PYRIMIDINE NUCLEOSIDE KINASE UCK1 AND TAM RECEPTOR TYROSINE KINASE MERTK CONVERGE ON THE UBIQUITIN-PROTEASOME PATHWAY TO REGULATE EGFR

Approved by supervisory committee

Michael A White, PhD

Michael Roth, PhD

Melanie Cobb, PhD

Kathlynn Brown, PhD

DEDICATION

I dedicate this work to all my family that came before me. I dedicate my time, and my effort to my husband and my children. I do this for them and their future, in an attempt to begin a tradition of educational excellence, of obtaining levels of knowledge beyond that of your peers, and seeking information about the world around you. Because ignorance is not bliss.

I thank my husband for his support and understanding. I also appreciate all the prayers and words of encouragement that that have been issued on my behalf.

Finally, I thank my Lord Jesus Christ, without whom I would not be here and would not have made it.

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by

VELEKA CASSIE IWUABA

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Veleka Cassie Iwuaba, PhD

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Supervising Professor: Michael A White, PhD

Oncogenic addiction to EGFR is observed in many tumor types often as a result of gene amplification and/or activating mutations. In this study, we are following up on two hits from a kinase screen, Receptor Tyrosine Kinase MerTK and Pyrimidine Nucleoside Kinase UCK1. We have discovered, that in addition to perturbing EGFR signaling and accumulation, they converge on the ubiquitin-degradation pathway in NSCLC (Non-Small Cell Lung Carcinoma). Loss of UCK1 reduces EGFR

accumulation by an EGF-independent mechanism but not other ErbB family members. Additionally, UCK1 depleted cells exhibit enhanced ubiquitin depletion, PARP inactivation, caspase-3 cleavage and increased BiP expression. Data from this study demonstrates that elevated BiP is likely due to depleted cytosolic ubiquitin pools and not induction of UPR. In contrast, Mertk loss results in significant EGFR accumulation, which appears to be enhanced by activating kinase mutations in EGFR, suggesting a trafficking defect in these cell lines. This data supports previous findings that EGFR mutants evade signal desensitization by prolonged residence in sorting endosomes and constitutive internalization/recycling.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF APPENDICES

LIST OF DEFINITIONS

UCK1 – Uridine Cytidine Kinase

MerTK – C-Mer proto oncogene

EGFR – Epidermal Growth Factor Receptor

EGFR – Epidermal Growth Factor

OS – Shed Photoreceptor Outer Segments

NSCLC – Non-Small Cell Lung Carcinoma

RTK – Receptor Tyrosine Kinase

ESCRT – Endosomal Sorting Complex Required for Transport

JAK/STAT – Janus Kinase/Signal Transducers and Activators of Transcription

PI3K/AKT – Phosphoinositide-3-kinase/Protein Kinase B

RAS/MAPK – Rat Sarcoma/Mitogen-Activated Protein Kinase

HUNK– Hormonally Up-Regulated Neu-Associated Kinase

PFKFB1 – 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 1

CSNK2A2 – Casein Kinase II Subunit Alpha

MGC16169 – TBC1 Domain Containing Kinase

TNK1 – Non-Receptor Tyrosine-Protein Kinase

DGUOK – Deoxyguanosine Kinase

GAS6 – Growth Arrest-Specific 6

PROTEIN S – Vitamin K-Dependent Plasma Glycoprotein

FAK – Focal Adhesion Kinase

TYPE I IFN – Type 1 Interferon

MIG6/RALT – Mitogen-Inducible Gene 6/ Receptor-Associated Late Transducer

C-CBL – CBL Proto Oncogene, E3 Ubiquitin Ligase

αvβ5 INTEGRIN – Integrin Alpha v Beta 5

NMII – Nonmuscle Myosin Heavy Chain IIA/ Nonmuscle Myosin Heavy Chain 9

PARP – Poly (ADP-ribose) polymerase 1

BiP – Binding Immunoglobulin Protein/GRP78

USP8/UBPY – Ubiquitin-Specific Protease 8

AMSH– STAM-binding Protein

CHAPTER ONE
INTRODUCTION

RECEPTOR TYROSINE KINASES

RTK Function

Protein Tyrosine Kinases (PTKs) are enzymes that phosphorylate on tyrosine residues. Within the class of PTKs are Receptor Tyrosine Kinases (RTK)s. The main function of receptor tyrosine kinases is to mediate the propagation of extracellular signals to the intracellular milieu where the nucleus is localized in order to exact a multitude of cellular changes including growth, proliferation, and survival. These kinases function as receptors for ligands such as growth factors, hormones, and cytokines. All RTKs have the following general architecture: N-terminal extracellular ligand-binding domains (except ErbB2), a single-pass transmembrane domain followed by a juxtamembrane region and a tyrosine kinase domain(Lax, 1991). Once bound by the ligand, the receptor can dimerize or oligomerize activating an autophosphorylation cascade or other proteins through phosphorylation of tyrosine residues. There are 58 RTKs, broken into 20 subfamilies (Figure 1) that are generally activated through ligand-mediated dimerization. Ligand binding induces a conformational change in the receptor, which initiates a series of autophosphorylation events along the intracellular tyrosine kinase domain.

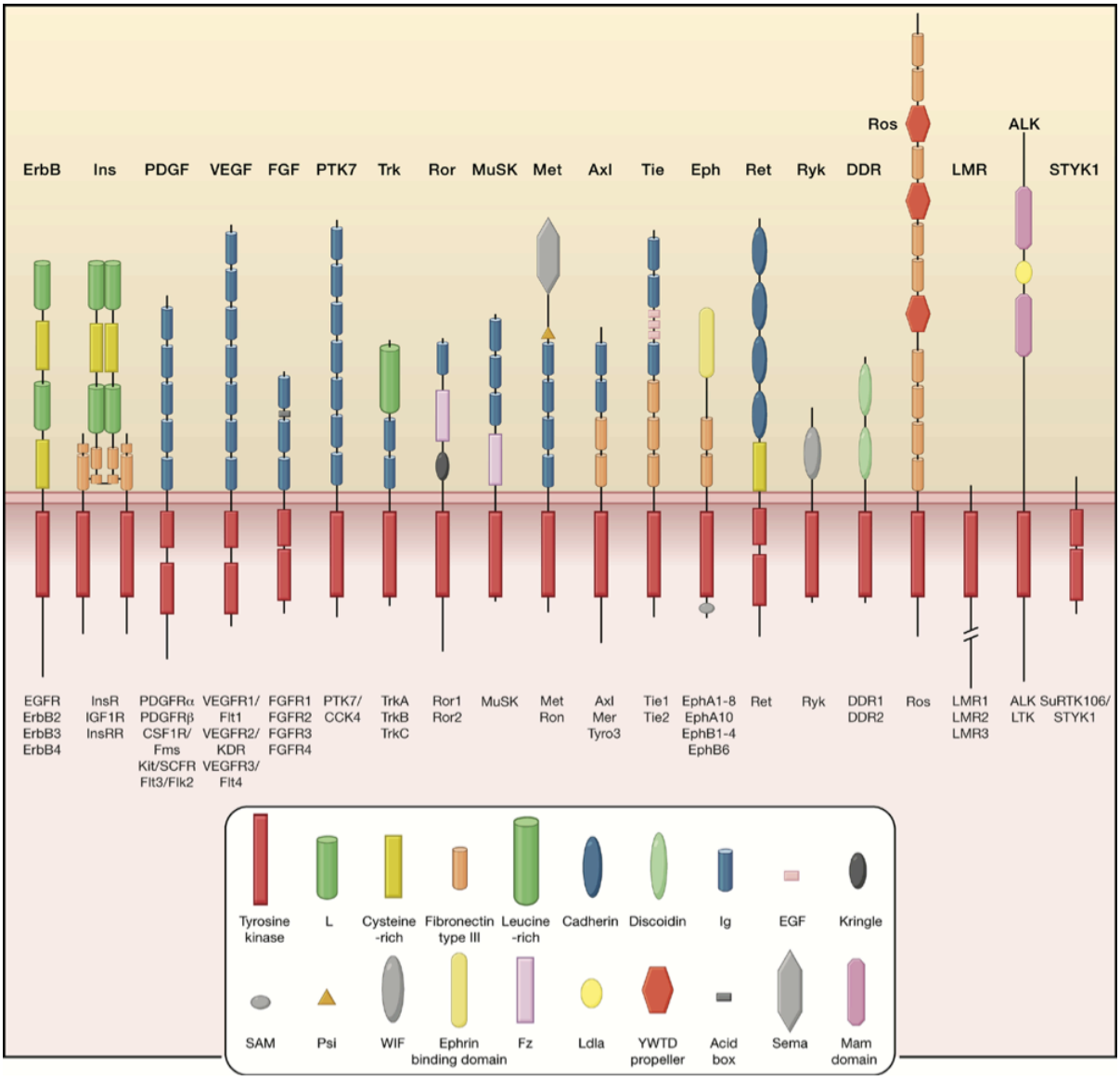


Figure 1. Receptor Tyrosine Kinase Subfamilies.
RTKs exhibit similar structural architecture consisting of an extracellular ligand binding domain and intracellular tyrosine kinase domain. Members of each RTK family are indicated below the respective receptor (Lemmon & Schlessinger, 2010).

These phosphorylation events signify the activation of the receptor and function as the nucleation center for signaling complexes. Activated receptors bind various adaptor/docking proteins that recruit effector molecules to further transduce signals

important for modulating protein synthesis/down regulation, cell proliferation, growth, survival, and motility.

RTK Signaling Pathways

RTKs signal through three main pathways including JAK/STAT, PI3K/AKT, and RAS/MAPK (Figure 2) to impact growth, survival, and proliferation (Hackel, Zwick, Prenzel, & Ullrich, 1999; Schlessinger, 2000). The PI3K/AKT cascade regulates many cellular functions including cell growth, cell survival, and cell cycle progression. This pathway can be stimulated by RTK activation, amplification, or overexpression in addition to alterations in the pathway itself (Tokunaga E, 2008). Activation of JAK/STAT results in a fairly direct delivery of an extracellular response to the cells transcriptional machinery to activate or repress target genes (Rawlings, Rosler, & Harrison, 2004; Yoh Dobashi, 2011). This can be achieved through RTK activation where STAT is phosphorylated by Src kinase or by crosstalk with the RAS/MAPK pathway (Rawlings et al., 2004). The RAS/MAPK signal transduction cascade is the major signaling pathway of RTKs and participates in extensive crosstalk whereby the pathway can activate or be activated by other RTK signaling pathways including

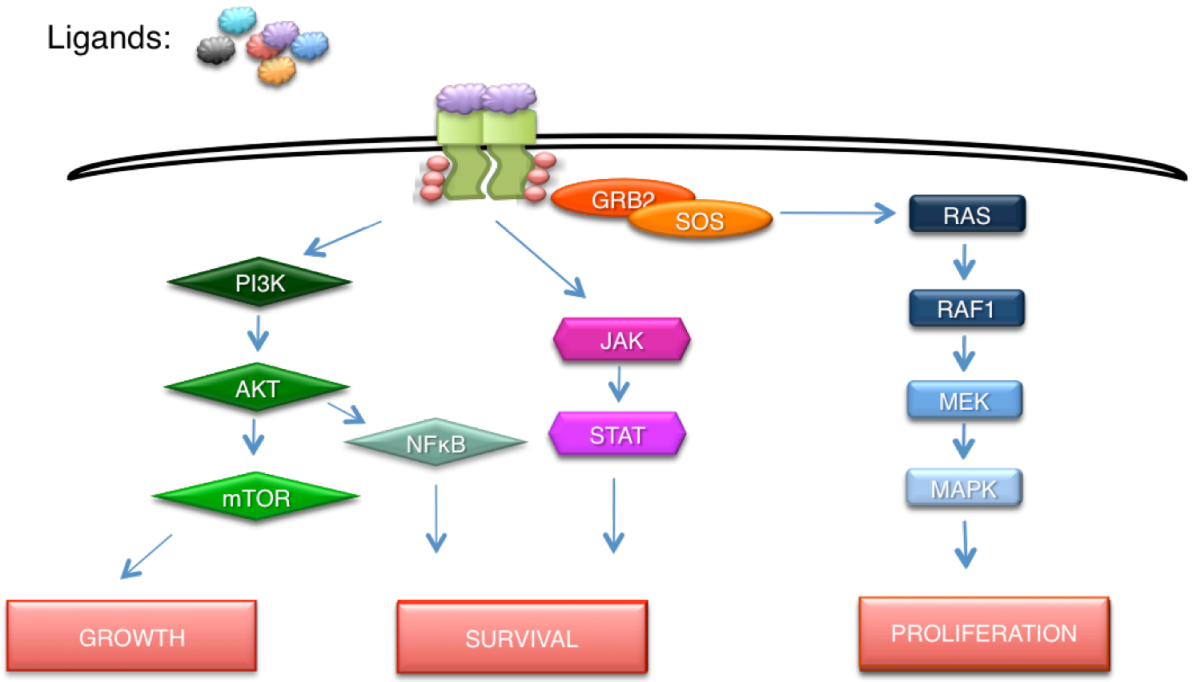


Figure 2. Receptor Tyrosine Kinase Signaling Pathway
Ligand activated RTKs recruit and activate SH- PTB-domain containing adaptor proteins to stimulate downstream effector pathways. PI3K/AKT, JAK/STAT, RAS/MAPK and others depending on the desired stimulatory effect. Through various effector proteins these pathways exhibit extensive inter-pathway crosstalk generating specific cellular changes

those mentioned above inducing a range of cellular modifications. Upon RTK activation by ligand, a series of autophosphorylation events followed by transphosphorylation take place on tyrosine residues in the TK domain increasing the receptors' kinase activity (Ullrich & Schlessinger, 1990). These phosphorylation events provide docking sites for Src homology 2(SH2) or phosphotyrosine binding (PTB) domain-containing substrates. Receptor activation recruits growth factor-bound 2 (Grb2) through its SH-domain which causes the translocation of the GEF

(guanine exchange factor) Sos (Son of sevenless) to the plasma membrane where it becomes constitutively bound to Grb2. This complex through Sos activation bridges the gap between RTKs and the RAS/MAPK pathway (Belov & Mohammadi, 2012; Lowenstein et al., 1992). Activation of the RAS/MAPK pathway results in the stimulation of multiple effector pathways including PI3K/AKT and phospholipase C-γ (PLCγ).

RTK Behavior in Disease States

Ligand/receptor autocrine loops, activating mutations or gene amplification leading to protein overexpression are the pathways through which many RTKs become constitutively active. In the absence of inhibitory brakes, these receptors propagate unrestrained signals to the nucleus. Signaling that is uncontrolled can lead to sustained cell proliferation and survival ultimately resulting in inflammatory malignancies that include cancer. Below I highlight a few receptor tyrosine kinases and how their aberrant expression leads to disease.

Anaplastic Lymphoma Kinase (ALK) Receptor Family

ALK Receptor family members include ALK and Leukocyte Receptor Tyrosine Kinase (LTK). This receptor family is reported to be important in neurogenesis (Dirks et al., 2002; Palmer, Vernersson, Grabbe, & Hallberg, 2009) . Chromosomal rearrangements, gene amplification, overexpression and mutations lead to oncogenic ALK. More than 20 ALK-fusion proteins are known in five different tumor

types often conferring enhanced oncogenic potential. Despite enhanced tumorigenesis these fusion proteins can serve as prognostic markers for patient outcome/survival as well as provide additional therapeutic targets. LTK is overexpressed in 14 types of cancers. Although, not much is known about the function of LTK, it has been reported to have a role in neural crest cell fate in mice and is highly expressed in leukemic cells and may be important for development (Bernards, 1990; Maru Y, 1990). Leveraging the high sequence homology between ALK and LTK, studies have demonstrated that LTK has transforming potential through activating mutations in it's kinase domain(Roll & Reuther, 2012).

