of desensitization of the epidermal growth factor receptor protein-tyrosine kinase. J Biol Chem *267*, 1129-1140.

Cruz, M., Velasco, E., and Kumate, J. (2004). Degradation of pro-insulin-receptor proteins by proteasomes. Arch Med Res *35*, 18-23.

Cui, X., Lazard, Z., Zhang, P., Hopp, T. A., and Lee, A. V. (2003). Progesterone crosstalks with insulin-like growth factor signaling in breast cancer cells via induction of insulin receptor substrate-2. Oncogene *22*, 6937-6941.

Danilczyk, U. G., and Williams, D. B. (2001). The lectin chaperone calnexin utilizes polypeptide-based interactions to associate with many of its substrates in vivo. J Biol Chem *276*, 25532-25540.

Del Prato, S., Leonetti, F., Simonson, D. C., Sheehan, P., Matsuda, M., and DeFronzo, R. A. (1994). Effect of sustained physiologic hyperinsulinaemia and hyperglycaemia on insulin secretion and insulin sensitivity in man. Diabetologia *37*, 1025-1035.

Denley, A., Wallace, J. C., Cosgrove, L. J., and Forbes, B. E. (2003). The insulin receptor isoform exon 11- (IR-A) in cancer and other diseases: a review. Horm Metab Res *35*, 778-785.

Dennis, P. B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S. C., and Thomas, G. (2001). Mammalian TOR: a homeostatic ATP sensor. Science *294*, 1102-1105.

Dunaif, A., Xia, J., Book, C. B., Schenker, E., and Tang, Z. (1995). Excessive insulin receptor serine phosphorylation in cultured fibroblasts and in skeletal muscle. A potential mechanism for insulin resistance in the polycystic ovary syndrome. J Clin Invest *96*, 801-810.

Easton, J. B., Kurmasheva, R. T., and Houghton, P. J. (2006). IRS-1: auditing the effectiveness of mTOR inhibitors. Cancer Cell *9*, 153-155.

Easton, R. M., Cho, H., Roovers, K., Shineman, D. W., Mizrahi, M., Forman, M. S., Lee, V. M., Szabolcs, M., de Jong, R., Oltersdorf, T.*, et al.* (2005). Role for Akt3/protein kinase Bgamma in attainment of normal brain size. Mol Cell Biol *25*, 1869-1878.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J. H., Masiarz, F., Kan, Y. W., Goldfine, I. D., and et al. (1985). The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signalling. Cell *40*, 747-758.

Eck, M. J., Dhe-Paganon, S., Trub, T., Nolte, R. T., and Shoelson, S. E. (1996). Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor. Cell *85*, 695-705.

Egawa, K., Nakashima, N., Sharma, P. M., Maegawa, H., Nagai, Y., Kashiwagi, A., Kikkawa, R., and Olefsky, J. M. (2000). Persistent activation of phosphatidylinositol 3-kinase causes insulin resistance due to accelerated insulin-induced insulin receptor substrate-1 degradation in 3T3-L1 adipocytes. Endocrinology *141*, 1930-1935.

Egawa, K., Sharma, P. M., Nakashima, N., Huang, Y., Huver, E., Boss, G. R., and Olefsky, J. M. (1999). Membrane-targeted phosphatidylinositol 3-kinase mimics insulin actions and induces a state of cellular insulin resistance. J Biol Chem *274*, 14306-14314.

Elchebly, M., Payette, P., Michaliszyn, E., Cromlish, W., Collins, S., Loy, A. L., Normandin, D., Cheng, A., Himms-Hagen, J., Chan, C. C.*, et al.* (1999). Increased insulin sensitivity and obesity resistance in mice lacking the protein tyrosine phosphatase-1B gene. Science *283*, 1544-1548.

Eldar-Finkelman, H., and Krebs, E. G. (1997). Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. Proc Natl Acad Sci U S A *94*, 9660-9664.

Elleman, T. C., Frenkel, M. J., Hoyne, P. A., McKern, N. M., Cosgrove, L., Hewish, D. R., Jachno, K. M., Bentley, J. D., Sankovich, S. E., and Ward, C. W. (2000). Mutational analysis of the N-linked glycosylation sites of the human insulin receptor. Biochem J *347 Pt 3*, 771-779.

Ellenberg, J., Siggia, E. D., Moreira, J. E., Smith, C. L., Presley, J. F., Worman, H. J., and Lippincott-Schwartz, J. (1997). Nuclear membrane dynamics and reassembly in living cells:

targeting of an inner nuclear membrane protein in interphase and mitosis. J Cell Biol *138*, 1193-1206.

Ellgaard, L., Molinari, M., and Helenius, A. (1999). Setting the Standards: Quality Control in the Secretory Pathway. Science *286*, 1882-1888.

Escobedo, J. A., and Williams, L. T. (1988). A PDGF receptor domain essential for mitogenesis but not for many other responses to PDGF. Nature *335*, 85-87.

Escribano, O., Fernandez-Moreno, M. D., Pina, M. J., Fueyo, J., Menor, C., Roman, I. D., and Guijarro, L. G. (2002). Pretreatment with FK506 up-regulates insulin receptors in regenerating rat liver. Hepatology *36*, 555-561.

Fernandez, J., Bode, B., Koromilas, A., Diehl, J. A., Krukovets, I., Snider, M. D., and Hatzoglou, M. (2002). Translation Mediated by the Internal Ribosome Entry Site of the cat-1 mRNA Is Regulated by Glucose Availability in a PERK Kinase-dependent Manner. J Biol Chem *277*, 11780-11787.

Fernandez, J., Yaman, I., Mishra, R., Merrick, W. C., Snider, M. D., Lamers, W. H., and Hatzoglou, M. (2001). Internal ribosome entry site-mediated translation of a mammalian mRNA is regulated by amino acid availability. J Biol Chem *276*, 12285-12291.

Fernandez, R., Tabarini, D., Azpiazu, N., Frasch, M., and Schlessinger, J. (1995). The Drosophila insulin receptor homolog: a gene essential for embryonic development encodes two receptor isoforms with different signaling potential. Embo J *14*, 3373-3384.

Fontanini, A., Chies, R., Snapp, E. L., Ferrarini, M., Fabrizi, G. M., and Brancolini, C. (2005). Glycan-independent role of calnexin in the intracellular retention of Charcot-Marie-tooth 1A Gas3/PMP22 mutants. J Biol Chem *280*, 2378-2387.

Fuller, W., and Cuthbert, A. W. (2000). Post-translational disruption of the delta F508 cystic fibrosis transmembrane conductance regulator (CFTR)-molecular chaperone complex with geldanamycin stabilizes delta F508 CFTR in the rabbit reticulocyte lysate. J Biol Chem *275*, 37462-37468.

Garofalo, R. S. (2002). Genetic analysis of insulin signaling in Drosophila. Trends Endocrinol Metab *13*, 156-162.

Gasparini, L., Racchi, M., Benussi, L., Curti, D., Binetti, G., Bianchetti, A., Trabucchi, M., and Govoni, S. (1997). Effect of energy shortage and oxidative stress on amyloid precursor protein metabolism in COS cells. Neurosci Lett *231*, 113-117.

George, S., Rochford, J. J., Wolfrum, C., Gray, S. L., Schinner, S., Wilson, J. C., Soos, M. A., Murgatroyd, P. R., Williams, R. M., Acerini, C. L.*, et al.* (2004). A family with severe insulin resistance and diabetes due to a mutation in AKT2. Science *304*, 1325-1328.

