INHIBITION OF COMPENSATORY SURVIVAL AND PROLIFERATIVE PATHWAY ACTIVATION INDUCED BY MTOR INHIBITION IN RENAL CELL CARCINOMA

Sean Thomas Bailey

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Genetics and Molecular Biology.

Chapel Hill
2014

Approved by:

William Y. Kim

Albert S. Baldwin

Victoria L. Bautch

Ian J. Davis

Wendy K. Rathmell

ABSTRACT

Sean T. Bailey: Inhibition of compensatory survival and proliferative pathway activation
induced by mTOR inhibition in Renal Cell Carcinoma
(Under the direction of William Y. Kim, M.D.)

The mammalian target of rapamycin (mTOR) is a key regulator of tumor

progression in a variety of cancers and has been shown to be dysregulated in renal cell

carcinoma (RCC). mTOR exists in two independent complexes. The mTORC1

complex consists of mTOR, Raptor, and GβL, while the mTORC2 complex consists of

mTOR, Rictor, and GβL. Currently there are two FDA approved rapamycin derivatives

(rapalogs) for the treatment of advanced renal cell carcinoma (RCC). Allosteric mTOR

inhibition (i.e. rapalogs) results in the release of negative feedback inhibition on the

PI3K/AKT survival-signaling pathway as well as upregulates the metabolically protective

process known as autophagy. Furthermore, catalytic mTOR inhibitors attenuate

mTORC1 downstream signaling nodes more completely, but also diminishes mTORC2

mediated AKT survival signals induced by allosteric mTOR inhibitors. Additionally, it

has been demonstrated, that mTOR inhibition results in activation of the MEK/MAPK

signaling cascade. Based on this information, we wished to interrogate the molecular

and biological consequences of inhibiting mTOR pharmacologically and genetically in

the context of RCC.

We first asked what was the effect of allosteric versus catalytic mTOR inhibition

in several conventional human RCC cell lines and novel patient derived xenograft cell

lines on mTORC1 and mTORC2 signaling. We saw that only 1) catalytic mTOR inhibition decreased $pAKT_{S473}$ expression, 2) that both allosteric and catalytic mTOR inhibition increase $pERK_{T202/Y204}$ expression, and 3) combined MEK and mTOR inhibition induced cell death better than single pathway inhibition alone. Finally, we noted that hierarchal clustering of KIRC_TCGA RPPA (reverse phase protein array) data by markers of mTOR and MEK activation revealed subclasses respective to mTOR and MEK/ERK signaling with significant differences in clinical outcome.

We next asked how allosteric versus catalytic mTOR inhibition affected the metabolically conserved process known as autophagy. While both allosteric and catalytic inhibition increased autophagic flux; catalytic mTOR inhibition did so to a greater degree. To determine if this was due to catalytic mTOR inhibitors' ability to attenuate mTORC2 signaling we assessed autophagic flux in cell lines stably expressing shRNAs targeting Raptor or Rictor, essential components for mTORC1 and mTORC2 signaling, respectively. Knock-down of Raptor results in an increase in autophagic flux, however knock-down of Rictor also increases autophagic flux demonstrating that the increased autophagic flux induced by catalytic mTOR inhibition is partly due to inhibition of mTORC2. Finally, we show that catalytic mTOR inhibition in conjunction with autophagy inhibition decreases cellular proliferation while augmenting apoptosis. Together these data support that there are differences in levels of activation of compensatory survival pathways in the context of allosteric and catalytic mTOR inhibition.

I dedicate this dissertation to Jennifer Streicker. Jenn is an unbelievable teacher, fighter, cancer survivor, and one aware of the opportunity to live life to the fullest. Words can't express how thankful I am for her instilling pride in the work I care about so much.