Discoidin Domain (DDR) Receptor Family

This DDR family is composed of two members DDR1 and DDR2 and unlike other RTKs are activated by an extracellular matrix protein, collagen. The DDR receptor family is important in regulating changes in the cellular matrix. However, when these receptors are overexpressed cancer can result. Since DDR receptors regulate tissue organization, deregulation in the signaling capacities of these receptors either through overexpression, upregulation, or mutation can significantly enhance the tumor cells' ability to proliferate, invade, and metastasize (Valiathan, Marco, Leitinger, Kleer, & Fridman, 2012).

Hepatocyte Growth Factor (HGF)/ MET Receptor Family.

c-MET (c-MET proto oncogene) and RON (*recepteur d'origine nantais)* of the MET receptor family are highly pathogenic factors in tumor progression (Benvenuti et al., 2011; Wang, Zhang, Zhou , & Yao, 2013). c-MET and its ligand HGF are essential in epithelial biology during both embryogenesis and adulthood. HGF-activated c-MET mediates cell scattering, mobility, differentiation, and morphogenesis in addition to cell survival and proliferation. Like many other RTKs c-MET can be aberrantly activated through multiple ways; c-MET can undergo chromosomal rearrangement which causes constitutive dimerization, gene amplification with overexpression and constitutive kinase activation and overexpression independently of amplification. Amplification of c-MET is observed in some NSCLC that have become refractory to EGFR inhibitors; this phenomena highlights the importance of c-MET in tumor progression and offers opportunities for c-MET inhibition or use as a diagnostic treatment marker (Organ & Tsao, 2011). c-MET crosstalk with other co-receptors or RTKs such as EGFR and RON has been found to amplify downstream signaling and, in some cases where the receptors are both activated by their cognate ligands, signal synergism can occur (Benvenuti et al., 2011; Organ & Tsao, 2011). Additionally, this interaction can result in ligandless transactivation of c-MET as observed when TGFα/EGF-stimulated EGFR interacts with c-MET (Jo et al., 2000). RON also crosstalks extensively with other RTKS including c-MET. Where MSP-stimulated RON transphosphorylates c-MET and HGF-activated c-met can

transphosphorylate RON, similar transactivation events have been described between RON and EGFR or PDGFR (M.-H. Wang et al., 2013). This kind of activation can enhance the transactivated receptors' kinase activity. Benvenuti et al. (2011) demonstrated that crosstalk between RON and c-MET is important in c-MET oncogene addicted tumors. RON is transactivated by constitutively active c-MET and supports its tumorigenic potential by enhancing signal transduction; this evidence is supported by reduction in cell proliferation, anchorage-independent growth and tumor burden in nude mice xenografts (Benvenuti et al., 2011).

c-Ros Oncogene 1 (ROS) Receptor Family

With no known ligand, ROS1 is one of two orphan RTKs. In the context of disease, not much is known about how ROS1 is activated. We do, however, know that this receptor's oncogenic potential is propagated by its activation of Shp-2, overexpression in an amplification-independent manner, and oncogenic gene fusion events in several cancers, most notably NSCLC (Acquaviva, Wong, & Charest, 2009; Bergethon et al., 2012; Kurtis D Davies & Doebele, 2013; Kurtis D. Davies et al., 2012).

Tyro3/Axl/Mer (TAM) Receptor Family

TAM Family members Tyro3, Axl, and MerTK and their ligands Gas6 and Protein S are critical in tissue homeostasis where regular clearance of apoptotic material is essential to the health and function of the tissue. The process of spermatogenesis is

cyclic, alternating between meiosis and apoptosis in the seminiferous tubules. When phagocytic Sertoli cells are depleted of Tyro3, Axl, and MerTK the result is a degenerative form of male sterility due to the accumulation of apoptotic corpses. A similar phenotype is observed in the retina when MerTK activity is lost due to mutated MerTK in the retinal pigment epithelium. In both cases, the apoptotic corpse accumulation was due to the phagocyte being unable to recognize the “eat me” signal, exposed phophatidylserine (PtdSer) (H. A. Anderson et al., 2003; Krahling, 1999; Lemke, 2013; Lemke & Burstyn-Cohen, 2010). Both TAM ligands contain special ϒ-carboxylated Gla-domains that bind PtdSer on the apoptotic corpse surface while “bridging” to and activating TAM expressing phagocytes (Lemke, 2013; Lemke & Burstyn-Cohen, 2010). Not surprisingly, TAM receptors are important for the engulfment and phagocytosis of apoptotic cell corpses by professional phagocytes such as dendritic cells and macrophages. In addition, to their role in apoptotic clearance, TAMs also regulate the innate immune response by feedback inhibition. They competitively bind the type I IFN receptor (IFNAR) over the type I IFN and promote the induction of suppressor of cytokine signaling (SOCS)1 and 3 in dendritic cells (Behrens et al., 2003; Cohen et al., 2002; Lemke, 2013; Lemke & Burstyn-Cohen, 2010; Scott et al., 2001) TAMs dampen cytokine signaling. The induction of cytokines is important in fighting pathogens; however, unrestrained cytokine signaling can result in chronic inflammation. If coupled with apoptotic

corpse accumulation and the formation of secondary necrosis, this can lead to a variety of autoimmune diseases.

In contrast, emerging data is beginning to uncover a role for TAM receptors in tumor progression, survival, migration and proliferation. Several types of cancers have been identified that have altered expression and activation of TAM receptors, specifically Axl and MerTK (Brandao et al., 2013; Cummings, Deryckere, Earp, & Graham, 2013; Linger et al., 2013; Tworkoski et al., 2013; Verma, Warner, Vankayalapati, Bearss, & Sharma, 2011; Y. Wang et al., 2013).

Erythroblastic Leukemia Viral (V-Erb-B) Oncogene Homolog/ Epidermal Growth Factor Receptor (EGFR) Family

The EGF Receptor family encompasses EGFR, HER2/Neu, ErbB3, and ErbB4. Gene amplification, protein overexpression, and tyrosine kinase domain mutations in the ErbB family all result in constitutive signaling that can lead to oncogenesis. This receptor family along with its 11 ligands can form 28 homo- or hetero-dimers and make 614 possible receptor combinations (Roskoski Jr, 2014). However, HER2:HER2 and ErbB3:ErbB3 homodimers are essentially non-functional. The HER2 receptor does not contain a functional ligand-binding domain and ErbB3 is kinase impaired, hence, these receptors must heterodimerize with other ErbB members to signal (King, 1988; Lonardo F1, 1990; Shi F, 2010; Stern, Heffernan, & Weinberg, 1986). Despite these structural differences in the receptor, HER2 binding to other ErbB members especially EGFR and ErbB3 results in potent signaling

events (Graus-Porta, Beerli, Daly, & Hynes, 1997). ErbB family members play an important role in the pathogenesis of many cancer types including: lung, breast, gastric, colorectal, head and neck, pancreatic, and glioblastoma (Roskoski Jr, 2014). In lung cancer, NSCLC accounts for 85% of the cases with approximately 40% being designated as the subtype adenocarcinomas (found in never-smokers)(Collins LG, 2007; Herbst, Heymach, & Lippman, 2008). The pathogenesis of NSCLC is mediated by deregulated EGFR. Gene amplified/overexpressed EGFR is observed in many NSCLC tumors and cell lines. Subsets of these NSCLC are EGFR addicted, through mutation-mediated constitutive activation of the tyrosine kinase domain. These tumors have somatic alterations occurring in exons 18-21 that correspond to the tyrosine kinase domain of EGFR (Lynch et al., 2004). Single mutations in this region render EGFR constitutively active and can be targeted by drugs that affect the ATP binding site in the tyrosine kinase domain. Tyrosine Kinase Inhibitors (TKI)s, Erolitinib and Gefitinib bind the ATP binding pocket preventing unrestrained signaling by these receptors. Unfortunately, these tumors can develop a secondary mutation, T790M in exon 20 that is believed to sterically block access to the ATP binding site by the TKI (Pao et al., 2005).

Regulation of RTKs

Receptor Tyrosine Kinases are tightly controlled proteins, managed by layers of regulation. Autoinhibition, negative feedback, and endocytosis are the primary

means of RTK regulation and are very important in maintaining appropriate signaling. Defects in any one of these mechanisms can result in excessive signaling.

Autoinhibition

Autoinhibition, whereby the RTK maintains an inactive conformation is one mechanism utilized by receptors to prevent activation through dimerization or oligomerization. Other forms of autoinhibition include 1)*cis*-autoinhibition by the activation loop of the tyrosine kinase domain where residues either directly block substrate-binding sites or specifically stabilize the inactive conformation, 2) juxtamembrane autoinhibition in which residues of the juxtamembrane region make extensive contact with several regions in the tyrosine kinase domain stabilizing the autoinhibited conformation and, similar in mechanism to the aforementioned modes of autoinhibition, is 3) c-terminal autoinhibition (Lemmon & Schlessinger, 2010). Two examples of autoinhibition through a closed conformation are observed in the regulation of RTKs, EGFR and FGFR. EGFR is maintained in the inactive state by a conformation that relies on a specific interaction between domain II and domain IV of the extracellular domain. This conformation restricts the interaction of domains I and III impeding high affinity binding of ligand (Ferguson et al., 2003; Schlessinger, 2003). The autoinhibition of FGFR involves a co-factor. Receptor tyrosine kinase FGFR participates in bivalent ligand-dependent activation where in addition to FGF, heparan sulfate proteoglycan (HPSP)/heparin is necessary for receptor activation(Schlessinger, 2000). The FGFR extracellular region is composed of three

Ig-like domains (D1-D3) with domains D1 and D2 separated by a stretch of negatively charged amino acids (acid box) and domains D2 and D3 separated by a linker region that houses the FGF binding site (Johnson & Williams, 1993; Plotnikov, Schlessinger, Hubbard, & Mohammadi, 1999). In monomeric FGFR the acid box binds to a stretch of positively charged amino acids in the D2 domain that corresponds to the heparin-binding site. This binding both blocks the heparin binding site and causes the receptor to adopt a closed conformation thus inhibiting ligand-dependent activation of the receptor (Lemmon & Schlessinger, 2010; Plotnikov et al., 1999; Schlessinger, 2000, 2003; Wesche, Haglund, & Haugsten, 2011).

Negative Feedback Mechanisms/ Transcriptional Changes

Negative feedback is a function of stoichiometry and can control signal amplitude, strength and longevity. Negative feedback mechanisms are important in signal attenuation and often lead to transcriptional changes that involve the activation of immediate early genes and late response genes. RTK-mediated signaling is a very potent mode of signal transduction having significant impact on cell survival, growth, proliferation, and migration. It has been proposed that RTK pre-dimers exist in concentrations up to 50%. In the absence of ligand, basal autophosphorylation may occur; this is kept in check by cytosolic protein tyrosine phosphatases that maintain unliganded receptor in a dephosphorylated and inactive state (Östman & Böhmer, 2001).

Activated RTKs actively participate in signal desensitization through the activation of kinases and signaling cascades that either directly interact with the receptor or generate transcriptional changes to reduce its signaling potential (Fiorini, Alimandi, Fiorentino, Sala, & Segatto, 2001; Gordus et al., 2009; Östman & Böhmer, 2001; Schlessinger, 2003; Zhang & Woude, 2013). Examples of these kinds of RTK inhibition include Mig6/RALT induction by activation of the RAS/MAPK pathway. Mig6 can attenuate EGFR signaling by physical interaction with the kinase domain of active EGFR sheparding it through clathrin-mediated endocytosis leading to degradation. Direct interaction between Mig6 and the receptor is specific to ErbB family members, however, Mig6 can dampen RTK signaling through binding downstream effector molecules like Grb2, PI3K, and CDC42 to attenuate signaling through their respective pathways (Ledda & Paratcha, 2007; Zhang & Woude, 2013). Sprouty, another negative feedback inhibitor, like Mig6 is transcriptionally induced by activated receptors and dampen RTK signaling by both direct receptor interaction and interaction with signaling molecules of the RAS/MAPK pathway reducing signal transduction (Zhang & Woude, 2013). PTEN is an early response gene that antagonizes PI3K by degrading PIP3 to PIP2, attenuating AKT signaling (Ledda & Paratcha, 2007).

Endocytosis-mediated degradation

Ligand-induced endocytosis is another method by which RTK signaling is attenuated.

Activated receptors are either destined for lysosomal degradation or recycled back to the plasma membrane. Upon ligand binding and receptor activation, RTKs recruit E3 ubiquitin ligase c-CBl through Grb2 where the RTK is ubiquitylated and internalized via clathrin-mediated endocytosis (Goh & Sorkin, 2013; Huang F, 2005; H. F. Jiang X, Marusyk A, Sorkin A, 2003; S. A. Jiang X, 2003; Sorkin A, 1993; Vieira, 1990). Ubiquitylated RTKs are then sorted to endocytic vesicles of decreasing pH based upon specific interactions or modifications to the receptor such as ubiquitination and the ligand:receptor affinity in low pH conditions (Alwan, van Zoelen, & van Leeuwen, 2003; Roepstorff et al., 2009). Upon internalization, receptors are first sorted to the early endosome. Early endosomes are mildly acidic, ~pH 6.5, so pH-sensitive ligands like TGFα disassociate from EGFR and the receptor is sorted to the limiting membrane of the endosome and recycled back to the cell surface (Skarpen et al., 1998; Wiley & Burke, 2001). pH-insensitive ligand pairs like EGF:EGFR while still bound by c-CBL, ubiquitin, and ESCRT complexes (proteins important for endosomal retention, sorting and degradation of the receptor/ ”cargo”) are sorted to intraluminal vesicles (ILV) (Katzmann, Odorizzi, & Emr, 2002). Intraluminal vesicles are formed by the inward invagination of the endosomal membrane; these invaginations result in the maturation of the late endosome or multivesicular body (MVB) (Babst, 2011; Huotari, 2011; Hurley, 2010). Prior to ILV scission and the formation of the MVB, deubiquitinases (DUBs) USP8/UBPY and AMSH are recruited to the ESCRT-III complex to remove ubiquitin (Ub) and replenish cytosolic Ub pools

(Alwan & van Leeuwen, 2007; Amerik, Nowak, Swaminathan, & Hochstrasser, 2000; Kimura et al., 2009; Piper, Dikic, & Lukacs, 2014; Row, Prior, McCullough, Clague, & Urbé, 2006). The resulting “cargo” can either be recycled or retained for degradation upon lysosomal acidification. Deubiquitination certainly can result in the recycling and continued signaling of receptors, however, that is counteracted by ESCRT (0,I,II, and III) complex’s use of multiple ubiquitin binding domains (UBD), in which receptor ubiquitination is not the deciding factor, but rather how ESCRT complex members and accessory proteins are modified (Piper et al., 2014).

The Study That Lead to My Thesis

Our lab was interested in how cell regulatory systems are organized to better identify drug targets, predict drug interaction, and understand the pathological regulatory environment. Using high-throughput reverse phase protein array, Komurov et al leveraged the stimulus-response relationship of EGFR and its cognate ligand EGF to interrogate the kinome, using changes in ERK1/2 and STAT3 activation as an indicator of pathway activation.

Major Findings

This screen identified two sets of modulators of EGF-induced signaling; gene products that are required for signal propagation via diminished ERK1/2 signaling (HUNK, PFKFB1, CSNK2A2, and MGC16169) or decreased STAT3 (CSN2A2, UCK1, MerTK, TNK1)(Komurov et al., 2010). Both UCK1 and MerTK significantly

reduced EGFR accumulation in an EGF-dependent manner. This was likely due to enhanced EGFR internalization and degradation, which suggests that MerTK and UCK1 may play a role in EGFR trafficking and turnover. Furthermore, Komurov et al. (2010) demonstrated that a positive correlation exists in breast cancer patients with both high HER2 and high UCK1 or DGUOK (another nucleoside kinase identified in the screen) and poor outcome.

Implications

This study has identified two kinases amongst others that modulate EGFR signal propagation. MerTK and UCK1 seem to do this by affecting EGFR accumulation. Pyrimidine nucleoside kinases UCK1 and DGUOK not only impact EGFR accumulation but also may have clinical importance in determining patient outcomes and possible treatment options (Komurov et al., 2010). This study begins to highlight the importance of nucleoside kinases and their role in tumor progression in addition to how an aberrantly expressed RTK may regulate ErbB proteins and modulate their stability.