Giraud, S., Greco, A., Brink, M., Diaz, J. J., and Delafontaine, P. (2001). Translation initiation of the insulin-like growth factor I receptor mRNA is mediated by an internal ribosome entry site. J Biol Chem *276*, 5668-5675.

Grako, K. A., Olefsky, J. M., and McClain, D. A. (1992). Tyrosine kinase-defective insulin receptors undergo decreased endocytosis but do not affect internalization of normal endogenous insulin receptors. Endocrinology *130*, 3441-3452.

Greer, E. L., and Brunet, A. (2005). FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene *24*, 7410-7425.

Gunn, M. D., Sen, A., Chang, A., Willerson, J. T., Buja, L. M., and Chien, K. R. (1985). Mechanisms of accumulation of arachidonic acid in cultured myocardial cells during ATP depletion. Am J Physiol *249*, H1188-1194.

Guo, S., Dunn, S. L., and White, M. F. (2006). The Reciprocal Stability of Foxo1 and Irs2 Creates a Regulatory Circuit That Controls Insulin Signaling*. Mol Endocrinol.

Haggie, P. M., Stanton, B. A., and Verkman, A. S. (2002). Diffusional mobility of the cystic fibrosis transmembrane conductance regulator mutant, delta F508-CFTR, in the endoplasmic reticulum measured by photobleaching of GFP-CFTR chimeras. J Biol Chem *277*, 16419-16425.

Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. (2003). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol *5*, 461-466.

Hahn-Windgassen, A., Nogueira, V., Chen, C. C., Skeen, J. E., Sonenberg, N., and Hay, N. (2005). Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity. J Biol Chem *280*, 32081-32089.

Hamman, B. D., Hendershot, L. M., and Johnson, A. E. (1998). BiP maintains the permeability barrier of the ER membrane by sealing the lumenal end of the translocon pore before and early in translocation. Cell *92*, 747-758.

Hammond, C., Braakman, I., and Helenius, A. (1994). Role of N-linked oligosaccharide recognition, glucose trimming, and calnexin in glycoprotein folding and quality control. Proc Natl Acad Sci USA *91*, 913-917.

Hampton, R. Y. (2002). ER-associated degradation in protein quality control and cellular regulation. Curr Opin Cell Biol *14*, 476-482.

Haruta, T., Uno, T., Kawahara, J., Takano, A., Egawa, K., Sharma, P. M., Olefsky, J. M., and Kobayashi, M. (2000). A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Mol Endocrinol *14*, 783-794.

Hay, N., and Sonenberg, N. (2004). Upstream and downstream of mTOR. Genes Dev *18*, 1926-1945.

Hebert, D. N., Foellmer, B., and Helenius, A. (1996). Calnexin and calreticulin promote folding, delay oligomerization and suppress degradation of influenza hemagglutinin in microsomes. Embo J *15*, 2961-2968.

Hebert, D. N., Zhang, J.-X., Chen, W., Foellmer, B., and Helenius, A. (1997). The Number and Location of Glycans on Influenza Hemagglutinin Determine Folding and Association with Calnexin and Calreticulin. J Cell Biol *139*, 613-623.

Helenius, A., and Aebi, M. (2001). Intracellular functions of N-linked glycans. Science *291*, 2364-2369.

Helenius, A., Trombetta, E., Hebert, D., and Simons, J. (1997). Calnexin, calreticulin and the folding of glycoproteins. Trends Cell Biol *7*, 193-200.

Hua, Q. X., Hu, S. Q., Jia, W., Chu, Y. C., Burke, G. T., Wang, S. H., Wang, R. Y., Katsoyannis, P. G., and Weiss, M. A. (1998). Mini-proinsulin and mini-IGF-I: homologous protein sequences encoding non-homologous structures. J Mol Biol *277*, 103-118.

Hua, Q. X., Nakagawa, S. H., Wilken, J., Ramos, R. R., Jia, W., Bass, J., and Weiss, M. A. (2003). A divergent INS protein in Caenorhabditis elegans structurally resembles human insulin and activates the human insulin receptor. Genes Dev *17*, 826-831.

Huang, C., Somwar, R., Patel, N., Niu, W., Torok, D., and Klip, A. (2002). Sustained exposure of L6 myotubes to high glucose and insulin decreases insulin-stimulated GLUT4 translocation but upregulates GLUT4 activity. Diabetes *51*, 2090-2098.

Hubbard, S. R., Mohammadi, M., and Schlessinger, J. (1998). Autoregulatory mechanisms in protein-tyrosine kinases. J Biol Chem *273*, 11987-11990.

Hubbard, S. R., and Till, J. H. (2000). Protein tyrosine kinase structure and function. Annu Rev Biochem *69*, 373-398.

Hubbard, S. R., Wei, L., Ellis, L., and Hendrickson, W. A. (1994). Crystal structure of the tyrosine kinase domain of the human insulin receptor. Nature *372*, 746-754.

Hughes, B. D., Pailthorpe, B. A., White, L. R., and Sawyer, W. H. (1982). Extraction of membrane microviscosity from translational and rotational diffusion coefficients. Biophys J *37*, 673-676.

Hwang, J. B., and Frost, S. C. (1999). Effect of alternative glycosylation on insulin receptor processing. J Biol Chem *274*, 22813-22820.

Hwang, J. B., Hernandez, J., Leduc, R., and Frost, S. C. (2000). Alternative glycosylation of the insulin receptor prevents oligomerization and acquisition of insulin-dependent tyrosine kinase activity. Biochim Biophys Acta *1499*, 74-84.

Inoki, K., Zhu, T., and Guan, K. L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577-590.

Iozzo, P., Pratipanawatr, T., Pijl, H., Vogt, C., Kumar, V., Pipek, R., Matsuda, M., Mandarino, L. J., Cusi, K. J., and DeFronzo, R. A. (2001). Physiological hyperinsulinemia impairs insulin-stimulated glycogen synthase activity and glycogen synthesis. Am J Physiol Endocrinol Metab *280*, E712-719.

Joazeiro, C. A. P., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T., and Liu, Y. (1999). The Tyrosine Kinase Negative Regulator c-Cbl as a RING-Type, E2-Dependent Ubiquitin-Protein Ligase. Science *286*, 309-312.

Kabani, M., Kelley, S. S., Morrow, M. W., Montgomery, D. L., Sivendran, R., Rose, M. D., Gierasch, L. M., and Brodsky, J. L. (2003). Dependence of endoplasmic reticulum-associated degradation on the peptide binding domain and concentration of BiP. Mol Biol Cell *14*, 3437-3448.

Kahn, C. R., Neville, D. M., Jr., and Roth, J. (1973). Insulin-receptor interaction in the obese-hyperglycemic mouse. A model of insulin resistance. J Biol Chem *248*, 244-250.

Khamzina, L., Veilleux, A., Bergeron, S., and Marette, A. (2005). Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. Endocrinology *146*, 1473-1481.

Kim, S., Nollen, E. A., Kitagawa, K., Bindokas, V. P., and Morimoto, R. I. (2002). Polyglutamine protein aggregates are dynamic. Nat Cell Biol *4*, 826-831.

King, N., and Carroll, S. B. (2001). A receptor tyrosine kinase from choanoflagellates: molecular insights into early animal evolution. Proc Natl Acad Sci U S A *98*, 15032-15037.

Kolterman, O. G., Gray, R. S., Griffin, J., Burstein, P., Insel, J., Scarlett, J. A., and Olefsky, J. M. (1981). Receptor and postreceptor defects contribute to the insulin resistance in noninsulin-dependent diabetes mellitus. J Clin Invest *68*, 957-969.