ACKNOWLEDGEMENTS

First, I would like to thank UNC Chapel Hill's Biological and Biomedical Sciences Program for giving the opportunity to pursue my Ph.D. I'm extremely grateful for the opportunity that my mentor and P.I., Dr. William Y. Kim gave me to work in his laboratory. Some of my most exciting times during the program have been conversing with him about data and experimental design. He taught me how important it is to stay in tune with the current literature and to always push to conduct clean and thoughtful science, and most importantly to never sell myself short.

I would like to thank my parents, Harold and Denise, for being extremely supportive, as I have made this journey. Their continuous support and confidence in me to not only reach for high goals, but also obtain them is something I will carry with me and pass on within my family. I would also like to thank my sister, Lauren, for her support and listening ear when times were tough, but also times of reward and thankfulness. I thank her for living a lifestyle that portrays the notion that nothing is impossible as long as you work hard and are passionate about what you do. I also want to give credit and infinite thanks to my wife, Carly, who believed in me and supported me regardless of the ups and downs that came along the way. She fueled my thinking with nutritious food, and helped teach me important values that have allowed me to focus when times are rough. She helps me remember why I love science and I am so thankful that she believes in me and has been with me through thick and thin. I am

extremely excited to about our opportunity to achieve our carrier and life goals in Los Angeles, CA.

My opportunity as an undergraduate at North Carolina State University (NCSU) to work in Dr. Russell Borski's lab was an experience that fueled my curiosity about molecular biology. My time at NCSU gave me my first independent research project and the confidence to pursue a graduate level education in science.

My opportunity to attend graduate school largely comes from the opportunity given by Beth Hollister at Piedmont Research Center (PRC). PRC saw potential in me and gave me my first job in pre-clinical drug development for oncology therapeutics. The skill set engrained into me by excellent training plays a pivotal role in why I am able to share this dissertation.

Finally, I would like to thank my many friends, colleagues, and lab members at UNC Chapel Hill. Specifically, I would like to thank Pat Phelps and Ashalla Freeman of IMSD for creating a group capable of not only career support, but also social comfort as well. I would like to give an enormous amount of gratitude to the GMB administrative staff for providing aid when needed the most. I also would like to thank Brian Gibbs, Daniel Dominguez, and Nick Gomez for being close friends and keeping the vision that hard work and commitment equal positive results. I would also like to thank all my close friends for trying to understand why I have been in school for so long and that the journey to learn and the energy directed to achieving one's goals is never over.

"Dreaming is for those not aware of opportunity"

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

4E-BP1: eukaryotic translation initiation factor 4E (eiF4E)-binding protein 1

AGC: protein kinase A/protein kinase G/protein kinase C

AMPK: AMP-activated protein kinase

ATG: autophagy regulating protein

BAD: BCL2-associated agonist of cell death

BEZ: BEZ235

BNIP3: BCL2/Adenovirus E1B 19kDa Interacting Protein 3

CA9: carbonic anhydrase 9

ccRCC: clear cell renal cell carcinoma

CQ: chloroquine

DEPTOR: DEP domain-containing mTOR-interacting protein

EGFR: epidermal growth factor receptor

EPO: erythropoietin

FDA: Food and Drug Administration

FKBP12: FK506-binding protein of 12 kDa

GAP: GTPase-activating protein

GEMM: genetically engineered mouse model

GLUT1: glucose transporter protein type 1

GSK: GSK212

HIF1α: hypoxia-inducible factor 1 alpha

HIF2α: hypoxia-inducible factor 2 alpha

HMOX-1: heme oxygenase decycling 1

HRE: hypoxia response element

IL-2: interleukin-2

INF-α: interferon alpha

IRS-1: insulin receptor substrate 1

KD: knock-down

KIRC: Kidney Renal Cell Clear Cell Carcinoma

LDHA: lactate dehydrogenase A (LDHA)