CHAPTER TWO
REVIEW OF THE LITERATURE

URIDINE CYTIDINE KINASE 1 (UCK1)

Human Uridine Cytidine Kinase 1 is located on chromosome 9 and has 7 exons that encode a 277 amino acid gene product with a molecular mass of 31 kDa (A. R. Van Rompay, Norda, Linden, Johansson, & Karlsson, 2001). Well conserved, human UCK1 shares 92% amino acid sequence similarity with mouse and 37% with *Caenorhabditis elegans*. UCK1 expresses two isoforms, a ubiquitously expressed 2.7kb mRNA transcript and a smaller 1.8kb transcript found only in heart, liver, kidney, and skeletal muscle (A. R. Van Rompay et al., 2001).

UCK1 Function

UCK1 is a nucleoside kinase that is the rate-limiting step in the anabolism of uridine and cytidine into UMP and CMP, respectively, in the pyrimidine-nucleotide salvage pathway (Figure 3) (E. P. Anderson & Brockman, 1964; Hatse, De Clercq, & Balzarini, 1999). UCK1 has been shown to be important in the activation of several cytotoxic ribonucleoside analogs such as 5-fluorouridine, 5-fluorocytidine, 6-azauridine, 5-azacytidine and 2-thiocytidine (Liacouras & Anderson, 1975; An R. Van Rompay, Johansson, & Karlsson, 2003). UCK1 is negatively regulated by the

accumulation of UTP and CTP (E. P. Anderson & Brockman, 1964) which is believed to distort the active homo-tetrameric complex (Suzuki, Koizumi, Fukushima, Matsuda, & Inagaki, 2004). Van Rompay (2003), also demonstrated that only ATP/GTP act as phosphate donors for UCK1 and that this ribonucleoside kinase cannot phosphorylate deoxyribonucleosides or purines nucleosides.

A

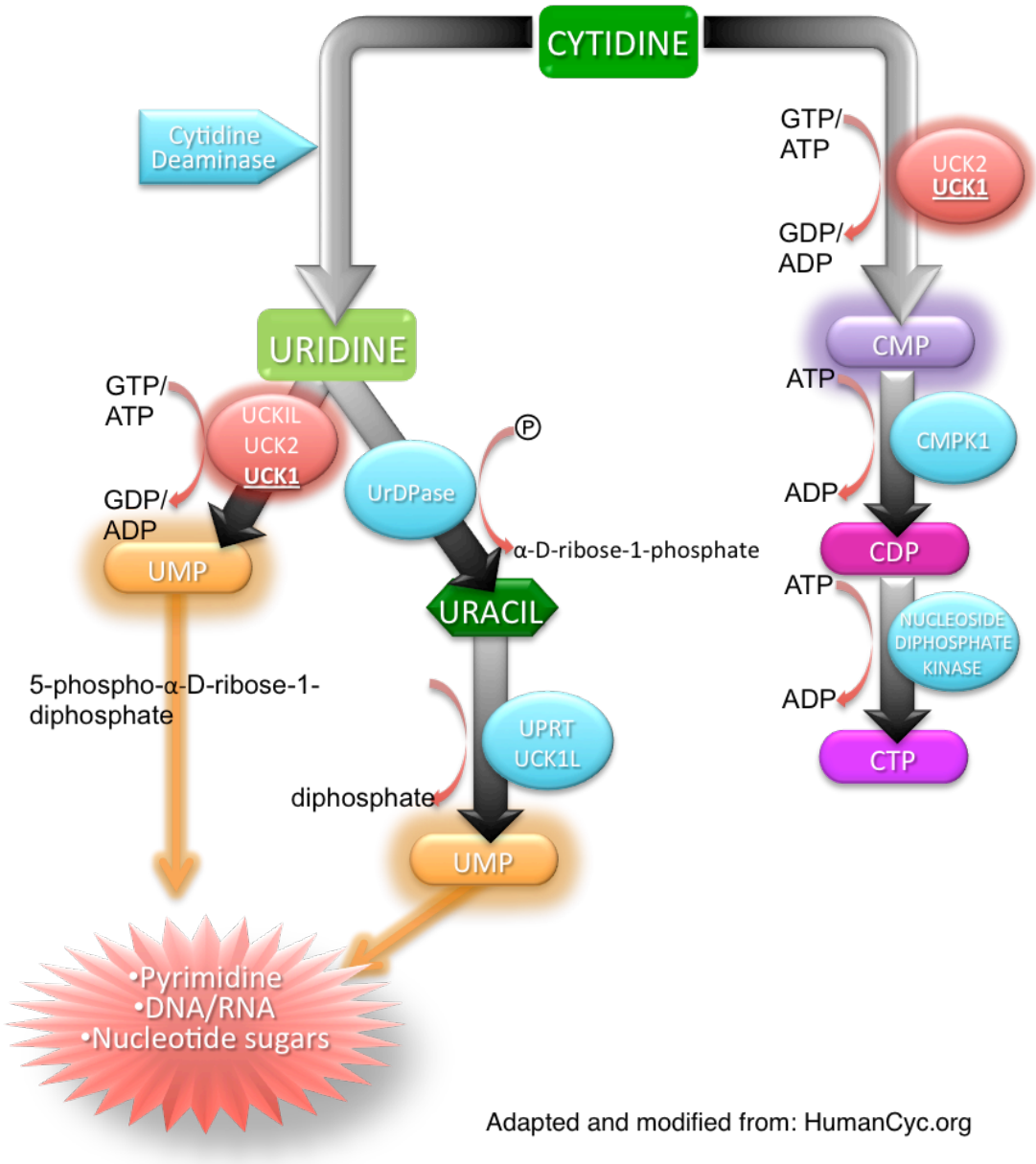


Figure 3. Pyrimidine Salvage Pathway
Uridine and Cytidine Metabolism by rate limiting Uridine Kinase molecules. UCK1 converts Uridine or Cytidine + ATP =>UMP or CMP + ADP. *UCK1 (Uridiidine Cytidine Kinase 1), UCK2 (Uridiidine Cytidine Kinase 2), UCK1L (Uridiidine Cytidine Kinase 1Like), UrDPase (Uridine Phosphorylase), UPRT (uracil phosphoribosyltransferase), CMPK1 (cytidine monophosphate (UMP-CMP) kinase 1).*

UCK1 in Cancer

Early studies suggested that UCK1 activity is increased in tumor cell versus normal cells and that UCK1 expression is increased in cell stimulated to divide (J. C. Cheng et al., 2004; N. Cheng & Traut, 1987; Greenberg, Schumm, Hurtubise, & Webb, 1977; F. Shen, Look, Yeh, & Weber, 1998). Much of the early work on UCK focused on determining UCK1 substrate specificity and phosphorylation of novel anticancer and antiviral nucleoside analogs; During this time it became increasing clear that UCK1 expression and/or activity is upregulated in neoplastic cells versus normal cells (Ahmed, 1982; Ahmed & Baker, 1980; Ahmed, Haggitt, & Welch, 1981; Ben-Kasus, Ben-Zvi, Marquez, Kelley, & Agbaria, 2005; J. C. Cheng et al., 2004; Cihak & Rada, 1976; Cihak, Seifertova, & Vesely, 1972; Connolly & Duley, 1999; Greenberg et al., 1977; Liacouras & Anderson, 1975; Liacouras, Garvey, Millar, & Anderson, 1975; Payne, Cheng, & Traut, 1985; F. Shen et al., 1998; Skold, Magnusson, & Revesz, 1962; A. R. Van Rompay et al., 2001; An R. Van Rompay et al., 2003; Winkler et al., 1964). Recent work by Komurov (2010), Hu (2012), and Cocoran (2013) has begun to reveal a role for UCK1 in cancer and its potential as a therapeutic target. Komurov (2010) demonstrated that EGFR, a known oncogene is significantly reduced in response to UCK1 depletion and that high UCK1 and high ErbB2 expression correlate with poor outcome in breast cancer patients. In another breast cancer study investigating triple negative breast cancer in SUM149 cells identified UCK1 as a hit and demonstrated that it's loss 1) inhibits growth in several

breast cancer cell lines including Trastuzumab-resistant cell line (HR5), 2) reduces $CD44^{high}$ sub population, and 3) specifically inhibits growth of sorted $CD44^{high}/CD24^{low}$ tumor initiating cells (TIC) suggesting that UCK1 may support cells with stem-like properties in a triple negative breast cancer model (Hu, Law, Fotovati, & Dunn, 2012). UCK1 was a top hit in a screen designed to identify genes that cooperate with MEK inhibitors to reduce cell viability in mutant Kras backgrounds using two cell lines with different sensitivities to MEK/PI3K inhibition (Corcoran et al., 2013). Ultimately, investigators followed up on BCL-XL as the top hit and found that, while MEK inhibition alone does not significantly induce apoptosis, nor does a small molecule inhibitor against BCL-XL, however, the combinatorial effect on cell viability are striking. Investigators attribute the synergistic effect of the inhibitors to the reduction of p-ERK leading to increased BIM, a pro-apoptotic protein, suggesting that MEK inhibition "primes" the cell for death but a second hit to the apoptotic pathway is required to induce cell death in KRAS mutant tumors (Corcoran et al., 2013).

C-MER PROTO-ONCOGENE (MERTK)

MerTK is a highly glycosylated single-pass transmembrane protein. The gene is located on chromosome 2 and contains 19 exons. MerTK shares three domains with TAM family members Axl and Tyro. Two serial immunoglobulin-like domains followed by two fibronectin type III domains on the extracellular, N-terminal side of

the protein define the TAM receptor family (Gal et al., 2000). The intracellularily localized tyrosine kinase domain contains the motif KW(I/L)A(I/L)ES which is has been described only for the TAM family.

MerTK Function

TAM family members including MerTK have been shown to regulate a number of cyclic processes to mediate tissue homeostasis and renewal (Lemke, 2013; Lemke & Burstyn-Cohen, 2010). MerTK is best known for its role in efferocytosis, the engulfment of apoptotic cells by phagocytes. Mutations in MerTK have been identified as a causative factor in early on-set retinal degeneration (D'Cruz et al., 2000; Gal et al., 2000). In MerTK-deficient Royal College of Surgeons (RCS) rats, a model for inherited retinal dystrophy, mutations in the MerTK gene result in defective phagocytosis of photoreceptor outer segment (OS) membranes by retinal pigment epithelial (RPE) cells (Feng, Yasumura, Matthes, LaVail, & Vollrath, 2002; Gal et al., 2000; Gallagher & LeRoith, 2010). In normal photoreceptor cells the OS are generated and cleared by phagocytosis on a daily basis to maintain proper length and function. When RPE are MerTK deficient, shed OS accumulate and trigger apoptosis leading to retinal degeneration.

In RPE-mediated phagocytosis, the shed OS are bound by apically localized αvβ5 integrin and FAK in polarized RPE (Anderson DH, 1995; Finnemann SC, 1997; Nandrot, Silva, Scelfo, & Finnemann, 2012; SC, 2003). The αvβ5 integrin and FAK

complex relocalizes to sites of shed OS and binds those particles. The RPE through αvβ5 integrin and FAK, activate MerTK allowing internalization of the bound OS particles. Nandrot et al. (2012) found that in MerTK-deficient or reduced RPE, αvβ5 integrin and FAK continue to bind OS cells in an enhanced unregulated manner, whereas in MerTK expressing RPE all αvβ5 integrin receptors at the cell surface are not actively binding photoreceptor OS. This suggests that MerTK may function as a negative regulator of αvβ5 integrin by limiting its binding potential of OS at the phagocytic surface (Nandrot et al., 2012). Further, Strick et al. demonstrated by coimmunprecipitation and co-localization experiments that NMII-A and NMII-B redistribute to sites of OS ingestion. NMII redistribution from the cell periphery in response to OS challenge was observed to be temporally and spatially MerTK-dependent (D. J. Strick & Vollrath, 2010; David J. Strick, Feng, & Vollrath, 2009). MerTK-dependent actin cytoskeletal reorganization in phagocytosis was later determined to be mediated through the activation of FAK and PLCγ2 by the specific activation of MerTK at Y867 (D. J. Strick & Vollrath, 2010; Tibrewal et al., 2008).

MerTK in Cancer

Although MerTK is known for its role in apoptotic corpse clearance, emerging data suggests that MerTK has a role in tumor survival and metastasis. In an attempt to identify novel B-cell tyrosine kinases, Graham et al. (1994) screened a B-

lymphoblastoid complementation library where MerTK was first isolated and cloned. Graham found that MerTK was expressed in a spectrum of tissues with the highest levels found in ovary, prostate, testis, lung, retina, and kidney. MerTK expression was also enriched within hematopoietic lineages (macrophages, dendritic cells, NK cells, NKT cells, megakaryocytes, and platelets). Initial observations revealed that MerTK was not expressed in mature or resting lymphocytes but in neoplastic B- and T-cell lines and other cancer cell lines including glioblastoma, lung, bladder, breast, colon and epidermoid carcinoma. This suggested to Graham that MerTK expression participates in tumor transformation. Later studies using a chimera receptor (EGFR ecto and transmembrane domain fused to MerTK tyrosine kinase domain) revealed that MerTK modulates actin cytoskeletal reorganization and blocks apoptosis (Guttridge et al., 2002). Additional work demonstrated that MerTK is important in the survival and metastatic potential of glioblastoma multiforme and melanoma (Rogers et al., 2012; Tworkoski et al., 2013; Y. Wang et al., 2013). Further, several groups have shown that MerTK is phosphorylated in different tumor types and when MerTK signaling is inhibited, migration, cell survival and growth are all attenuated (Brandao et al., 2013; Linger et al., 2013; Rogers et al., 2012; Schlegel et al., 2013; Tworkoski et al., 2013; Y. Wang et al., 2013).

CHAPTER THREE
Methodology

MATERIALS AND METHODS

Reagents and Antibodies

The following reagents were used in this study: MG132, Leupeptin, Tunicamycin, Thapsigargin, and Brefeldin A (Sigma-Aldrich), Cell TiterGlo reagent (Promega); hEGF (Peprotech), RNAiMAX (Life Technologies), and Dharmafect (Thermo Scientific).

The following antibodies were used: anti-UCK1(Aviva Systems Biology); anti-Ub(P4D1) (Santa Cruz Biotechnology), anti-β-actin and anti-β-tubulin (Sigma-Aldrich); all other antibodies used were from Cell Signaling Technology. All primary antibodies were diluted in 5% BST solution.

Cell Culture

Cell lines used: H358, H3255, HCC827, HCC366, H820, H1975, H1155, HCC95. All NSCLC cell lines used in this study were maintained in RPMI-1640 (Gibco;Life Technologies) supplemented with 5% FBS (Atlanta Biologicals) and 1% Penicilin-Streptomycin (Life Technologies).

siRNA Transfection

Forward Transfection

Cells were seeded to approximately ~200k/well in 6-well cluster plates in serum and antibiotic-free media. 24-hours later cells were transfected with 100nM siRNA with either Dharmafect (Thermo Scientific) or RNAiMAX (Life Technologies) transfection reagents in Opti-MEM (Life technologies) for 6-hours. Opti-Mem was replaced with complete conditioned media. Cells were transfected for 72-hours.

Reverse Transfection

Transfection reagent and siRNA was diluted in Opti-MEM for 5 minutes, separately. The transfection reagent and siRNA were then incubated together for 15 minutes after which trypsinized and counted cells were added to the siRNA and transfection reagent complexes and incubated overnight. The following day Opti-MEM was changed to complete conditioned media. Cells were seeded approximately ~7.5-10K/well in 96-well format or 200k-300k for 6-well cluster plates and transfected for 72-hours.

siRNA Oligos

UCK1 depletion was performed using pooled oligos from Dharmacon's siGENOME library. MerTK depletion was performed using either pooled oligos from Dharmacon's siGENOME library or Sigma-Aldrich.