Lamorte, L., and Park, M. (2001). The receptor tyrosine kinases: role in cancer progression. Surg Oncol Clin N Am *10*, 271-288, viii.

Le Marchand-Brustel, Y., Jeanrenaud, B., and Freychet, P. (1978). Insulin binding and effects in isolated soleus muscle of lean and obese mice. Am J Physiol *234*, E348-358.

Leach, M. R., Cohen-Doyle, M. F., Thomas, D. Y., and Williams, D. B. (2002). Localization of the lectin, ERp57 binding, and polypeptide binding sites of calnexin and calreticulin. J Biol Chem *277*, 29686-29697.

Leconte, I., Carpentier, J. L., and Clauser, E. (1994). The functions of the human insulin receptor are affected in different ways by mutation of each of the four N-glycosylation sites in the beta subunit. J Biol Chem *269*, 18062-18071.

Lee, A. V., Schiff, R., Cui, X., Sachdev, D., Yee, D., Gilmore, A. P., Streuli, C. H., Oesterreich, S., and Hadsell, D. L. (2003). New mechanisms of signal transduction inhibitor action: receptor tyrosine kinase down-regulation and blockade of signal transactivation. Clin Cancer Res *9*, 516S-523S.

LeRoith, D., and Roberts, C. T., Jr. (2003). The insulin-like growth factor system and cancer. Cancer Lett *195*, 127-137.

Levin, M. H., Haggie, P. M., Vetrivel, L., and Verkman, A. S. (2001). Diffusion in the endoplasmic reticulum of an aquaporin-2 mutant causing human nephrogenic diabetes insipidus. J Biol Chem *276*, 21331-21336.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R.*, et al.* (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science *275*, 1943-1947.

Li, W., Kennedy, S. G., and Ruvkun, G. (2003). daf-28 encodes a C. elegans insulin superfamily member that is regulated by environmental cues and acts in the DAF-2 signaling pathway. Genes Dev *17*, 844-858.

Lin, K., Dorman, J. B., Rodan, A., and Kenyon, C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science *278*, 1319-1322.

Lippincott-Schwartz, J., Snapp, E., and Kenworthy, A. (2001). Studying protein dynamics in living cells. Nat Rev Mol Cell Biol *2*, 444-456.

Liu, H., Hu, Y. P., Savaraj, N., Priebe, W., and Lampidis, T. J. (2001). Hypersensitization of tumor cells to glycolytic inhibitors. Biochemistry *40*, 5542-5547.

Liu, W., James, C. D., Frederick, L., Alderete, B. E., and Jenkins, R. B. (1997). PTEN/MMAC1 mutations and EGFR amplification in glioblastomas. Cancer Res *57*, 5254-5257.

Loo, M. A., Jensen, T. J., Cui, L., Hou, Y., Chang, X. B., and Riordan, J. R. (1998). Perturbation of Hsp90 interaction with nascent CFTR prevents its maturation and accelerates its degradation by the proteasome. EMBO J *17*, 6879-6887.

Lou, M., Garrett, T. P., McKern, N. M., Hoyne, P. A., Epa, V. C., Bentley, J. D., Lovrecz, G. O., Cosgrove, L. J., Frenkel, M. J., and Ward, C. W. (2006). The first three domains of the insulin receptor differ structurally from the insulin-like growth factor 1 receptor in the regions governing ligand specificity. Proc Natl Acad Sci U S A *103*, 12429-12434.

Lowell, B. B., and Shulman, G. I. (2005). Mitochondrial dysfunction and type 2 diabetes. Science *307*, 384-387.

Markussen, J., Jorgensen, K. H., Sorensen, A. R., and Thim, L. (1985). Single chain des-(B30) insulin. Intramolecular crosslinking of insulin by trypsin catalyzed transpeptidation. Int J Pept Protein Res *26*, 70-77.

Marr, M. T., 2nd, D'Alessio, J. A., Puig, O., and Tjian, R. (2007). IRES-mediated functional coupling of transcription and translation amplifies insulin receptor feedback. Genes Dev *21*, 175-183.

Marshall, S. (1985). Kinetics of insulin receptor internalization and recycling in adipocytes. Shunting of receptors to a degradative pathway by inhibitors of recycling. J Biol Chem *260*, 4136-4144.

Matsumoto, M., Pocai, A., Rossetti, L., Depinho, R. A., and Accili, D. (2007). Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor foxo1 in liver. Cell Metab *6*, 208-216.

Mayor, S., and Pagano, R. E. (2007). Pathways of clathrin-independent endocytosis. Nat Rev Mol Cell Biol *8*, 603-612.

McCracken, A. A., and Brodsky, J. L. (2005). Recognition and delivery of ERAD substrates to the proteasome and alternative paths for cell survival. Curr Top Microbiol Immunol *300*, 17-40.

McKern, N. M., Lawrence, M. C., Streltsov, V. A., Lou, M. Z., Adams, T. E., Lovrecz, G. O., Elleman, T. C., Richards, K. M., Bentley, J. D., Pilling, P. A.*, et al.* (2006). Structure of the insulin receptor ectodomain reveals a folded-over conformation. Nature *443*, 218-221.

Meusser, B., Hirsch, C., Jarosch, E., and Sommer, T. (2005). ERAD: the long road to destruction. Nat Cell Biol *7*, 766-772.

Molinari, M., Eriksson, K. K., Calanca, V., Galli, C., Cresswell, P., Michalak, M., and Helenius, A. (2004). Contrasting functions of calreticulin and calnexin in glycoprotein folding and ER quality control. Mol Cell *13*, 125-135.

Morris, J. B., Kenney, B., Huynh, H., and Woodcock, E. A. (2005). Regulation of the proapoptotic factor FOXO1 (FKHR) in cardiomyocytes by growth factors and alpha1-adrenergic agonists. Endocrinology *146*, 4370-4376.

Mosesson, Y., Shtiegman, K., Katz, M., Zwang, Y., Vereb, G., Szollosi, J., and Yarden, Y. (2003). Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation. J Biol Chem *278*, 21323-21326.

Motta, M. C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bultsma, Y., McBurney, M., and Guarente, L. (2004). Mammalian SIRT1 represses forkhead transcription factors. Cell *116*, 551-563.

Mukherjee, S., Tessema, M., and Wandinger-Ness, A. (2006). Vesicular trafficking of tyrosine kinase receptors and associated proteins in the regulation of signaling and vascular function. Circ Res *98*, 743-756.

Myers, M., and White, M. (2002). The molecular basis of insulin action. In Insulin Signaling: From cultured cells to animal model, G. Grunberger, and Y. Zick, eds. (New York, Taylor and Francis, Inc), pp. 55-87.

Nakae, J., Biggs, W. H., 3rd, Kitamura, T., Cavenee, W. K., Wright, C. V., Arden, K. C., and Accili, D. (2002). Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. Nat Genet *32*, 245-253.

Nakashima, N., Sharma, P. M., Imamura, T., Bookstein, R., and Olefsky, J. M. (2000). The tumor suppressor PTEN negatively regulates insulin signaling in 3T3-L1 adipocytes. J Biol Chem *275*, 12889-12895.

Nave, B. T., Ouwens, M., Withers, D. J., Alessi, D. R., and Shepherd, P. R. (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J *344 Pt 2*, 427-431.