MAPK/ERK: mitogen activated protein kinase

MEK: mitogen-activated protein kinase kinase

mLST8: mammalian lethal with sec-13 protein beta

mSin1: mammalian stress-activated map kinase-interacting protein1

mTOR: mechanistic target of rapamycin

NDRG1: N-myc downstream regulated gene 1 protein

PDH1: pyruvate dehydrogenase kinase 1

PEPCK: phosphoenolpyruvate carboxykinase

PKCα: protein kinase C-alpha

PRAS40: proline-rich AKT substrate 40 kDa

PROTOR: protein observed with rictor protein

PTEN: phosphatase and tensin homolog

Rap: rapamycin

Raptor: regulatory-associated protein of mTOR

RCC: renal cell carcinoma

REDD1: regulated in development and DNA damage responses 1 protein

Rictor: rapamycin-insensitive companion of mTOR

RTK: receptor tyrosine kinase

S6K1 - S6 kinase 1

SGK1: serum/glucocorticoid regulated kinase 1

SLC2A1: solute carrier family 2 (facilitated glucose transporter), member 1

TCGA: The Cancer Genome Atlas

TGFa: transforming growth factor alpha

TKI: tyrosine kinase inhibitor

TSC1/2: tuberous sclerosis 1/2

ULK1: Unc-51 like autophagy activating kinase 1

VEGF-R: vascular endothelial growth factor receptor

VEGF: vascular endothelial growth factor

VHL: von Hippel-Lindau

VLDL-R: low-density lipoprotein receptor

CHAPTER 1

INTRODUCTION: TARGETING MTOR IN RENAL CELL CARCINOMA

1.1   Renal cell carcinoma

Renal cell carcinoma (RCC) is a heterogeneous disease of epithelial renal origin

and ranks 6th and 8th amongst other cancer types in men and women, respectively [1].

In 2013, RCC occurrences accounted for an estimated 40,000 and 25,000 new cases in

men and women, respectively [1]. Together, estimated deaths from RCC tallied near

14,000 for men and women combined [1]. RCC typically presents as one of several

histological subtypes: clear cell, type I or II papillary, chromophobe, or oncocytoma [2].

The heterogeneous nature of RCC reflects the differences in genetics and biology, and

these differences can be linked to unique genetic lesions [2]. Interestingly, the genetic

basis for the different histological subtypes of RCC can be linked not only through

inherited cancer syndromes (Table 1.1), but also occurrences of sporadic mutations of

the same loci coupled with the corresponding familial cancer syndromes (Table 1.1). Of

the different subtypes of RCC, clear cell renal cell carcinoma (ccRCC) encompasses

~65% of reported cases [3]. Histological features consist of hyperchromatic nuclei and

a distinct clear cytoplasm caused by an accumulation of cholesterol with a recent

molecular link to hypoxia-inducible factor 1 α (HIF1α) transcriptional regulation of very

low-density lipoprotein receptor (VLDL-R) [4,5]. Furthermore, ccRCC development is

tightly associated with mutations in the von Hippel-Lindau (*VHL*) tumor suppressor gene, and will be the main RCC subtype focused on within this document [3,6].

Sporadic RCC			Hereditary RCC	
Histology	**Incidence (%)**	**Gene**	**Inherited Syndrome**	**Gene**
ccRCC	~65	*VHL*	VHL disease	*VHL*
Papillary	~15-20	*MET*	HPRC HLRCC	*MET FH*
Chromophobe	5		Birt-Hogg-Dubé syndrome	*BHD*
Oncocytoma	4		Birt-Hogg-Dubé syndrome	*BHD*
Collecting Duct	<1			

Table 1.1: Sporadic and hereditary RCC characteristics: Histological, incidence, genetic lesion, and inherited syndrome correlation of RCC

1.1.1 *VHL* and ccRCC relevance

The *VHL* gene has been shown to function as a tumor suppressor through both functional and genetic evidence [7]. VHL disease effects 1:35,000 individuals and is an autosomal dominant cancer syndrome consisting of a spectrum of tumors, but most commonly ccRCC, hemangioblastoma, and pheochromocytoma [6]. Interestingly, there are direct genotype-phenotype correlations for VHL disease patients. Specifically, Type1 or Type 2B VHL disease patients present with clinical manifestations associated with RCC. It's important to note, in the case of inherited ccRCC, that loss of heterozygocity of the remaining wild-type allele results in renal cysts followed by tumor initiation and these lesions are multifocal and can occur bilaterally in both kidneys [8].