Immunoprecipitation

Cells were lysed in Modified RIPA Buffer (25mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 1%NP-40) with Halt protease and phosphatase inhibitor cocktail (Pierce) on ice with rocking for 20 minutes. Lysates were then clarified by centrifugation for 15 minutes. Supernatant were collected and protein concentration determined by BCA (micro BCA). Supernatants were then incubated with antibody for 4 hours at 4 degrees Celsius with end over end rotation. Following antigen capture, agarose beads (Protein A/G; Santa Cruz Biotechnology) were added to each sample and incubated for 1 hour at 4 degrees Celsius. Samples were washed three times in modified RIPA buffer and proteins were eluted in sample buffer.

Apoptosis Assay

Apoptosis in MNT1 cells was quantitatively evaluated by flow cytometry. Cells were transfected for 72 hours. They were then harvested and fixed overnight in 80% ethanol at 4°C. Cells were then washed and stained with propidium iodide (BD

Pharmigen) for 15 minutes. Approximately 10,000 events were collected. Assays were kindly analyzed by Banu Eskiocak using Cell Quest Software.

Cell Viability

Cell viability experiments were carried out in 96-well format. Each sample was plated in triplicate. Transfected cells were incubated with CellTiter-Glo (Promega) for a total of 12 minutes (2 minute mixing by orbital shaker followed by 10 minute incubation) after a 30-minute room temperature equilibration period. The plates were then read on a PheraStar plate reader and analyzed in Excel or Prism.

Western Blot

Transfected samples were washed two times in PBS and harvested in 2% SDS-TRIS. Protein concentrations were determined by BCA and adjusted to 2ug/ul. Samples were then resuspended in 2x sample buffer and separated by according to size by SDS-PAGE. Proteins were then wet-transferred to PVDF membranes. Membranes were blocked in 5% Milk for 1 hour, washed 3 times in 0.1% TBST and incubated with primary antibodies overnight followed by 3 washes in TBST and a final 1-hour incubation with secondary antibodies.

Ubiquitination Assay

For the simple detection of ubiquitinated proteins, samples were processed as indicated for Western Blot with the addition of freshly prepared 10mM NEM (N-Ethylmaleimide) to both PBS and lysis buffers. For immunoprecipitated proteins, fresh 10mM NEM was added to PBS and IP lysis buffer throughout IP protocol including final wash steps prior to protein elution.

Inhibition of Degradative Pathways

Transfected H3255 cells were treated with DMSO, 25uM MG132, or 100uM Leupeptin for 4 hours in conditioned complete media. Whole cell lysates were then analyzed by Western blot.

Inhibition of Glycosylation

Transfected H3255 cells were treated overnight with 10ug/ml Tunicamycin (or as otherwise noted) or DMSO in complete conditioned media. Samples were then probed for total EGFR.

EGF-Stimulation

Stimulated samples were serum starved for either 4 hours or over night during the 72-hour transfection period. Samples were incubated with 100ng/ul EGF for the times indicated at 37°C and immediately placed on ice at the end of the incubation time point for lysis.

Serum-Deprivation and PARP Assay

Post-transfection H3255 cells were either fed fresh media containing serum daily, fed fresh media containing serum for 24 followed by serum-free media every 24-hours for 2 cycles (48-hour time point) or fed fresh media containing serum every 24-hours for 2 cycles followed by a final cycle of serum-free media for 24-hours (24-hour time point). For PARP analysis, media was collected by centrifugation and pooled with SDS-TRIS harvested lysate for each individual sample.

CHAPTER FOUR
Results

UCK1

Here we present data that demonstrates how UCK1 supports cell survival by enhancing EGFR stability through modulating the ubiquitin-proteasome pathway.

UCK1 Primarily Affects EGFR Accumulation

In a previous study by our lab we identified two kinases that significantly affected EGFR accumulation: MerTK and UCK1(Komurov et al., 2010) (Appendix A). This finding was observed in A431, an epidermoid carcinoma cell line that overexpresses gene amplified WT EGFR. We wanted to determine whether this phenotype is dependent on EGFR status and if this effect is ErbB-centric or EGFR-specific. Loss of UCK1 generally results in decreased EGFR accumulation and altered downstream signaling with the degree to which these changes occur depending on the genetic background of the cell line (Figure 4). Importantly, reduction of EGFR is not dependent on EGF-stimulated activation as observed in 4 of 5 NSCLC tested. We also investigated if other ErbB family members were similarly sensitive to UCK1 depletion (Figure 4B). HER2 levels were not altered in response to

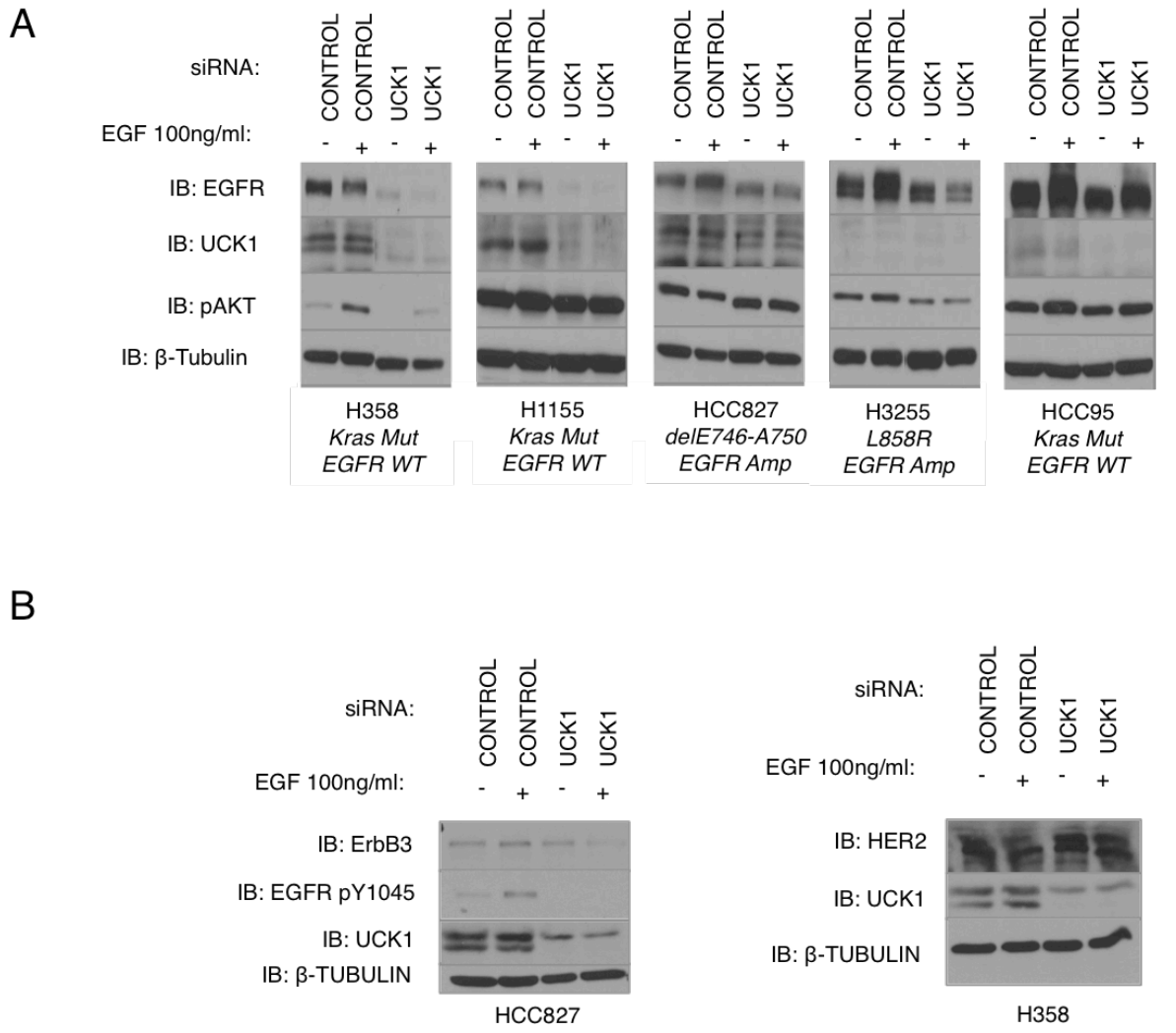


Figure 4. UCK1 Depletion Primarily Effects EGFR Accumulation
(A) NSCLC cell lines were incubated for 5 minutes with or without 100 ng/ml EGF, post overnight serum starvation. EGFR and phospho-AKT expression was assessed by immunoblotting whole-cell lysates. (B) NSCLC cell lines were incubated for 5 minutes with or without 100 ng/ml EGF, post overnight serum starvation. Additional ErbB family members, ErbB3 and HER2/Neu, and phospho-EGFR Y1045 expression was assessed by immunoblotting whole-cell lysates. Tubulin was used as a loading control n=2+.

UCK1 depletion in NSCLC H358, which expresses moderate to high levels of HER2. ErbB3 expressing cell line HCC827 shows a less than moderate reduction of ErbB3 in an EGF-dependent manner. Although accumulation changes in ErbB3 are negligible, EGFR activation and phosphorylation of Y1045 is decreased.

Loss of UCK1 does not result in a glycosylation defect

Pyrimidines are important in nucleic acid metabolism and the generation of nucleotide sugars for glycosylation, therefore we hypothesized that loss of UCK1 may compromise protein glycosylation. EGFR is a heavily glycosylated protein and inadequate glycosylation could result in its retention in the ER and subsequent degradation. Hypo-glycosylated proteins will migrate faster on polyacrylamide gels and exhibit a mobility shift at a lower than expected molecular weight. In UCK1-depleted cells EGFR is found at the correct molecular weight. Upon treatment with Tunicamycin, an inhibitor of the first step of the dolichol pathway for N-glycosylation, UCK1 depletion does not affect glycosylation of EGFR. However, it is interesting that fully glycosylated EGFR is depleted, as seen in (Figure 5). This suggests that UCK1 supports EGFR stability and possibly its ability to be localized to the cell surface. Since only the glycosylated form was degraded this further highlights the importance of appropriate and specific post-translational modifications to support protein stability. Further more, the presence of immature EGFR versus mature EGFR posits the possibility that UCK1 also supports membrane bound factors important for EGFR

stability and retention in the membrane, a Triton-X 100 insoluble fraction (Ling, Li, Perez-Soler, & Haigentz, 2009).

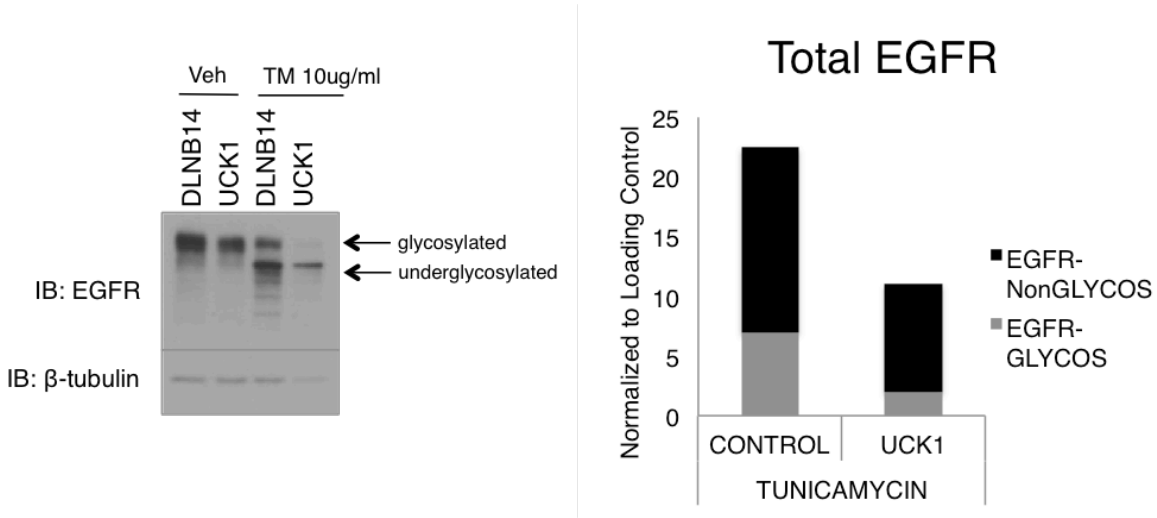


Figure 5. Loss of UCK1 does not generate a glycosylation defect
(A) H3255 cells were treated overnight with 10ug/ml Tunicamycin or DMSO. Total EGFR accumulation quantified by measuring blot density. Total EGFR (A) expression was detected by immunoblot of whole-cell lysate. Tubulin was used as loading control n=2.

UCK1 depletion results in baseline induction of apoptosis and enhanced BiP expression

In an effort to explore whether loss of EGFR is due to induction of the unfolded protein response (UPR), I challenged H3255 cells with Tunicamycin, an ER stress inducer. Loss of UCK1 did not differentially induce ER stress under these conditions, (data not shown), however, PARP is inactivated (Figure 6A). The use of high dosage Tunicamycin, to induce ER stress can be harsh and disrupting to many processes vital for cell survival. To simplify and clarify whether loss of UCK1 inactivates PARP, H3255 cells were serum starved in a time dependent manner. In the presence of

constant serum UCK1 depleted cells have baseline inactivated PARP. This suggests that UCK1 supports cell survival and its loss “primes” the cell for cell death. It is not clear how UCK1 does this. Loss of UCK1 may severely impact RNA/DNA synthesis and, therefore, genomic stability. Further more, the H358 cell line under identical conditions to (Figure 6B) demonstrates that UCK1 depletion alone generates baseline BiP above control samples. Consistent with (Figure 6A) and (Figure 6B), (Appendix C) demonstrates that in H358 that baseline PARP cleavage is caspase 3 dependent. Moreover, H358 treated with 1uM tunicamycin has been reported to cause a reduction in cell viability of not greater than 20% after 72-hours treatment and does not generate active cleaved caspase 3 after 48hrs of treatment (Ling et al., 2009). These data suggest that UCK1 supports survival and that baseline induction of apoptosis may be BiP-independent.

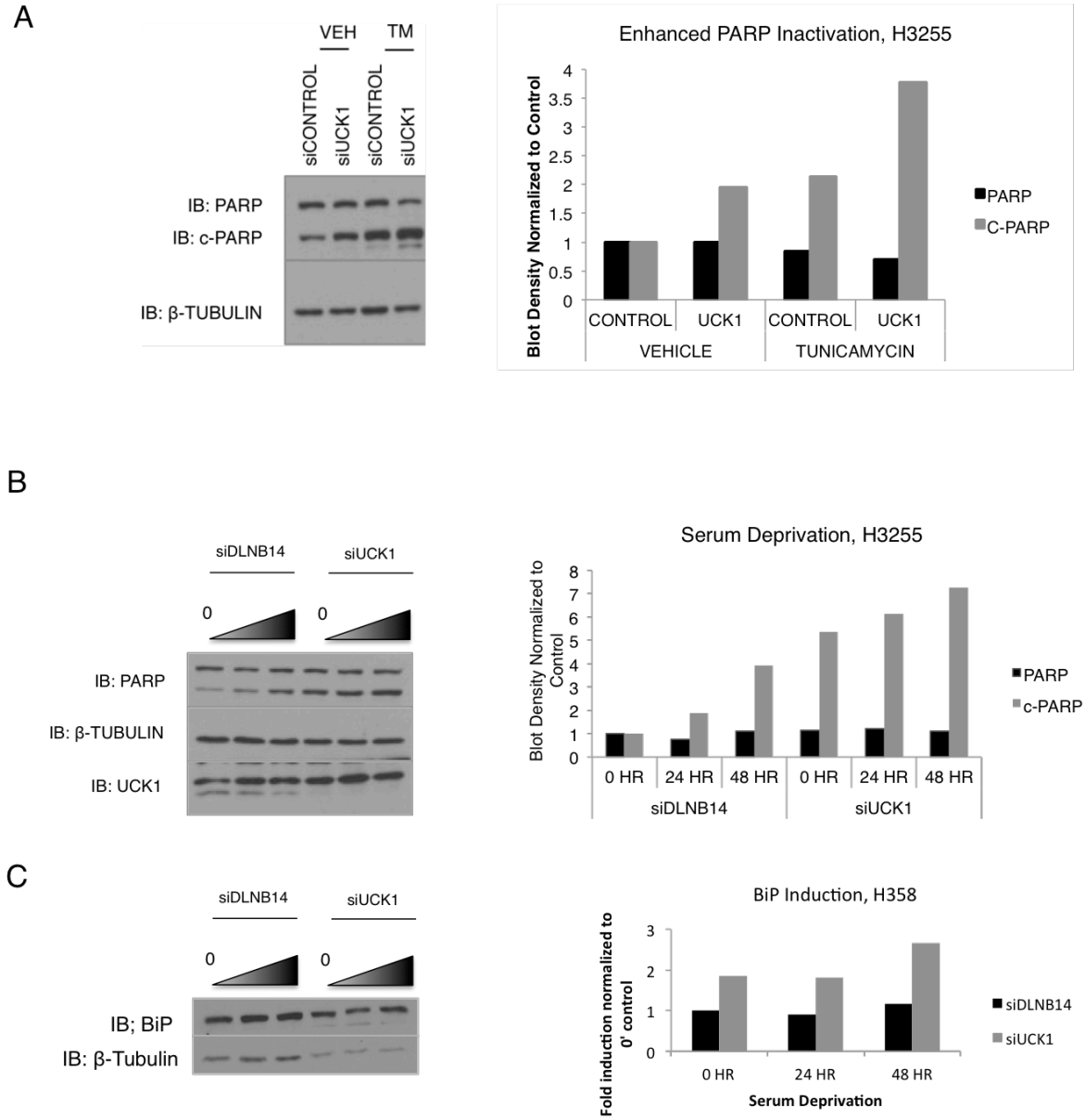


Figure 6. UCK1 loss elevates baseline PARP inactivation and BiP levels
(A) NSCLC cell line H3255 treated for 16 hours with Veh(Vehicle), TM(Tunicamycin, 5ug/ml). (B) NSCLC cell line H3255 serum starved for 0, 24, or 48 hours. (C) NSCLC cell line H358 treated as (B) and probed with BiP antibody. Tubulin used as loading control. Westerns were resolved using SDS-PAGE and immunoblotted for PARP (recognizes total and cleaved PARP species) or UCK1. Cells were transfected for 72 hours. Proteins were quantified by measuring blot density in Photoshop n=2.