Nehls, S., Snapp, E. L., Cole, N. B., Zaal, K. J., Kenworthy, A. K., Roberts, T. H., Ellenberg, J., Presley, J. F., Siggia, E., and Lippincott-Schwartz, J. (2000). Dynamics and retention of misfolded proteins in native ER membranes. Nat Cell Biol *2*, 288-295.

Nishikawa, S. I., Fewell, S. W., Kato, Y., Brodsky, J. L., and Endo, T. (2001). Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. J Cell Biol *153*, 1061-1070.

Nishiyama, T., Shirotani, T., Murakami, T., Shimada, F., Todaka, M., Saito, S., Hayashi, H., Noma, Y., Shima, K., Makino, H., and et al. (1994). Expression of the gene encoding the tyrosine kinase-deficient human insulin receptor in transgenic mice. Gene *141*, 187-192.

Ogg, S., Paradis, S., Gottlieb, S., Patterson, G. I., Lee, L., Tissenbaum, H. A., and Ruvkun, G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature *389*, 994-999.

Oh, S. W., Mukhopadhyay, A., Dixit, B. L., Raha, T., Green, M. R., and Tissenbaum, H. A. (2006). Identification of direct DAF-16 targets controlling longevity, metabolism and diapause by chromatin immunoprecipitation. Nat Genet *38*, 251-257.

Okabayashi, Y., Maddux, B. A., McDonald, A. R., Logsdon, C. D., Williams, J. A., and Goldfine, I. D. (1989). Mechanisms of insulin-induced insulin-receptor downregulation. Decrease of receptor biosynthesis and mRNA levels. Diabetes *38*, 182-187.

Olson, T. S., Bamberger, M. J., and Lane, M. D. (1988). Post-translational changes in tertiary and quaternary structure of the insulin proreceptor. Correlation with acquisition of function. J Biol Chem *263*, 7342-7351.

Pap, M., and Cooper, G. M. (1998). Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J Biol Chem *273*, 19929-19932.

Parodi, A. J. (2000a). Protein glucosylation and its role in protein folding. Annu Rev Biochem *69*, 69-93.

Parodi, A. J. (2000b). Role of N-oligosaccharide endoplasmic reticulum processing reactions in glycoprotein folding and degradation. Biochem J *348 Pt 1*, 1-13.

Pawson, T. (1995). Protein modules and signalling networks. Nature *373*, 573-580.

Paz, K., Hemi, R., LeRoith, D., Karasik, A., Elhanany, E., Kanety, H., and Zick, Y. (1997). A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. J Biol Chem *272*, 29911-29918.

Pederson, T. M., Kramer, D. L., and Rondinone, C. M. (2001). Serine/threonine phosphorylation of IRS-1 triggers its degradation: possible regulation by tyrosine phosphorylation. Diabetes *50*, 24-31.

Peng, X. D., Xu, P. Z., Chen, M. L., Hahn-Windgassen, A., Skeen, J., Jacobs, J., Sundararajan, D., Chen, W. S., Crawford, S. E., Coleman, K. G., and Hay, N. (2003). Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev *17*, 1352-1365.

Pessin, J. E., and Saltiel, A. R. (2000). Signaling pathways in insulin action: molecular targets of insulin resistance. J Clin Invest *106*, 165-169.

Petersen, K. F., Befroy, D., Dufour, S., Dziura, J., Ariyan, C., Rothman, D. L., DiPietro, L., Cline, G. W., and Shulman, G. I. (2003). Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science *300*, 1140-1142.

Pierce, S. B., Costa, M., Wisotzkey, R., Devadhar, S., Homburger, S. A., Buchman, A. R., Ferguson, K. C., Heller, J., Platt, D. M., Pasquinelli, A. A.*, et al.* (2001). Regulation of DAF-2 receptor signaling by human insulin and ins-1, a member of the unusually large and diverse C. elegans insulin gene family. Genes Dev *15*, 672-686.

Pinkston-Gosse, J., and Kenyon, C. (2007). DAF-16/FOXO targets genes that regulate tumor growth in Caenorhabditis elegans. Nat Genet *39*, 1403-1409.

Plas, D. R., and Thompson, C. B. (2003). Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. J Biol Chem *278*, 12361-12366.

Plemper, R. K., Bohmler, S., Bordallo, J., Sommer, T., and Wolf, D. H. (1997). Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. Nature *388*, 891-895.

Proud, C. G. (2002). Regulation of mammalian translation factors by nutrients. Eur J Biochem *269*, 5338-5349.

Puig, O., Marr, M. T., Ruhf, M. L., and Tjian, R. (2003). Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes Dev *17*, 2006-2020.

Puig, O., and Tjian, R. (2005). Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. Genes Dev *19*, 2435-2446.

Puig, O., and Tjian, R. (2006). Nutrient availability and growth: regulation of insulin signaling by dFOXO/FOXO1. Cell Cycle *5*, 503-505.

Qiao, L. Y., Goldberg, J. L., Russell, J. C., and Sun, X. J. (1999). Identification of enhanced serine kinase activity in insulin resistance. J Biol Chem *274*, 10625-10632.

Ramos, R. R., Swanson, A. J., and Bass, J. (2007). Calreticulin and Hsp90 stabilize the human insulin receptor and promote its mobility in the endoplasmic reticulum. Proc Natl Acad Sci U S A *104*, 10470-10475.

Rodriguez, M., Li, S. S., Harper, J. W., and Songyang, Z. (2004). An oriented peptide array library (OPAL) strategy to study protein-protein interactions. J Biol Chem *279*, 8802-8807.

Ronnett, G. V., Knutson, V. P., Kohanski, R. A., Simpson, T. L., and Lane, M. D. (1984). Role of glycosylation in the processing of newly translated insulin proreceptor in 3T3-L1 adipocytes. J Biol Chem *259*, 4566-4575.

Rossetti, L. (2000). Perspective: Hexosamines and nutrient sensing. Endocrinology *141*, 1922-1925.

Rouard, M., Bass, J., Grigorescu, F., Garrett, T. P., Ward, C. W., Lipkind, G., Jaffiole, C., Steiner, D. F., and Bell, G. I. (1999). Congenital insulin resistance associated with a conformational alteration in a conserved beta-sheet in the insulin receptor L1 domain. J Biol Chem *274*, 18487-18491.

Rouard, M., Macari, F., Bouix, O., Lautier, C., Brun, J. F., Lefebvre, P., Renard, E., Bringer, J., Jaffiol, C., and Grigorescu, F. (1997). Identification of two novel insulin receptor mutations, Asp59Gly and Leu62Pro, in type A syndrome of extreme insulin resistance. Biochem Biophys Res Commun *234*, 764-768.

Saito, Y., Ihara, Y., Leach, M. R., Cohen-Doyle, M. F., and Williams, D. B. (1999). Calreticulin functions in vitro as a molecular chaperone for both glycosylated and non-glycosylated proteins. EMBO J *18*, 6718-6729.

Saitoh, T., Yanagita, T., Shiraishi, S., Yokoo, H., Kobayashi, H., Minami, S., Onitsuka, T., and Wada, A. (2002). Down-regulation of cell surface insulin receptor and insulin receptor substrate-1 phosphorylation by inhibitor of 90-kDa heat-shock protein family: endoplasmic reticulum retention of monomeric insulin receptor precursor with calnexin in adrenal chromaffin cells. Mol Pharmacol *62*, 847-855.

Sakagami, M., Morrison, P., and Welch, W. J. (1999). Benzoquinoid ansamycins (herbimycin A and geldanamycin) interfere with the maturation of growth factor receptor tyrosine kinases. Cell Stress Chaperones *4*, 19-28.