Sporadic mutations and hypermethylation of *VHL* may also occur in individuals that are wild-type for both *VHL* alleles, and interestingly occurs in ~90% of patients with

sporadic RCC [9]. As with inherited ccRCC, loss of the remaining wild-type allele is necessary for sporadic tumor formation. However, unlike hereditary ccRCC's multi-focal presentation, sporadic ccRCC tumors form as focal lesions in a unilateral fashion [8].

1.1.2   VHL and HIFα regulation[1]

The *VHL* gene is composed of 3 exons located on the short arm of chromosome 3 and codes for the protein VHL (pVHL). pVHL expression is not restricted to tissues effected by VHL disease [10]. pVHL functions in a ubiquitin ligase complex consisting of several proteins: elongin B, elongin C, Cul2, and Rbx1 [7]. When functional, and in normoxic cellular environments, this complex targets HIFα proteins for proteosomal degradation [11] (Figure 1.1).

---

[1] 1.1.2-1.1.4 modified from Jonasch E, Futreal A, Davis I, Bailey S, Kim WY, Brugarolas J, Giaccia AJ, Kurban G, Pause A, Frydman J, Zurita A, Rini BI, Sharma P, Atkins M, Walker C, Rathmell. *State-of-the-science: An update on renal cell carcinoma*. Molecular Cancer Research. (2012)
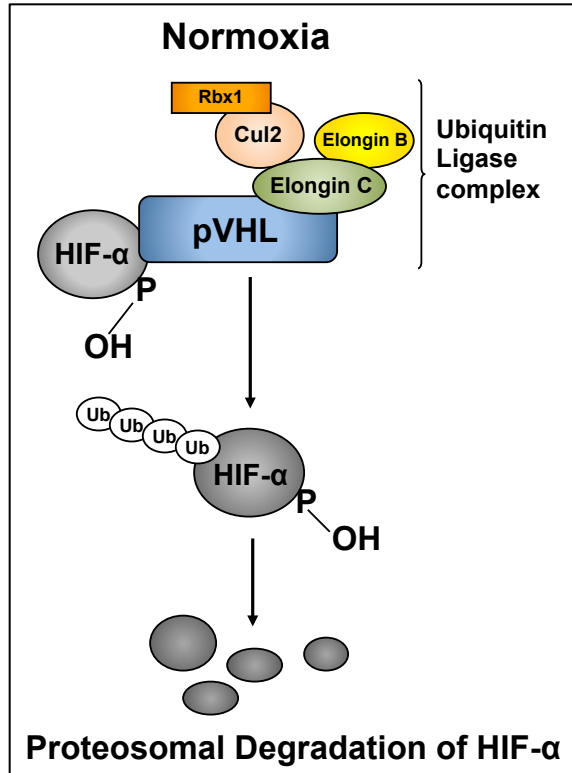
Figure 1.1: VHL regulation of HIFα. Functional pVHL post transcriptionally ubiquitinates HIFα targeting it for proteosomal degradation.

However, in the context of hypoxia, hereditary VHL disease associated RCC or sporadic