UCK1 depletion impacts protein ubiquitination and proteasomal-directed degradation

UCK1-dependent loss of EGFR appears to be a general phenotype across NSCLC cell lines. In order to address the decreased accumulation of EGFR in the cell lines in response to siUCK1, we observed how EGFR responds to blockage of the proteasome using MG-132 and inhibition of lysosome hydrolase activity by Leupeptin. Inhibition of both pathways resulted in a partial restoration of EGFR and down stream signaling, with a more potent rescue by proteasomal inhibition (Figure 7). Unexpectedly, we observed a significant reduction in global protein ubiquitination upon UCK1 loss. These data suggest that (1) UCK1 supports the stability of EGFR and other proteins as well. (2) There may be a subset of proteins that UCK1 protects from ubiquitination. Additionally, there maybe a smaller pool of proteins that UCK1 regulates directly and independently from ubiquitination since there was not a complete rescue by degradation inhibitors.

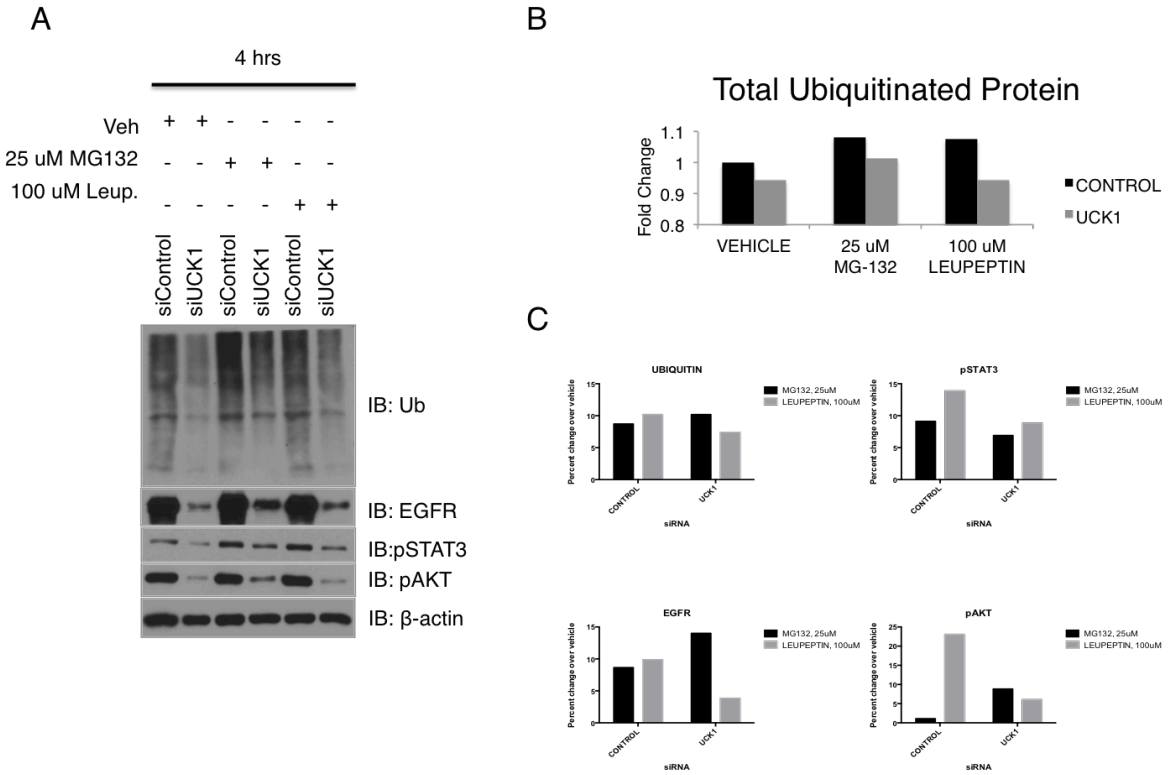


Figure 7. UCK1 depletion impacts protein ubiquitination and proteasomal directed degradation

(A) NSCLC cell line H3255 was harvested 72-hours post transfection and 4-hour incubation with either DMSO, 25uM MG132, or 100 uM Leupeptin. Proteins were resolved by SDS-PAGE and immunoblotted for UB (ubiquitin), EGFR, pSTAT3, or pAKT expression. Tubulin was used as the loading control. (B) Quantification of total ubiquitinated proteins for each treatment by blot density. (C) Quantification and comparison of protein rescue between proteasomal inhibitor MG132 and Lysosomal inhibitor Leupeptin as measured by blot density. All blot density measurements were done using Photoshop n=2.

UCK1 loss depletes low molecular weight ubiquitin.

UCK1 exquisitely affects ubiquitination. Here we examine ubiquitin deficiency in closer detail by looking at two major ubiquitin linkage sites: 1) K48-linkages which direct proteins towards proteasomal degradation and 2) K63-linkages that are

important in signaling (Nguyen, Kolch, & Kholodenko, 2013; Palombella, Rando, Goldberg, & Maniatis, 1994), DNA repair (Bennett & Clarke, 2006; Komander & Rape, 2012; Sobhian et al., 2007), and vesicle trafficking (Acconcia, Sigismund, & Polo, 2009; Adhikari & Chen, 2009; Amerik et al., 2000; Nathan, Tae Kim, Ting, Gygi, & Goldberg, 2013). In two different NSCLC cell lines with varying genetic backgrounds, HCC827 and H358, we see that loss of UCK1 strongly impacts low molecular weight K63-linked proteins (Figure 8). UCK1 depletion causes a change in the amount of K63- and K48-ubiquitin linkages, especially the mono- or free-ubiquitin pools. This suggests that a portion of free ubiquitin is bound by substrates as indicated by the existence of high molecular weight ub-linkages and is not readily recycled, as we don't observe a concomitant increase in high molecular weight ubiquitinated proteins. Ubiquitin stores are depleted.

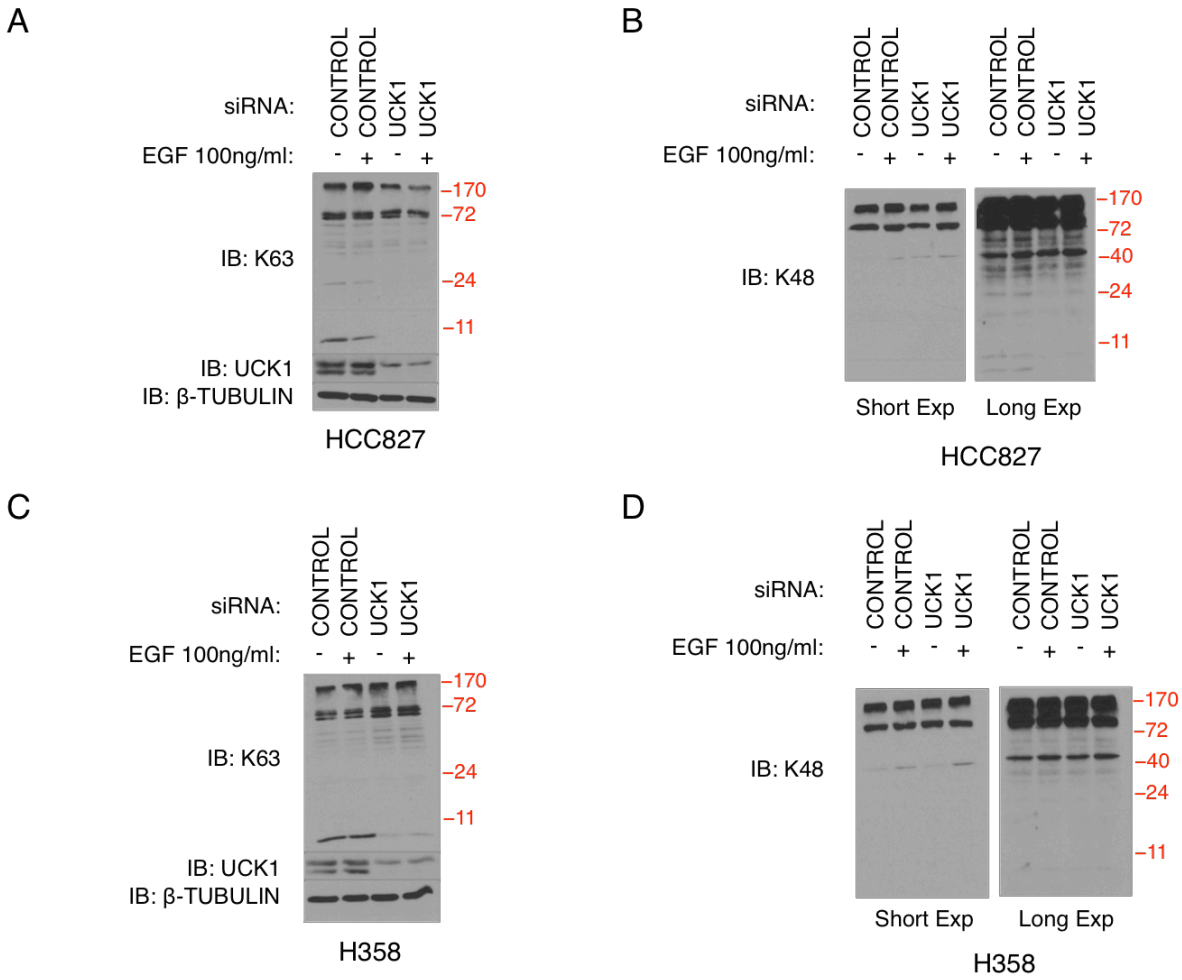


Figure 8. UCK1 loss depletes low molecular weight ubiquitin and ubiquitylated proteins

(A-B) NSCLC HCC827 cell line harvested 72-hours post transfection. Cells were serum starved overnight and stimulated with 100ng/ml EGF for 15 minutes. (C-D) NSCLC H358 cell line harvested 72-hours post transfection. Cells were serum starved overnight and stimulated with 100ng/ml EGF for 15 minutes. Proteins resolved by SDS-PAGE and immunoblotted for UCK1, K63, or K48. Tubulin used as loading control n=1.

UCK1 supports cell survival in cell lines harboring a single EGFR activating kinase mutation

We have shown that loss of UCK1 decreases EGFR, reduces ubiquitination, and poises the tumor cell for death. Next we assessed the impact of siUCK1 on cell viability in two sets of NSCLC, those with a single mutation in the TK domain and those that have acquired a secondary mutation (T790M) (Figure 9). Our results indicate that UCK1 supports cell survival in cell lines that have amplified, and over expressed EGFR leading to oncogene addiction. In contrast, cell lines that do not over express or have amplified EGFR and/or have a secondary TK mutation are resistant to UCK1-dependent death. Mutations *delE746-A750* and *L858R* are single mutations in the EGFR tyrosine kinase domain that confer sensitivity to TK inhibitors gefitinb and erlotinib (Lynch et al., 2004; Pao et al., 2005). Gatekeeper mutations like T790M are secondary mutations that block these inhibitors from the ATP binding site on the TK domain by steric hindrance or by increasing the receptor's affinity for ATP (Pao et al., 2005). One reason that UCK1 depletion may not reduce viability in these cell lines may be because the T790M causes EGFR to have inefficient degradation (Han, Zhang, Yu, Foulke, & Tang, 2006). A reduction in ubiquitylation by siUCK1 coupled to a mutation that confers resistance to degradation would result in EGFR that is not efficiently downregulated and prone to continued recycling and signaling. Therefore, use of UCK1 as a target in EGFR addicted tumors may be important as a first line therapeutic, but should be reevaluated once secondary mutations occur.

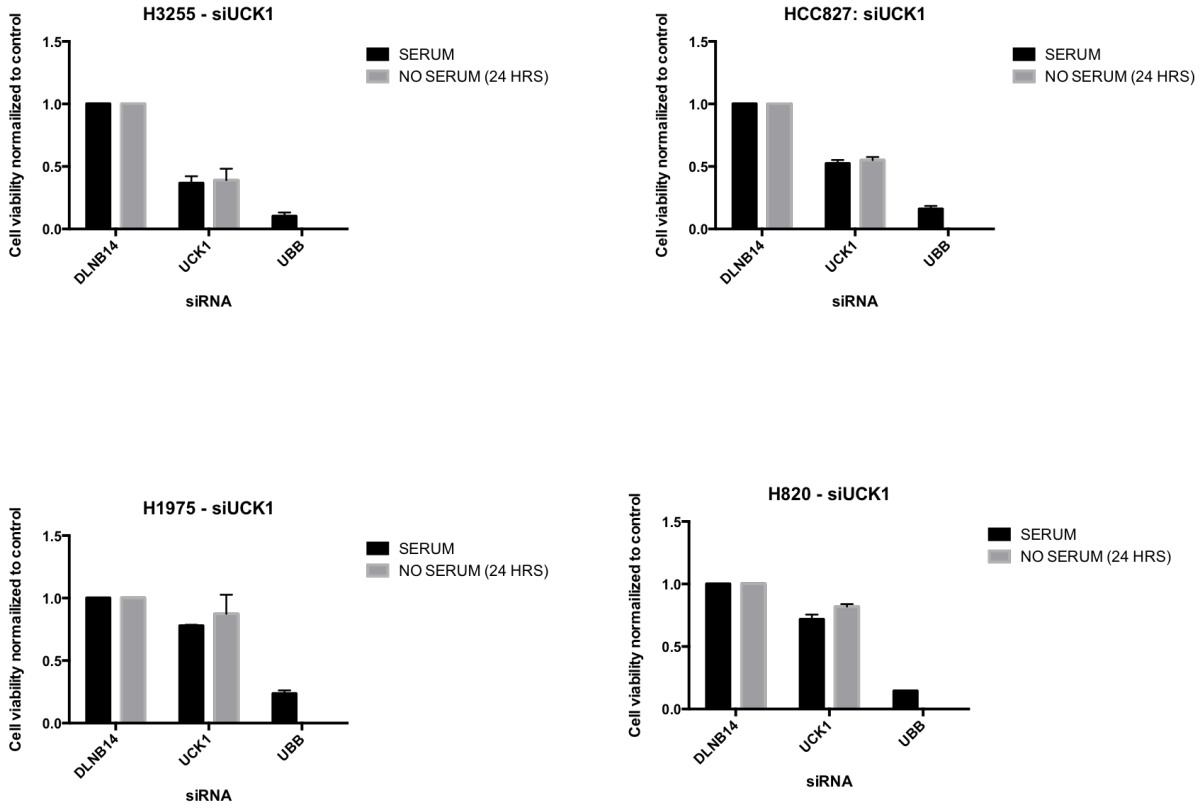


Figure 9. UCK1 supports cell survival in NSCLC harboring single TK activating mutations

(A-B) NSCLC cell lines harboring single mutations HCC827 (*delE746-A750*) and H3255 (*L858R*) assayed for cell viability using CellTiter-Glo upon siRNA-mediated knock down of Control versus UCK1. (C-D) NSCLC cell lines with secondary gatekeeper mutation T790M H820 (*delE746-A750*) and H1975 (*L858R*) assayed for cell viability using CellTiter-Glo upon siRNA-mediated knock down of Control versus UCK1. All cells transfected for 72 hours and serum starved for the last 24 hours as indicated n=2.