Saltiel, A., and Pessin, J. (2002). Insulin signaling pathways in time and space. Trends in Cell Biology *12*, 65-71.

Sarbassov dos, D., Guertin, D. A., Ali, S. M., and Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098-1101.

Sayeed, A., and Ng, D. T. (2005). Search and destroy: ER quality control and ER-associated protein degradation. Crit Rev Biochem Mol Biol *40*, 75-91.

Scanga, S. E., Ruel, L., Binari, R. C., Snow, B., Stambolic, V., Bouchard, D., Peters, M., Calvieri, B., Mak, T. W., Woodgett, J. R., and Manoukian, A. S. (2000). The conserved PI3'K/PTEN/Akt signaling pathway regulates both cell size and survival in Drosophila. Oncogene *19*, 3971-3977.

Schlessinger, J. (2000). Cell signaling by receptor tyrosine kinases. Cell *103*, 211-225.

Schrag, J. D., Bergeron, J. J., Li, Y., Borisova, S., Hahn, M., Thomas, D. Y., and Cygler, M. (2001). The Structure of calnexin, an ER chaperone involved in quality control of protein folding. Mol Cell *8*, 633-644.

Seino, S., and Bell, G. I. (1989). Alternative splicing of human insulin receptor messenger RNA. Biochem Biophys Res Commun *159*, 312-316.

Seoane, J., Le, H. V., Shen, L., Anderson, S. A., and Massague, J. (2004). Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell *117*, 211-223.

Sepp-Lorenzino, L., Ma, Z., Lebwohl, D. E., Vinitsky, A., and Rosen, N. (1995). Herbimycin A induces the 20 S proteasome- and ubiquitin-dependent degradation of receptor tyrosine kinases. J Biol Chem *270*, 16580-16587.

Shah, O. J., Wang, Z., and Hunter, T. (2004). Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. Curr Biol *14*, 1650-1656.

Shi, Y., Sharma, A., Wu, H., Lichtenstein, A., and Gera, J. (2005a). Cyclin D1 and c-myc internal ribosome entry site (IRES)-dependent translation is regulated by AKT activity and

enhanced by rapamycin through a p38 MAPK- and ERK-dependent pathway. J Biol Chem *280*, 10964-10973.

Shi, Y., Yan, H., Frost, P., Gera, J., and Lichtenstein, A. (2005b). Mammalian target of rapamycin inhibitors activate the AKT kinase in multiple myeloma cells by up-regulating the insulin-like growth factor receptor/insulin receptor substrate-1/phosphatidylinositol 3-kinase cascade. Mol Cancer Ther *4*, 1533-1540.

Shulman, G. I. (2000). Cellular mechanisms of insulin resistance. J Clin Invest *106*, 171-176.

Snapp, E. L., Sharma, A., Lippincott-Schwartz, J., and Hegde, R. S. (2006). Monitoring chaperone engagement of substrates in the endoplasmic reticulum of live cells. Proc Natl Acad Sci USA *103*, 6536-6541.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., and et al. (1993). SH2 domains recognize specific phosphopeptide sequences. Cell *72*, 767-778.

Suga, H., Katoh, K., and Miyata, T. (2001). Sponge homologs of vertebrate protein tyrosine kinases and frequent domain shufflings in the early evolution of animals before the parazoan-eumetazoan split. Gene *280*, 195-201.

Summers, S. A., and Birnbaum, M. J. (1997). A role for the serine/threonine kinase, Akt, in insulin-stimulated glucose uptake. Biochem Soc Trans *25*, 981-988.

Supino-Rosin, L., Yoshimura, A., Yarden, Y., Elazar, Z., and Neumann, D. (2000). Intracellular Retention and Degradation of the Epidermal Growth Factor Receptor, Two Distinct Processes Mediated by Benzoquinone Ansamycins. J Biol Chem *275*, 21850-21855.

Svitkin, Y. V., Herdy, B., Costa-Mattioli, M., Gingras, A. C., Raught, B., and Sonenberg, N. (2005). Eukaryotic translation initiation factor 4E availability controls the switch between cap-dependent and internal ribosomal entry site-mediated translation. Mol Cell Biol *25*, 10556-10565.

Swanton, E., High, S., and Woodman, P. (2003). Role of calnexin in the glycan-independent quality control of proteolipid protein. Embo J *22*, 2948-2958.

Takano, A., Usui, I., Haruta, T., Kawahara, J., Uno, T., Iwata, M., and Kobayashi, M. (2001). Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. Mol Cell Biol *21*, 5050-5062.

Tatu, U., and Helenius, A. (1997). Interactions between newly synthesized glycoproteins, calnexin and a network of resident chaperones in the endoplasmic reticulum. J Cell Biol *136*, 555-565.

Taylor, S. I. (2002). Mutations in the insulin receptor gene. In Insulin signaling: from cultured cells to animal models, G. Grunberger, and Y. Zick, eds. (New York, Taylor and Francis), pp. 1-17.

Teng, D. H., Hu, R., Lin, H., Davis, T., Iliev, D., Frye, C., Swedlund, B., Hansen, K. L., Vinson, V. L., Gumpper, K. L.*, et al.* (1997). MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines. Cancer Res *57*, 5221-5225.

Theroux, S. J., Taglienti-Sian, C., Nair, N., Countaway, J. L., Robinson, H. L., and Davis, R. J. (1992). Increased oncogenic potential of ErbB is associated with the loss of a COOH-terminal domain serine phosphorylation site. J Biol Chem *267*, 7967-7970.

Tikhomirov, O., and Carpenter, G. (2000). Geldanamycin induces ErbB-2 degradation by proteolytic fragmentation. J Biol Chem *275*, 26625-26631.

Tremblay, F., Gagnon, A., Veilleux, A., Sorisky, A., and Marette, A. (2005). Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. Endocrinology *146*, 1328-1337.

Trischitta, V., Wong, K. Y., Brunetti, A., Scalisi, R., Vigneri, R., and Goldfine, I. D. (1989). Endocytosis, recycling, and degradation of the insulin receptor. Studies with monoclonal antireceptor antibodies that do not activate receptor kinase. J Biol Chem *264*, 5041-5046.

Ueki, K., Kondo, T., and Kahn, C. R. (2004). Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. Mol Cell Biol *24*, 5434-5446.

Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y. C., Tsubokawa, M., and et al. (1985). Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. Nature *313*, 756-761.

Vander Haar, E., Lee, S. I., Bandhakavi, S., Griffin, T. J., and Kim, D. H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat Cell Biol *9*, 316-323.

Vanhaesebroeck, B., and Alessi, D. R. (2000). The PI3K-PDK1 connection: more than just a road to PKB. Biochem J *346 Pt 3*, 561-576.

Verdu, J., Buratovich, M. A., Wilder, E. L., and Birnbaum, M. J. (1999). Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. Nat Cell Biol *1*, 500-506.

Wan, X., Harkavy, B., Shen, N., Grohar, P., and Helman, L. J. (2006). Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. Oncogene.

Wang, X., Venable, J., LaPointe, P., Hutt, D. M., Koulov, A. V., Coppinger, J., Gurkan, C., Kellner, W., Matteson, J., Plutner, H.*, et al.* (2006). Hsp90 cochaperone Aha1 downregulation rescues misfolding of CFTR in cystic fibrosis. Cell *127*, 803-815.

Werner, E. D., Brodsky, J. L., and McCracken, A. A. (1996). Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. Proc Natl Acad Sci U S A *93*, 13797-13801.

Whiteman, E. L., Cho, H., and Birnbaum, M. J. (2002). Role of Akt/protein kinase B in metabolism. Trends Endocrinol Metab *13*, 444-451.

Wullschleger, S., Loewith, R., and Hall, M. N. (2006). TOR signaling in growth and metabolism. Cell *124*, 471-484.

Xu, W., Mimnaugh, E., Rosser, M. F., Nicchitta, C., Marcu, M., Yarden, Y., and Neckers, L. (2001). Sensitivity of mature Erbb2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. J Biol Chem *276*, 3702-3708.

Xuan, Z., and Zhang, M. Q. (2005). From worm to human: bioinformatics approaches to identify FOXO target genes. Mech Ageing Dev *126*, 209-215.

Youker, R. T., Walsh, P., Beilharz, T., Lithgow, T., and Brodsky, J. L. (2004). Distinct roles for the Hsp40 and Hsp90 molecular chaperones during cystic fibrosis transmembrane conductance regulator degradation in yeast. Mol Biol Cell *15*, 4787-4797.

Zabolotny, J. M., Haj, F. G., Kim, Y. B., Kim, H. J., Shulman, G. I., Kim, J. K., Neel, B. G., and Kahn, B. B. (2004). Transgenic overexpression of protein-tyrosine phosphatase 1B in muscle causes insulin resistance, but overexpression with leukocyte antigen-related phosphatase does not additively impair insulin action. J Biol Chem *279*, 24844-24851.

Zhang, H., Cicchetti, G., Onda, H., Koon, H. B., Asrican, K., Bajraszewski, N., Vazquez, F., Carpenter, C. L., and Kwiatkowski, D. J. (2003). Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR. J Clin Invest *112*, 1223-1233.

Zhang, J. X., Braakman, I., Matlack, K. E., and Helenius, A. (1997). Quality control in the secretory pathway: the role of calreticulin, calnexin and BiP in the retention of glycoproteins with C-terminal truncations. Mol Biol Cell *8*, 1943-1954.

Zhang, L., Gjoerup, O., and Roberts, T. M. (2004). The serine/threonine kinase cyclin G-associated kinase regulates epidermal growth factor receptor signaling. Proc Natl Acad Sci U S A *101*, 10296-10301.

Zhao, J. J., Cheng, H., Jia, S., Wang, L., Gjoerup, O. V., Mikami, A., and Roberts, T. M. (2006). The p110alpha isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation. Proc Natl Acad Sci U S A *103*, 16296-16300.

APPENDIX

Table A.1. Sequences of optimized primers used in real-time PCR analysis for 293T HEK and HepG2 cell lines.

(Optimum concentrations are 10 uM except otherwise indicated.)

Name	Forward primer	Reverse primer
hGAPDH	5'-CCACATCGCTCAGACACCAT-3'	5'-CCAGGCGCCCAATACG-3'
hIR_3	5'-CGGAAGACAGTGAGCTGTTCG-3'	5'-CTCGAGGGCAGCTTCAGC-3'
hErbB2	5'-CTGCTGGACATTGACGAGACA-3'	5'-GCCGGCGGAGAATGG-3'
hTrfRec1	5'-GCTTTCCCTTTCCTTGCATATTC-3'	5'-GGTGGTACCCAAATAAGGATAATCTG-3'
hAKT1	5'-AGCGACGTGGCTATTGTGAAG-3'	5'-GCCGCCAGGTCTTGATGTAC-3'
hAKT2	5'- TGGCGGTCAGCAAGGCACG-3'	5' - CGGCCAGTGGCCTTCTCCC - 3'
hAKT3	5'-CTGTGGCACTCCAGAATATCTG-3'	5'- TGCTCGGCCATAGTCATTATC - 3'
hmTOR (5uM)	5'-TCCCCAAAGTGCTGCAGTACTAC-3'	5'- CTCATCGCGGGCTTGGT - 3'
hIRS-1	5'-CCAGAAGCAGCCAGAGGAC-3'	5'-GTCTGGGTACCCATGAGTTAGAAGAG-3'
hp70S6K-1	5'-CGGGCTCTGAGGATGAG-3'	5'-GCCAAGTTCATATGGTCCAACTCC-3'
h4EBP1	5'-GGCGGCACGCTCTTCA -3'	5'- TCAGGAATTTCCGGTCATAGATG -3'

Table A.2. Sequences of optimized primers used in real-time PCR analysis for MEFs from wild-type and AKT1$^{-/-}$ mice.

(*Optimum concentrations are 10 uM except otherwise indicated.)*

Name	Forward primer	Reverse primer
mGAPDH	5'-CAAGAAGTAAGAAACCCTGGACC-3'	5'-CGAGGTGGGATAGGGCCTCT-3'
mIR	5'-CATGGATGGAGGCTATCTGGAT-3'	5'-GGGTTGAACTGCCAGCACAT-3'
mEGFR-2	5'-GAGGCAGGGAGTGCGTGG-3'	5'-GATGTTCATGGCCTGGGGC-3'
mTrfRec1-1	5'-CCCGTTGTTGAGGCAGACC-3'	5'-CCCTGATGACTGAGATGGCGG-3'
mAKT1-1 (5uM)	5'-CTTCCATGTGGAAACGCC-3'	5'-CTTGAGTCCATCTGCCAC-3'
mAKT2-1	5'-GGATGAAGTCGCCCACAC-3'	5'-CGGTCATGGGTCTGGAAG-3'
mAKT3-1	5'-CCCCGAACACTCTCTTCAGATGC-3'	5'-ATCTGGCCCTCCACCAAGG-3'
mFOXO1-1	5'-GAGCGTGCCCTACTTCAAGG-3'	5'-GCTGTGAAGGGACAGATTGTGG-3'
mmTOR #3	5'-CGCCTGGCTGCATTCC-3'	5'-CACAGCTCAGGACATGGTTCA-3'
mpI3Kp85a-2	5'-GCAGGGCTGCTATGCCTG-3'	5'-CAGGTTGTAGGGCTCGGC-3'
mpI3Kp110a-2	5'-CCAATTGGTCCGTGTCCCG-3'	5'-CCCGGGTGCAAAGTGCTC-3'
mp70S6K-1 (5uM)	5'-GCCAGAAGATGCAGGCTCTGA-3'	5'-TCCCCCATGGTCCATGCTTTC-3'
mp85S6K-1	5'-CACAGCTGGGTGTCAGGC-3'	5'-GGGCATCTTTGGACTGCTCC-3'
m4EBP1 (5uM)	5'-CCACAGGTGAGTTCCGACACT-3'	5'-GCACGCTCTTCAGCACCAC-3'
mIRS1-1	5'-AGGTGGGACTGACTCGGAGAT-3'	5'-ACTGATGCTGGACGGGACAT-3'
mIRS2-1	5'-GCCGCTCCATGTCCATGC-3'	5'-GGTGATGCAGTGGATGAGGCA-3'

Table A.3. Sequences of optimized primers used in real-time PCR analysis for liver samples from wild-type and AKT1$^{-/-}$ mice.