CHAPTER FIVE
Results

MERTK

EGFR behavior due to loss of MertK is heavily dependent on genetic background. In response to MerTk depletion, EGFR is not degraded but accumulates. This is due to inadequate and delayed receptor phosphorylation at Y1045.

Highly variable EGFR accumulation outcomes with loss of MerTK

Here we demonstrate how MerTK depletion elicits three very different affects on EGFR accumulation in NSCLC (Figure 10). H358 and H1155 cell lines mutant for KRAS have an EGF-dependent reduction in EGFR. However, MerTK loss in H1155 cells results in an overall increase in EGFR accumulation. Interestingly, MerTK loss in H358 cells significantly reduced EGFR within 5 minutes of EGF stimulation, which is interesting as this cell line has been reported to have a slower rate of EGFR degradation (Feng Shen, Lin, Gu, Childress, & Yang, 2007). H3255 cells that have an activating mutation in the tyrosine kinase domain do not display a receptor accumulation phenotype. Taken together these findings suggest that MerTK expression in these cell lines may have different roles to play depending on the genetic background. This data also suggests that MerTK may regulate EGFR stability such that its loss results in rapid reduction in EGFR as observed in cell line

H358 or conversely, MerTK may negatively regulate proteins involved in EGFR stability in other cell lines since MerTK depletion leads to protein accumulation and enhanced AKT signaling observed in H1155 cells.

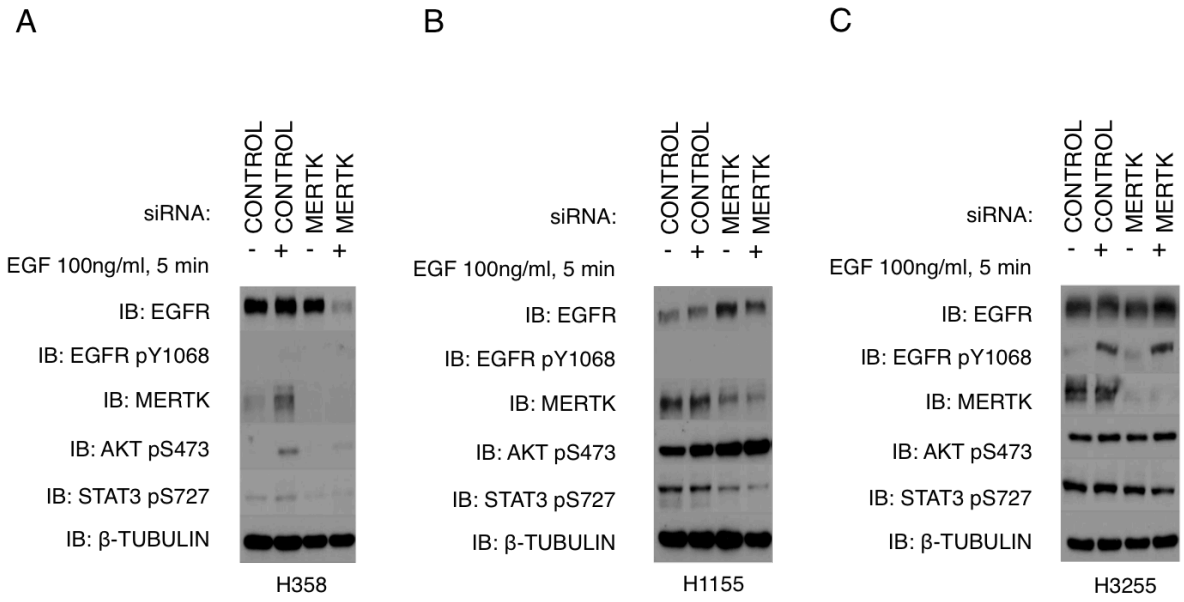


Figure 10. MerTK depletion elicits highly variable accumulation outcomes on EGFR in NSCLC

(A) NSCLC cell lines H358 (Kras mut, EGFR WT), (B) H1155 (Kras mut, EGFR WT), (C) H3255 (L858R, (EGFR Amp). Samples harvested 72-hrs post transfection, with overnight serum starvation prior to 5 minute stimulation with 100ng/ml EGF. Proteins were resolved by SDS-PAGE and expression detected by immunoblot n=2+.

Loss of MerTK causes reduced and delayed activation of EGFR at Y1045 resulting in decreased EGFR ubiquitination

Observations in (Figure 10) reveal that MerTK loss has differential affects on EGFR stability. Here, we examine the contributions of MerTk on EGFR trafficking. Figure 11A and B demonstrate that MerTK depletion impacts EGF-dependent activation of EGFR by hypophosphorylating Y1045 and consequently hypo-ubiquitylating EGFR. The reduction in phosphorylated Y1045 and hypo-ubiquitination suggests inefficient trafficking of EGFR. Therefore, receptor trafficking was examined through Y1045 activation in (Figure 11C). In control cells phosphorylation proceeds precipitously. In contrast, MerTK depleted cells experience a delay in receptor phosphorylation at residue Y1045; the activation slope is reduced and flat until 15 minutes of EGF stimulation. Although MerTK depletion affects EGFR ubiquitination, it is not a global effect as observed in UCK1 (Figure 11D). These results suggest that EGFR that is not properly ubiquitylated may be inefficiently degraded and can result in receptor recycling; leading to increased surface EGFR accumulation and enhanced signaling as observed in some MerTK depleted cell lines.

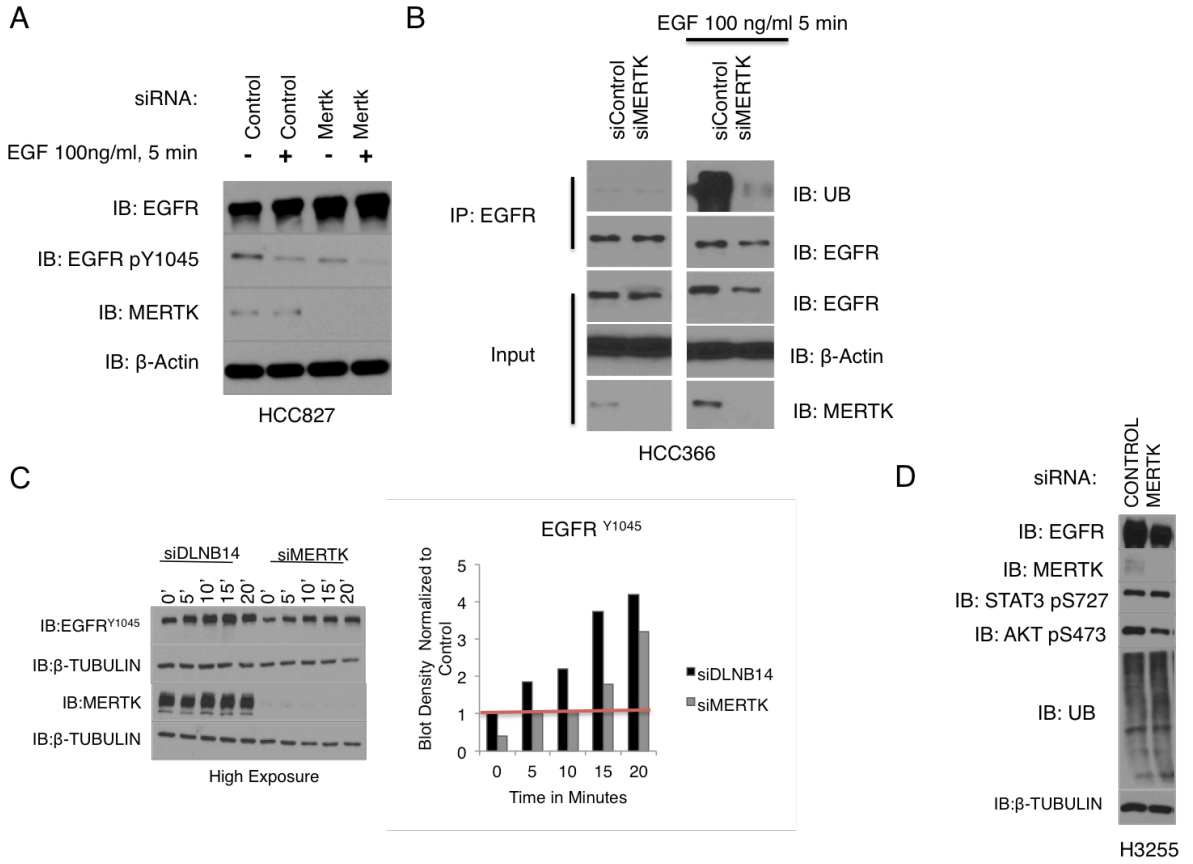


Figure 11. MerTK depletion results In reduced and delayed activation of EGFR at Y1045 resulting in decreased EGFR ubiquitination

(A) Cell line HCC827 was treated or not with 100ng/ml EGF for 5 mins. Proteins were resolved by SDS-PAGE from whole cell lysates and detected by immunoblot. Actin was used as the loading control. (B). Cell line HCC366 was treated or not with 100ng/ml EGF for 5 mins. Whole cell lysates were subjected to immunoprecipitation of ubiquitin or EGFR. Proteins were resolved by SDS-PAGE and detected by immunoblot. Actin was used as the loading control. (C) H3255 whole cell lysates were treated with 100ng/ml EGF for the indicated times. Proteins were resolved by SDS-PAGE and detected by immunoblot. Quantification was assessed by measuring blot density using Photoshop. (D) Whole cell lysates were treated with 100 ng/ml EGF for 15 minutes. Proteins were resolved and immunoblotted as previously indicated. All transfections were for 72-hours and include an overnight serum starvation prior to EGF stimulation n=2.

Loss of MerTK does not reduce cell viability in NSCLC mutant for EGFR

Recent reports have demonstrated strong evidence for the involvement of TAM receptors, in tumor progression and survival (Linger et al., 2013; Schlegel et al., 2013; Tworkoski et al., 2013; Y. Wang et al., 2013). MerTK has been shown to significantly reduce nude mice xenograft tumor growth and cell viability by the induction of apoptosis in a panel of NSCLC cell lines (Linger et al., 2013). Therefore, we wanted to investigate whether our NSCLC with enhanced EGFR accumulation could phenocopy the results obtained in that study upon MerTK knock down. MerTK depletion does not reduce cell viability in all NSCLC cell lines (Figure 12). This is not in complete contrast with the report from Linger et al. (2013). In their study, all of the cell lines used were wild type for EGFR, which is in contrast to ours. Our finding is not surprising since these cell lines are (1) oncogene addicted to EGFR, and (2) often have constitutive AKT signaling, which is also enhanced in some cell lines upon MerTK loss. Consistent with enhanced EGFR accumulation and AKT signaling, MerTK loss in cell lines mutant for EGFR does not result in cell death.

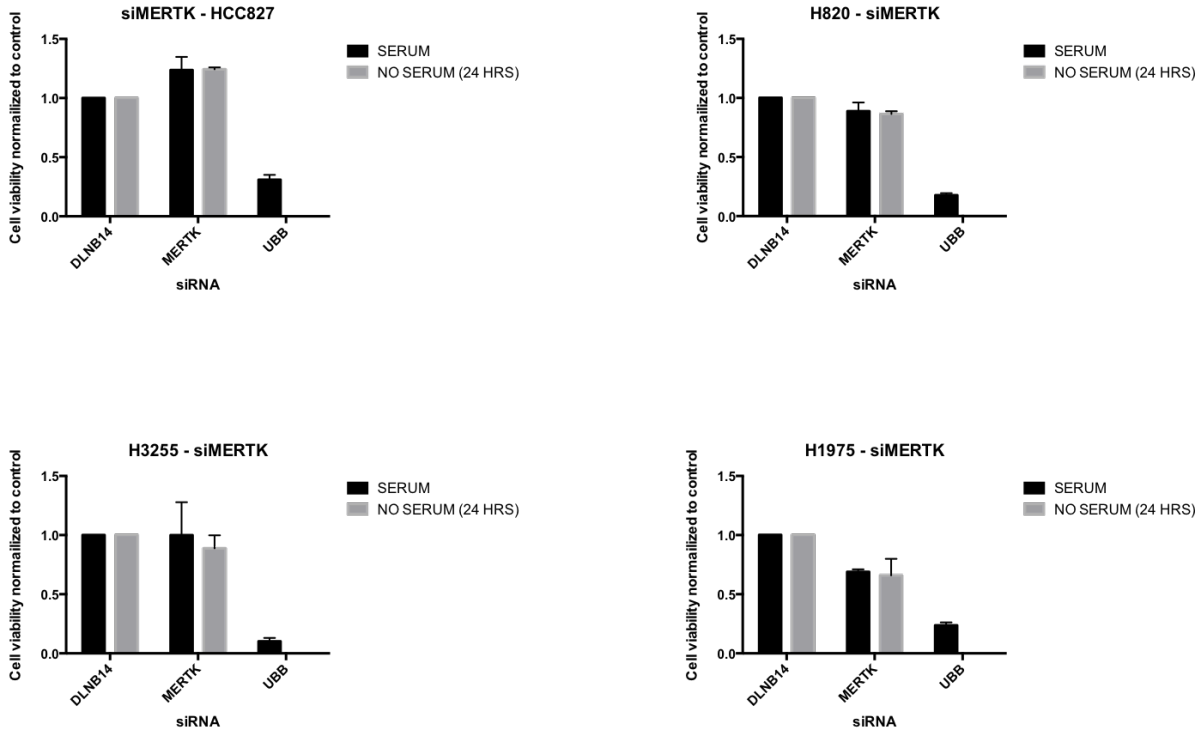


Figure 12. MerTK depletion does not reduce cell viability in NSCLC cell lines mutant for EGFR

(A-B) NSCLC cell lines harboring single mutations HCC827 (*delE746-A750*) and H3255 (*L858R*) assayed for cell viability using CellTiter-Glo upon siRNA-mediated knock down of Control versus MerTK. (C-D) NSCLC cell lines with secondary gatekeeper mutation T790M H820 (*delE746-A750*) and H1975 (*L858R*) assayed for cell viability using CellTiter-Glo upon siRNA-mediated knock down of Control versus MerTK. All cells transfected for 72 hours and serum starved for the last 24 hours as indicated n=2.

CHAPTER SIX
Conclusions and Recommendations

UCK1 AND MERTK DIFFERENTIALLY AFFECT EGFR STABILITY THROUGH THE MODULATION OF UBIQUITYLATION

The aim of this study was contribute to identifying clues into how UCK1, a ribonucleoside kinase, and MerTK, a mediator of phagocystosis, impact EGFR accumulation and signaling. We have identified ubiquitlyation as the process that mediates the stability of EGFR in UCK1 and MerTK depleted cells.