(*Optimum concentrations are 10 uM except otherwise indicated.)*

Name	Forward primer	Reverse primer
mGAPDH	5'-CAAGAAGTAAGAAACCCTGGACC-3'	5'-CGAGGTGGGATAGGGCCTCT-3'
mIR-2	5'- GAT CAT CAA CAT CCG AGG GGG C -3'	5'- AGA GCA TAG GAG CGG CGG -3'
mEGFR-1	5'-GCTGGGGAGGAGGAGAGG-3'	5'-GGC AGC AGT CAC TGG GG-3
mTrfRec1-1	5'-CCCGTTGTTGAGGCAGACC-3'	5'-CCCTGATGACTGAGATGGCGG-3'
mAKT1-1 (5uM)	5'-CTTCCATGTGGAAACGCC-3'	5'-CTTGAGTCCATCTGCCAC-3'
mAKT2-1	5'-GGATGAAGTCGCCCACAC-3'	5'-CGGTCATGGGTCTGGAAG-3'
mAKT3-1	5'-CCCCGAACACTCTCTTCAGATGC-3'	5'-ATCTGGCCCTCCACCAAGG-3'
mFOXO1-1	5'-GAGCGTGCCCTACTTCAAGG-3'	5'-GCTGTGAAGGGACAGATTGTGG-3'
mmTOR #3	5'-CGCCTGGCTGCATTCC-3'	5'-CACAGCTCAGGACATGGTTCA-3'
mpI3Kp85a-2	5'-GCAGGGCTGCTATGCCTG-3'	5'-CAGGTTGTAGGGCTCGGC-3'
mpI3Kp110a-2	5'-CCAATTGGTCCGTGTCCCG-3'	5'-CCCGGGTGCAAAGTGCTC-3'
mp70S6K-1	5'-GCCAGAAGATGCAGGCTCTGA-3'	5'-TCCCCCATGGTCCATGCTTTC-3'
mp85S6K-1	5'-CACAGCTGGGTGTCAGGC-3'	5'-GGGCATCTTTGGACTGCTCC-3'
m4EBP1	5'-CCACAGGTGAGTTCCGACACT-3'	5'-GCACGCTCTTCAGCACCAC-3'
mIRS1-1	5'-AGGTGGGACTGACTCGGAGAT-3'	5'-ACTGATGCTGGACGGGACAT-3'
mIRS2-1	5'-GCCGCTCCATGTCCATGC-3'	5'-GGTGATGCAGTGGATGAGGCA-3'

CURRICULUM VITAE

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Education

Northwestern University Evanston, IL
Ph.D. Interdepartmental Biological Sciences (IBiS) (GPA 4.0/4.0) December 2007
- Coursework included: obesity, cancer biology, Alzheimer's disease, RNAi, biomedical marketing (taken at Kellogg School of Management)
- Dissertation topic: Molecular mechanisms of insulin receptor homeostasis
- American Association of University Women (AAUW) International Fellow

University of the Philippines Quezon City, Philippines
B. S. Molecular Biology and Biotechnology, *magna cum laude* March 1999
- Oblation Scholar (top 50 applicants to the University), Most Outstanding Student (Science & Technology), AT&T Student Leadership Awardee for Asia

Research and Teaching Experience

Northwestern University Evanston, IL
Dissertation research, Advisor: Dr. Joseph Bass 2001 - present
- Designed novel bioluminescent insulin receptor chimeras to enable live-cell imaging of protein transport in the cell to investigate the role of chaperone proteins in mobility and homeostasis of the insulin receptor
- Determined the roles of nutrient availability and insulin signaling in insulin receptor expression
- Actively mentored undergraduate students' independent research to enable them to successfully obtain competitive summer research grants from Northwestern University and the international Endocrine Society

Volunteer, Chute and Northwestern Science Education (CHANSE) Program Fall, 2007
- Engaged middle school students in an after-school program designed to give them a fun hands-on experience in different scientific experiments while providing encouragement and support

Teaching Assistant, Advanced Cell Biology and Biochemistry 2002 - 2005
- Designed lectures and quizzes for laboratory exercises and objectively evaluated students' reports and exams
- Facilitated small study group discussions to deepen students' understanding of lecture and laboratory topics

Mentor, Science and Engineering Research and Teaching Synthesis Program 2001-2004
- Introduced non-science undergraduates to scientific research and elucidated its significance through actual experiments and in-depth discussions of current breakthroughs in science

Science Fair Judge: local Chicago public schools 2006-2007
Intel International Science and Engineering Fair (Indianapolis, IN) May 10, 2006
Chicago Public School's Annual Science Fair, 7th-9th grade 2006-2007
Science Academy of Chicago's Science Fair for 3rd-8th Graders Dec. 2003
- Engaged the students in a discussion about their science projects, giving them constructive feedback and encouraging them to continue their scientific exploration

University of the Philippines Quezon City, Philippines
Research Assistant, Antibody Molecular Oncology Research Laboratory 1999-2000
- Cloned DNA constructs of humanized antibodies against specific antigens for breast cancer therapy; supervised several undergraduate students' thesis projects

Tutor, Krus na Ligas School 1996-1998
- Designed lessons and programs for underprivileged 3rd-6th grade students in science, math, and reading, and provided a fun and comfortable learning environment for them

Leadership Experience

Association for Women in Science (AWIS), Chicago chapter Chicago, IL
Vice-President, Service May, 2006 – Aug. 2007
- Strengthened collaborations with local groups and increased AWIS members' participation in various outreach programs including being resource speakers for the Chicago Science Expedition and various career talks (Argonne National Labs, public/private schools) and volunteering as judges in Chicago schools' science fairs
- Provided documentation of AWIS meetings and events
- Contributed feature articles to AWIS newsletter

Northwestern University Evanston, IL
Senior Community Assistant, Engelhart Graduate Residence May, 2006 - present
- Directed weekly meetings with other community assistants and supervised planning and implementation of academic, social and cultural programs for 300+ graduate students, scholars and their families
- Interviewed and selected new Community Assistants (CA)
- Edited and produced "Engelhart News", a bi-quarterly newsletter, to keep residents up-to-date about Engelhart activities and events and opportunities in school and in the Chicago area
- Coordinated with other CAs to produce a monthly duty schedule

Community Assistant, Engelhart Graduate Residence August, 2003 – May, 2006
- Conceptualized and implemented various academic, social and cultural programs for 300+ graduate students, scholars and their families
- Designed two more user-friendly handbooks for incoming residents with information about the Evanston community and living in either Engelhart Hall or McManus Hall (for NU Kellogg School of Management students)
- Edited and produced "Engelhart News", a bi-quarterly newsletter
- Maintained the Engelhart listserve and distributed weekly e-mails with announcements about events in Engelhart and Northwestern that would be of interest to residents

Student Volunteer, BIO 2006 Annual International Convention (Chicago) April 9-12, 2006
- Assisted in setting up venues for plenary speakers and coordinated with major sponsors to arrange requirements for private meetings

Volunteer Activities Chair, Graduate Student Association (GSA) 2003 - 2005
- Organized GSA's participation in various community service activities within Evanston and in the greater Chicago area (Project Pumpkin, Fairy Tale Trail, OASIS' Community Action Day)
- Wrote an article about GSA's volunteer activities which was featured in Northwestern University's "The Graduate School Quarterly" newsletter
- Helped GSA win the award for Most Outstanding Graduate Student Organization in 2003-2004 at Northwestern University

GSA representative, IBiS Student Organization (ISO) 2002-2004
- Coordinated with NU graduate student associations to organize an inter-campus faculty seminar series
- Provided feedback at GSA meetings and encouraged involvement of ISO members in GSA activities

Member, Orientation Committee/Advisory Board, International Office 2001 - 2004
- Conceptualized, organized and implemented various activities for the benefit of the international student population such as International Education Week, New Student Orientation and Tax Preparation workshops

Member, Graduate Young Adults (GYA), Sheil Center, Northwestern University 2000-2004
- Assisted in implementing various activities in the Sheil Center, including Mardi Gras, the Center's major annual fundraiser

University of the Philippines Quezon City, Philippines
Secretariat Committee, 10th Philippine-American Association of Scientists and Engineers Meeting July, 2000
- Coordinated with various speakers, poster presenters and attendees to ensure an efficient registration process both prior to and during the meeting

President, Molecular Biology and Biotechnology Society 1998-1999
- Organized and implemented various activities (i.e. "Science in the Movies" exhibit, Science Trivia contests, and inter-school quiz bees) to promote awareness and appreciation of scientific research by the general public

Publications

Hua, Q, Nakagawa, S., Wilken, J., **Ramos, RR**., Jia, W., Bass, J. Weiss, M. 2003. A divergent INS protein in *Caenorhabditis elegans* structurally resembles human insulin and activates the human insulin receptor. Genes and Development *17*, 826-831 (Perspective p 813-818).

Ramos, RR, Swanson, AJ, Bass, J. 2007. Calreticulin and Hsp90 stabilize the human insulin receptor and promote its mobility in the endoplasmic reticulum. PNAS *104*, 10470-10475.

Ramos, RR, Sakamoto, Y, Hay, N, Bass, J. *In vivo* regulation of receptor tyrosine kinase metabolism by PI3K/AKT/mTOR signaling. *In preparation.*

Presentations

Oral:
Ramos, M.R., Swanson, A.J., Bass, J. Dynamic Regulation of Membrane Protein Quality Control: Buffering by ER Calreticulin and Cytosolic Hsp90. 10th Annual Midwest Stress Response and Molecular Chaperone Meeting, Jan. 15, 2005. Evanston, IL.

Ramos, M.R., Calacal, G., Halos, S. Effects of Time and Environmental conditions on DNA Extracted from Human Bloodstains for PCR Amplification of STR loci. 15th Philippine Chemical Congress. May 26-29, 1999. Cebu City, Philippines

Poster:
Ramos, M.R., Sakamoto, Y., Bass, J. The role of PI3K/AKT/mTOR Signaling in Insulin Receptor Homeostasis. 20th Chicago Signal Transduction Symposium. May 24, 2007. Chicago, IL.

(Best poster) Ramos, M.R., Swanson, A. J., Bass, J. Calreticulin and Hsp90 Stabilize and Promote Insulin Receptor Mobility in the ER. 2nd Annual Chicago Diabetes Day. May 12, 2007. Chicago, IL.

Ramos, M. R., Swanson, A. J., Bass, J. Dynamic Regulation of Membrane Protein Quality Control by Calreticulin and Hsp90. American Association for the Advancement of Science' Annual Meeting. Feb. 16-20, 2006. St. Louis, MO.

Ramos, M.R., Swanson, A. J., Bass, J. Nutrient Regulation of Insulin Receptor Homeostasis. American Diabetes Association's 65th Scientific Sessions. June 10-14, 2005. San Diego, CA.

Ramos, M.R., Swanson, A. J., Bass, J. Role of Metabolism and Cell Signaling in Insulin Receptor Homeostasis. Endocrine Society's 86th Annual Meeting. June 15-19, 2004. New Orleans, LA.

Ramos, M.R., Swanson, A. J., Bass, J. Identification of Subtilisin-Like Protease Furin In Congenital Insulin Resistance And Nutrient Signaling Response. 17th Chicago Signal Transduction Symposium. May 28, 2004. Chicago, IL

Ramos, M.R., Bass, J. Identification of Subtilisin-Like Protease Furin In Congenital Insulin Resistance And Nutrient Signaling Response. NIDDK's Protein Misfolding and Misprocessing in Disease Symposium. May 4-5, 2004. Rockville, Maryland.

Ramos, M.R., Swanson, A.J., Bass, J. Role of PI3K and and Redox Flux in IR Biogenesis and Degradation. 9th Annual Midwest Stress Response and Molecular Chaperone Meeting, Jan 10, 2004. Evanston, IL.
Ramos, M.R., Swanson, A.J., Cheng, D. Bass. J. Receptor Tyrosine Kinase Degradation Involves Exofacial Cleavage and Phoshorylation. American Society for Cell Biology's 42nd Annual Meeting. Dec. 14-18, 2003. San Francisco, CA.

Ramos, M.R., Bass, J. Role of PI3K and mTOR in ER-Associated Degradation of the Insulin Receptor. 16th Chicago Signal Transduction Symposium. May 7, 2003. Chicago, IL

Ramos, M.R., Swanson, A.J., Cheng, D., Bass, J. Receptor Kinase Degradation Is Coupled To Glucose Metabolism And Involves ER-Golgi Transport. 8th Annual Midwest Stress Response and Molecular Chaperone Meeting, Jan 18, 2003. Evanston, IL.

Ramos, M.R., Cheng, D. Bass. J. Molecular Determinants of Receptor Kinase Trafficking in Living Cells. 15th Chicago Signal Transduction Symposium. May 23, 2002. Chicago, IL

Ramos, M.R., Cheng, D., Bass, J. Molecular Determinants of Receptor kinase Trafficking in Living Cells. 7th Annual Midwest Stress Response and Chaperone Meeting, Jan, 19, 2002. Evanston, IL.

Ramos, M.R., Calacal, G., Halos, S. Effects of Time and Environmental conditions on DNA Extracted from Human Bloodstains for PCR Amplification of STR loci. 21st Annual Scientific Meeting of the National Academy of Science and Technology. July 7-8, 1999. Manila, Philippines.

Skills

Laboratory: bacterial transformation and plasmid amplification, design and construction of mammalian expression vectors, mammalian cell culture, transfection, protein radiolabelling, western blotting, immunochemistry, widefield and laser confocal microscopy, live cell imaging (fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP)), RNAi, quantitative real-time polymerase chain reaction (RT-PCR)

Computer: proficient in both Windows and Mac platforms, Microsoft Office (Word, Powerpoint, Excel, Access, Publisher), Adobe publishing (InDesign, Illustrator, Pagemaker, Photoshop), EndNote, FileMaker, GraphPad Prism 4.0 (statistical software for sciences)

Awards and Fellowships

May, 2007	Best Poster, Chicago Diabetes Day
Feb. 2006, June 2005	Travel Grant, Graduate School, Northwestern University
2003 – 2004	American Association of University Women (AAUW) International Fellowship
2002	Fujisawa (Astellas) Research grant
Dec. 2000	AT&T Student Leadership Award for Asia
Oct. 1999	Chancellor's Award for Most Outstanding Student in the Science and Technology Cluster, University of the Philippines
April, 1999	*Magna cum laude*, B.S. Molecular Biology and Biotechnology, University of the Philippines
1995-1999	Oblation scholar (top 50 applicants to the University of the Philippines)
1995	College scholarship, Dept. of Science and Technology *(declined in favor of the Oblation scholarship)*

Professional Society Memberships

2007 - present	National Association of Science Writers (NASW)
2003 - present	American Association of University Women (AAUW)
2003 - present	Vice-President, Service (2006-2007), Association for Women in Science (AWIS)-Chicago Chapter
2003 - present	American Association for the Advancement of Science (AAAS)
2002 - present	American Society for Cell Biology (ASCB)
1999 - present	Phi Kappa Phi Honor Society, University of the Philippines, Diliman Chapter
1999 - present	Phi Sigma Honor Society, University of the Philippines, Diliman Chapter