UCK1

Consistent with results published by Komurov et al. (2010) UCK1 depletion reduces EGFR in most cell lines. However, UCK1 depletion independent of stimulation significantly impacts AKT signaling in some cell lines and others not at all. This maybe due to constitutive AKT activation, loss of PTEN, or PI3KCA mutations (Kandasamy & Srivastava, 2002). Our observations that loss of UCK1 does not impact HER2 accumulation may seem at odds with recent work describing a role for UCK1 in supporting breast cancer tumor initiating cells (Hu et al., 2012) or the association of high UCK1 and high HER2 expression in breast cancer patients with poor outcome (Komurov et al., 2010), however targeting EGFR in some HER2 overexpressing breast cancer cells has been found to be efficacious (Moulder et al.,

2001), suggesting that although UCK1 may not directly target HER2, its apparent impact on EGFR stability may compensate. Further, UCK1 maybe important for the stability of many other proteins not investigated here, as data on roles outside of nucleoside phosphorylation does not exist.

Global reduction in ubiquitin was unexpected. Both K63-linked and K48-linked ubiquitinated proteins were affected but surprisingly, ubiquitin-linked low molecular weight proteins and free ubiquitin pools were significantly depleted. Also, K63-linkages appeared to be preferentially targeted over K48-linkages. Generally, when ubiquitin pools are depleted there is a concomitant increase in high molecular weight ub, which was not overtly observed here. This posits the possibility of DUB involvement. DUBs (Deubiquitnases) are enzymes that remove ub from its substrate prior to degradation thereby preventing the ubiqiuitin from being destroyed in the lysosome or proteasome (Amerik et al., 2000; Swaminathan, Amerik, & Hochstrasser, 1999).DUBs like POH1 or hUSP8 (UBPY)/*DOA4* from *S.cerevisiae* and others directly contribute to cytosolic ubiquitin stores by regulating ubiquitin homeostasis keeping conjugated substrates and free ubiquitin pools in balance (Appendix I) (Haas & Bright, 1987; Kimura et al., 2009; Row et al., 2006; Swaminathan et al., 1999). K63-linked Ub chains function in a variety of cellular processes including, DNA repair, trafficking, signal transduction, and stress response. Loss of UCK1 seemingly impacts each of these processes, it is not

surprising that there is a global ubiquitin defect and K63-linkages are greatly affected.

Glycosylation of EGFR is important for protein folding, function, and cell membrane localization. Although UCK1 loss does not appear to affect EGFR glycosylation, Tunicamycin treatment enhanced UCK1-dependent EGFR degradation of the fully glycosylated form over the non-glycosylated EGFR. Additionally, baseline BiP expression is elevated in UCK1 depleted cells in complete media conditions. These results suggest that UCK1 supports EGFR stability through appropriate posttranslational modifications and that BiP maybe increased not necessarily because UPR has been induced but because global ubiquitin levels are depleted and BiP elevation is a consequence of altered ubiquitin turnover.

Baseline PARP inactivation leading to caspase-3 cleavage upon loss of UCK1 is consistent with reports showing that loss of UCK1 supports cell survival, however, in NSCLC cell lines that have acquired the T790M mutation, UCK1 does not seem to affect cell survival. It may be that UCK1 depletion sensitizes these cells to inhibition of other pathways. Second hits to the BCL2 protein family has been found to be sufficient to induce apoptosis when one hit to the pathway is not. It would have been interesting to determine which member(s) are affected upon UCK1 depletion in mutant EGFR NSCLC cell lines and which are potential pressure points.

MerTK

In contrast to UCK1, MerTk depletion largely resulted in the increased accumulation of EGFR with the exception of MerTK depletion in H358 cells, which resulted in very rapid down regulation of EGFR. Though these are opposing phenotypes, they both point to MerTK-mediated changes in EGFR stability. H1155 and H3255 cell lines both exhibited enhanced EGFR accumulation independently of EGF stimulation; here loss of MerTK suggests that MerTK expression may negatively regulate EGFR through proteins important for its stability. Many NSCLC cell lines have abundant EGFR, or activating mutations that are constitutively endocytosed (Chung et al., 2009) and recycled, averting degradation. In the case of increased EGFR accumulation the loss of MerTK may release a brake allowing for enhanced receptor recycling.

Further, our results have demonstrated that in addition to enhanced EGFR accumulation, and AKT signaling, MerTK loss is unable to reduce cell viability; cell survival is one of the main tumorigenic characteristics supported by MerTK that has been reported by others a as pressure point in several different tumors types in including NSCLC. This difference in outcomes is largely due to enhanced oncogenic signaling of mutant EGFR and AKT signaling (Tworkoski et al., 2013).

Closing Remarks

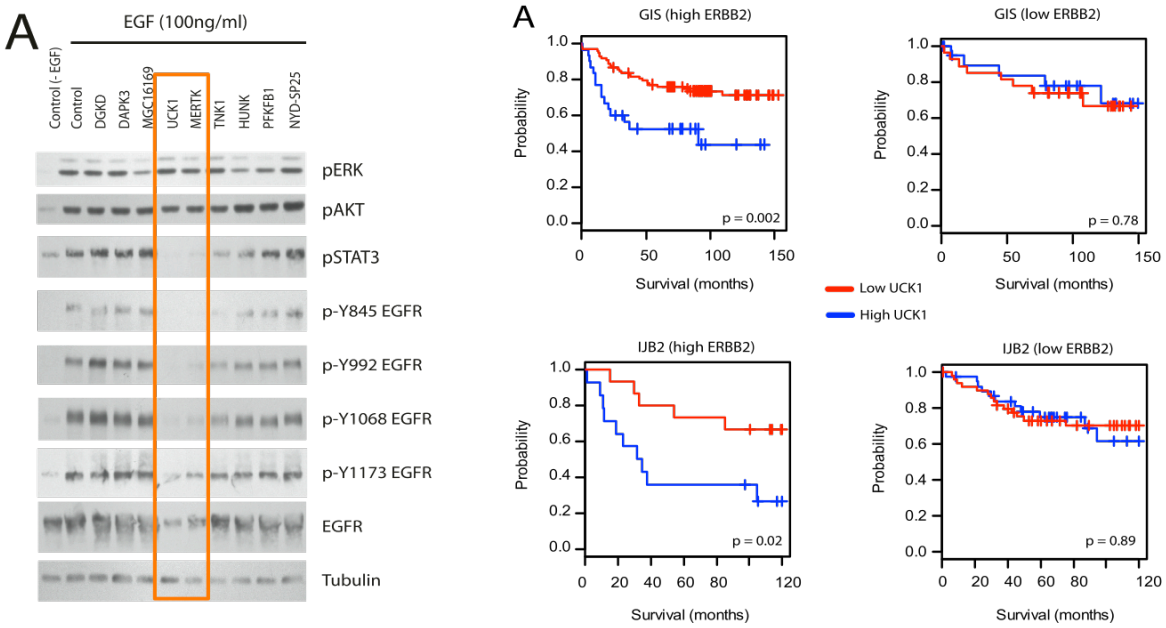
The use of NSCLC cell lines harboring EGFR mutations was not the best approach for this study. The activating mutations confer resistance to signal desensitization by evading degradation. Double mutants constitutively interact with c-CBL and are

ubiquitinated but are preferentially recycled to the surface. Single mutants have slower trafficking kinetics and thus prolonged signaling. Many of the phenotypes observed have been documented previously but are enhanced upon loss of MerTK. This observation is validated in HCC366 cell line that has wt-EGFR and cleanly recapitulates some of the observations made in cell lines with mut-EGFR. Therefore utilizing these mutants may have masked important phenotypes related to MerTK function in NSCLC. The inherent trafficking defects of the EGFR mutants, made visualizing how MerTK depletion altered trafficking difficult to interpret. Instead, cell lines with wt-EGFR would have led to cleaner less ambiguous results. Regardless, with the recent work implicating MerTK in tumor survival, the use of AKT inhibitors in this study in concert with MerTK depletion or inhibition would have likely yielded interesting results. Also, the fact that a protein critical for the engulfment of apoptosis was identified to potentially affect EGFR trafficking; It would have been interesting to look harder at the role NMII, in a addition to other cytoskeletal proteins may play in EGFR trafficking.

Altered pyrimidine metabolism can affect many processes from transcription to glycosylation. Studying UCK1 function was difficult since the impact on many processes was great. In the case of EGFR, the use of a protein synthesis inhibitor would have clarified the effect on EGFR and Ub depletion. Additionally investigating the role of BCL2 family proteins would have given insight to how UCK1 supports cell survival.

All in all there is a great deal of work left to be done on this project as many questions remain.

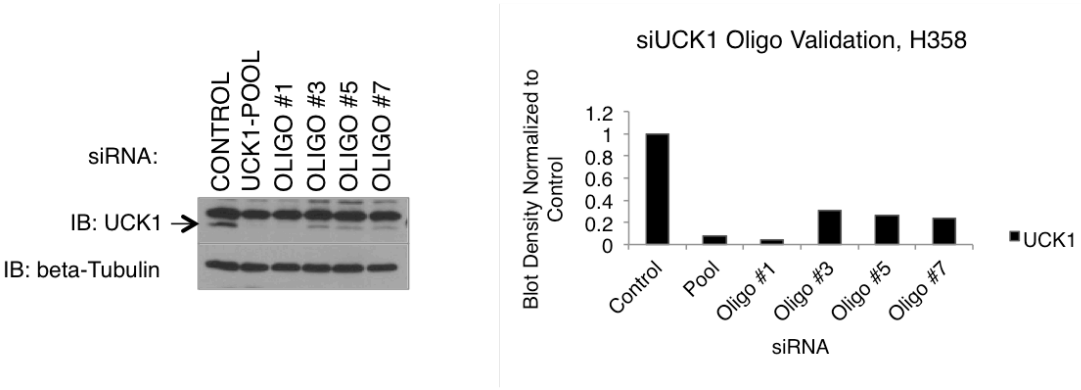
APPENDIX A
Background



Appendix A. UCK1 and MerTK are important hits in kinome screen
Taken and modified from Komurov et al. (2010), UCK1 and MerTK depletion reduce EGFR accumulation. High UCK1 and ErbB2 correlate with poor prognosis.

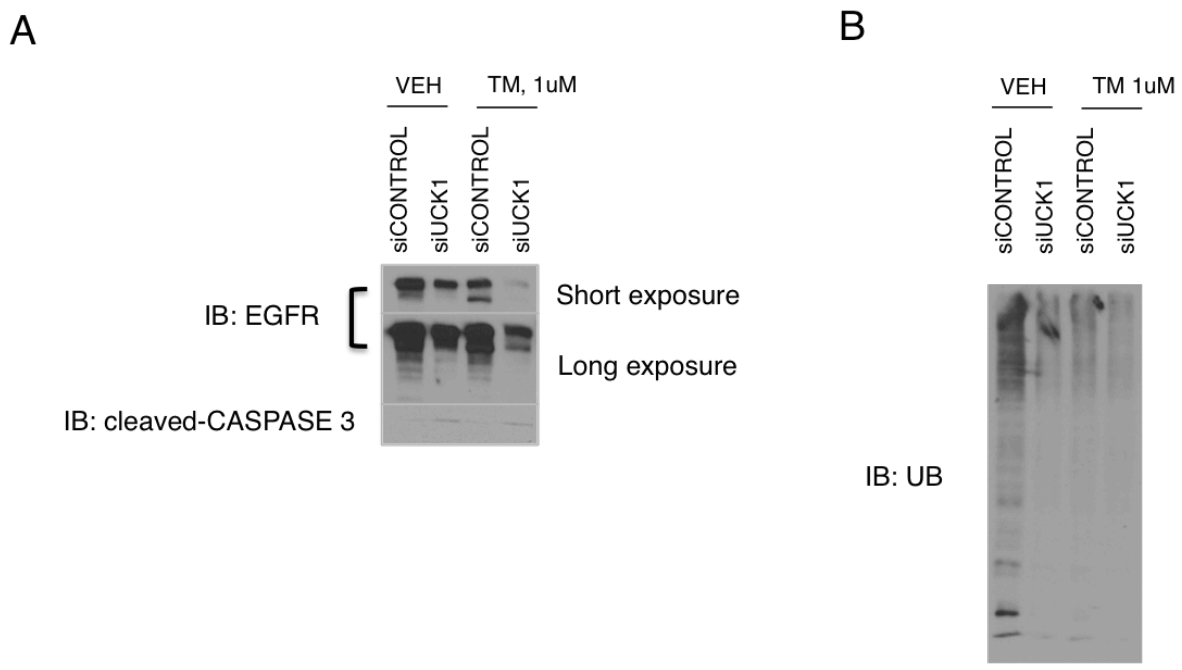
APPENDIX B
UCK1 – Oligo Validation

A



Appendix B. UCK1 oligo validation in H358
Dharmacon siGenome Oligo smart pool validation for UCK1 depletion. UCK1 oligo pool was deconvoluted to determine oligo on target depletion of UCK1.

APPENDIX C
UCK1 – Caspase-3 Activation



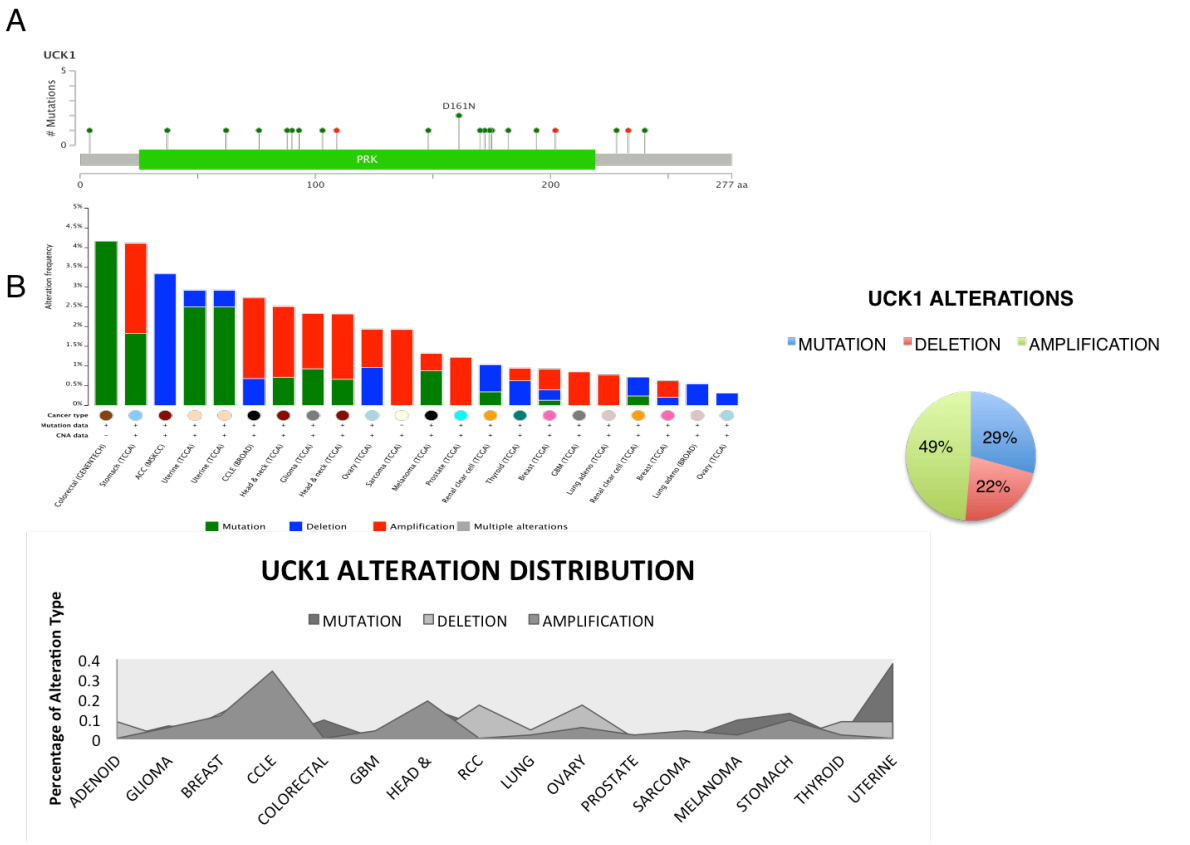
Appendix C. UCK1 depletion causes caspase-3 activation independently of ER stress
NSCLC H358 treated overnight with 1uM Tunicamycin or vehicle. (A-B) Whole cell lysates subjected to SDS-Page electrophoresis. Protein expression evaluated by immunoblot. Note: NEM was not used in the preparation of (B) n=1.

UCK1 depletion cause caspase-3 activation independently of ER stress

Treatment of NSCLC H358 with Tunicamycin revealed that UCK1 depletion causes baseline caspase-3 (panel A). This is consistent with the baseline elevation of inactivated PARP due to UCK1 loss. This suggests that UCK1 expression may have

a role in survival. Panel B, reiterates the phenotype observed in (Figure 7). UCK1 depletion reduces global ubiquitination in an additional cell line, H358.

APPENDIX D
UCK1 – MOLECULAR ABERRATIONS



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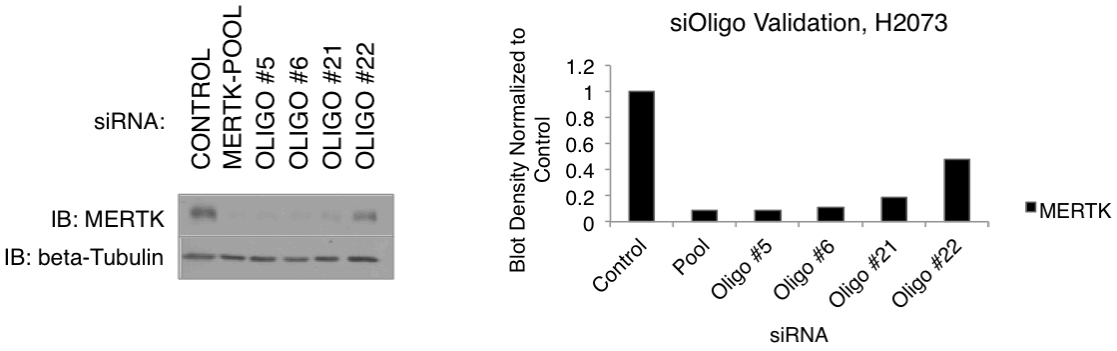
Appendix D. Molecular aberrations of Uridine-Cytidine Kinase 1 and its representation across tumor samples

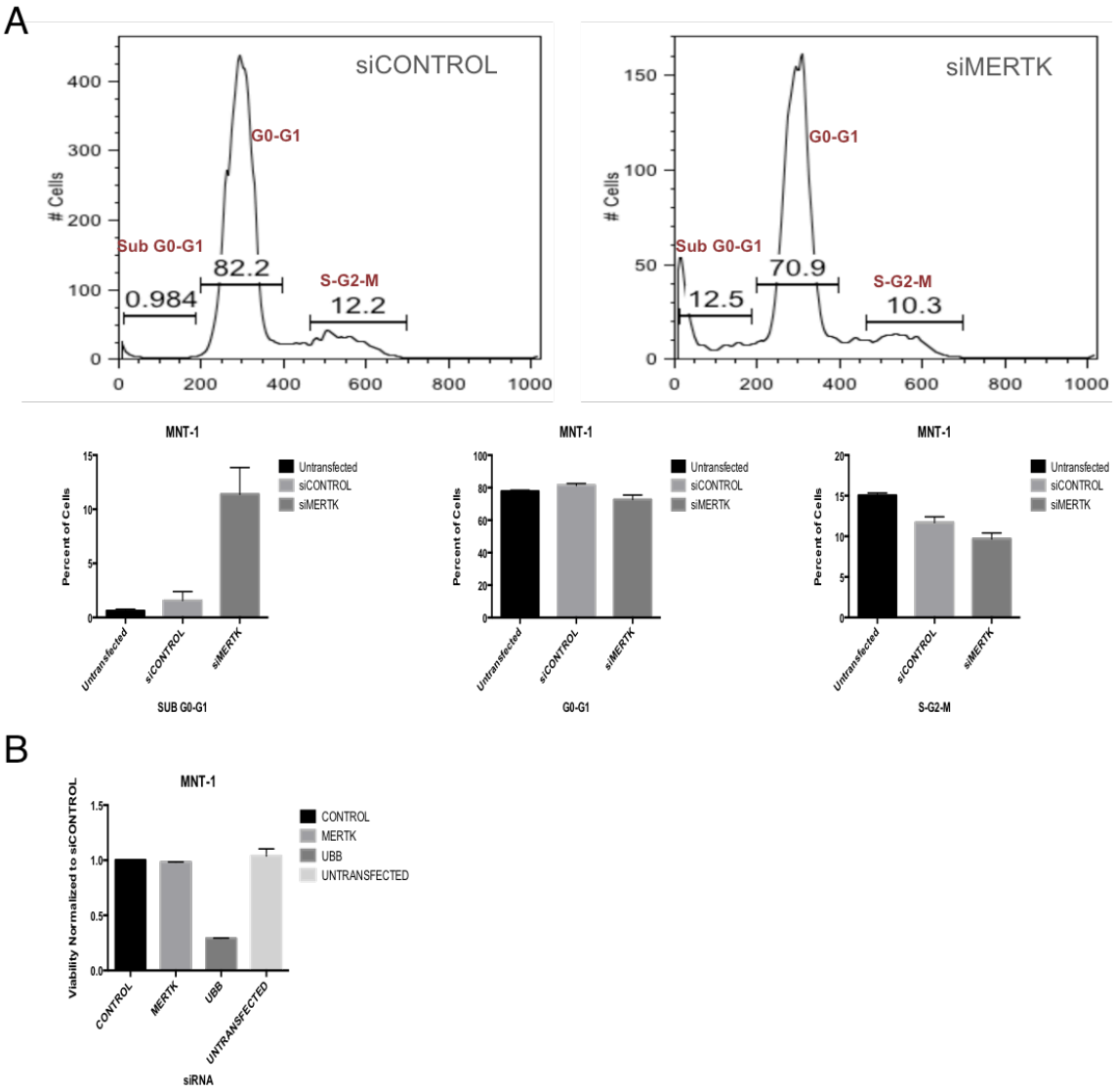
(A) Documented mutations for UCK1. (B) Distribution of UCK1 alterations across tumor samples. Adapted from (Cerami et al., 2012; Gao et al., 2013).

APPENDIX E
MerTK – Oligo Validation



Appendix E. MerTK Oligo Validation
H2073 cell was transfected for 72-hours with with either control, pooled, or individual siRNA oligos. MERTK knock down was validated by immunoblot of whole cell lysate. Tubulin was used as a loading control. Knock down of MERTK was quantified by measuring blot density in Photoshop.

APPENDIX F
MerTK – MNT1 Cell Viability



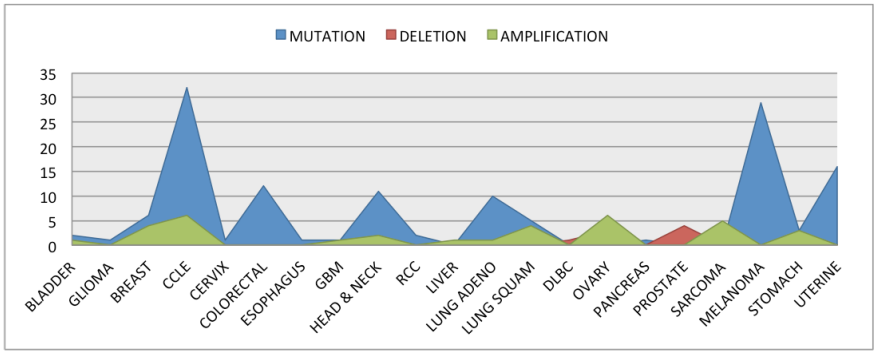
Appendix F. MERTK Depletion Causes an Increase in Apoptotic Cells in Human Melanoma cell line MNT1, but no decrease in cell viability
MNT1 cells transfected for 72 hours. (A) Cell evaluated by flow cytometry and sorted according to propidium iodide staining. (B) Cell viability assessed by CellTiter-Glo n=1.

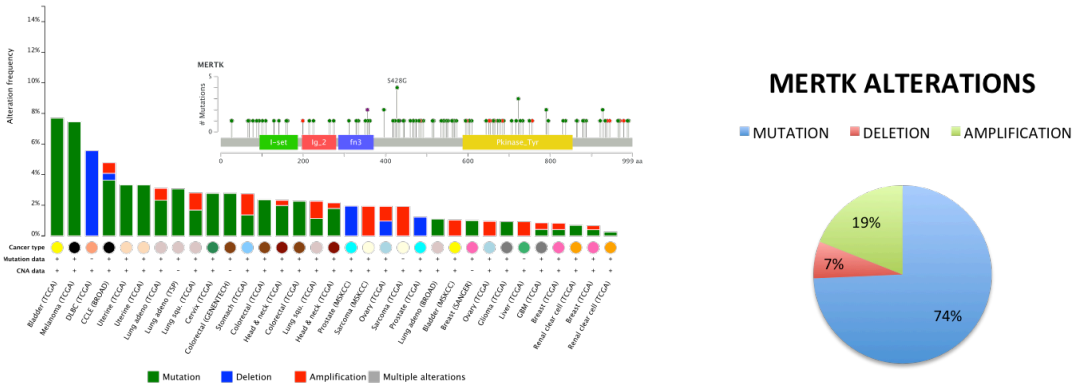
MerTK depletion causes an increase in apoptotic cells in human melanoma cell line MNT1, but no decrease in cell viability

Consistent with reports that MerTK is important to cell survival in Melanoma (Schlegel et al., 2013; Tworkoski et al., 2013), we demonstrate that MerTK depletion leads to apoptosis. Despite, the appearance of apoptotic cells we are unable to detect changes in cell viability by CellTiter-Glo. This discrepancy could be due to temporal differences in the induction of apoptosis versus actual cell death, transfection efficiency, or detection sensitivity differences between methods or devices.

APPENDIX G
MerTK – Molecular Aberrations

A



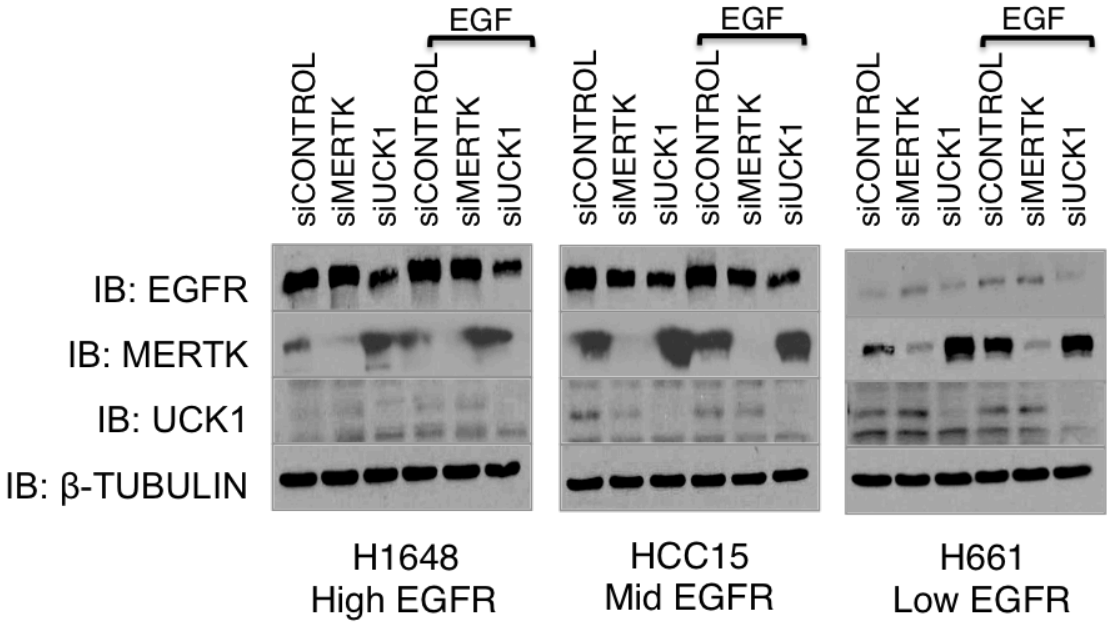


Appendix G. Molecular aberrations of MerTK and its representation across tumor samples

(A) Documented mutations for MerTK and distribution of MerTK alterations

across tumor samples. Adapted from (Cerami et al., 2012; Gao et al., 2013).

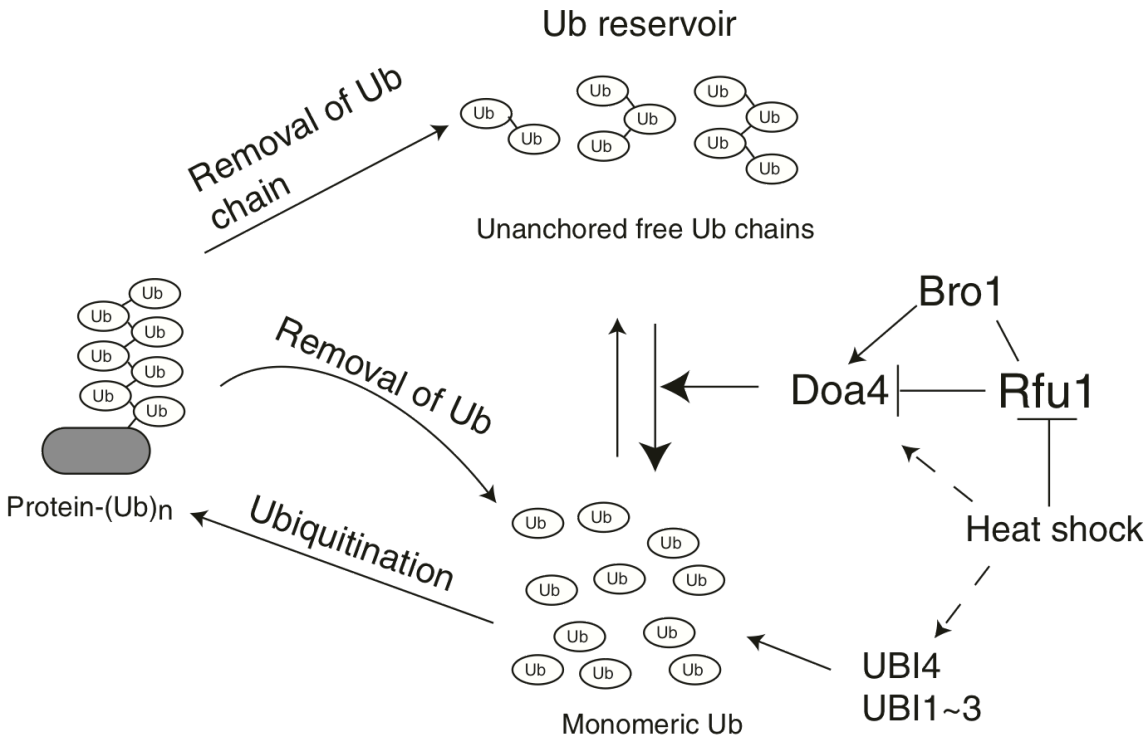
APPENDIX H
MerTK and UCK1 – Similar phenotypes in wt-EGFR versus mt-EGFR NSCLC



Appendix H. Depletion of MerTK or UCK1 have similar effects on receptor accumulation in wt-EGFR as in mt-EGFR independently of high or low EGFR expression
NSCLC cell lines, H1648, HCC15, H661 are wild type for EGFR, expressing varying amounts of EGFR. Cell lines transfect for 72 hours and serum starved overnight before EGF stimulation.

Depletion of MerTK or UCK1 have similar effects on receptor accumulation in wt-EGFR as in mt-EGFR independently of high or low EGFR expression. Both MerTK and UCK1 exert the same relative affect on wild-type EGFR as mutant EGFR. This phenotype is independent of level of EGFR expression.

APPENDIX I
DOA4-Regulation of Ubiquitin Homeostasis



Appendix I. Ubiquitin Homeostasis in *S. cerevisiae.*
Ubiquitin homeostasis is regulated by DUB *DOA4* by removal of ubiquitin from substrates prior to Lysosomal degradation at the multivesicular body. This removal replenishes cytosolic ubiquitin stores.

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