ccRCC this negative regulation of HIFα is perturbed (Figure 1.2).
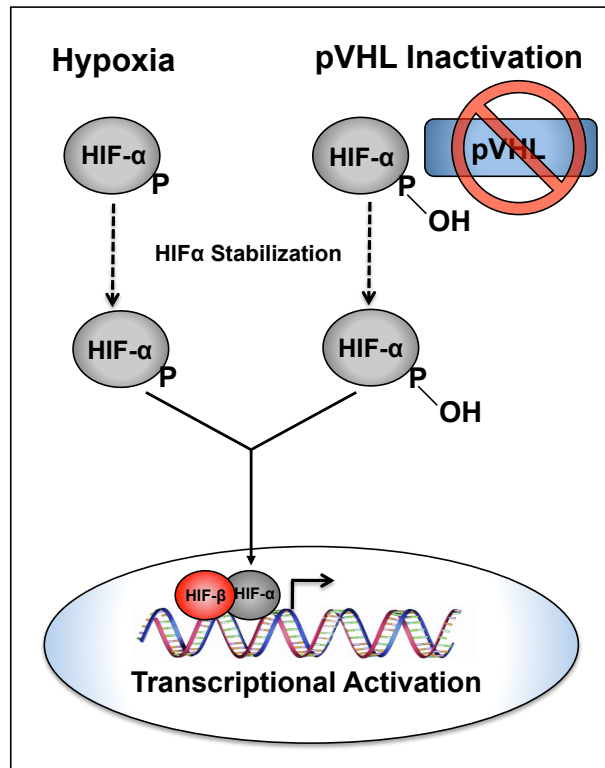
Figure 1.2: HIFα stabilization. Under hypoxic conditions or when pVHL is non-functional HIFα is not hydroxylated resulting in perturbation of pVHL binding and subsequent HIFα stabilization. Stabilized HIFα translocates to the nucleus to form a dimer with HIFβ and activate transcription of HIFα target genes.

The interaction between HIFα and pVHL is mediated by an enzymatic, post-translational hydroxylation of HIFα on conserved prolyl residues (by a family of HIFα prolyl hydroxylases (PHDs or EGLNs). In keeping with the notion that regulation of HIF is an important function of pVHL, the majority of disease associated VHL mutations are predicted to abolish the interaction between pVHL and HIFα resulting in an upregulation of HIFα target genes [12].

1.1.3   HIF and HIF target genes

The HIF proteins are a family of transcription factors that contain a basic helix-loop-helix domain and function in a heterodimeric complex [13]. There are three known

HIFα subunits (HIF1α, HIF2α, HIF3α) which heterodimerize with their binding partner ARNT (HIF1β) to transcriptionally regulate target genes containing hypoxia response elements (HREs). HIF1α and HIF2α, are best characterized and are known to regulate transcriptional programs associated with cellular and physiological adaptation to hypoxia such as erythropoietin (*EPO*), vascular endothelial growth factor (*VEGF*), and carbonic anhydrase 9 (*CA9*), amongst others [14,15]. While there is significant overlap in genes that are transcriptionally activated by HIF1α and HIF2α, each HIF family member is thought to also transactivate unique target genes [16]. For example, HIF1α has been linked to regulating genes in pathways associated with glycolytic metabolism and autophagy such as, solute carrier family 2 (facilitated glucose transporter), member 1 (*SLC2A1*) also known as glucose transporter protein type 1 (*GLUT1*), pyruvate dehydrogenase kinase1 (*PDK1*), lactate dehydrogenase A (*LDHA*), and BCL2/Adenovirus E1B 19kDa Interacting Protein 3 (*BNIP3*) [17]. HIF2α is uniquely responsible for transcriptionally activating genes associated with proliferation and de-differentiation, transforming growth factor alpha (*TGFα*), *CCND1* (Cyclin D1), and *Oct4*, respectively [13,18,19].

1.1.4 HIF1-α and HIF2-α contribution in ccRCC

Early *in vitro* and cell line xenograft studies suggested that while HIF2α is both necessary and sufficient for the growth of transformed ccRCC cell lines [20-22] HIF1α is not [23], indicating that HIF1α is dispensable for ccRCC growth. However, it appears that HIF1α is not merely dispensable in the context of RCC but actually functions as a tumor suppressor gene. Several lines of evidence support this hypothesis. First, targeted exon sequencing of ccRCC has demonstrated (albeit rarely) inactivating

mutations in HIF1α [24], while copy number analysis of ccRCC cell lines and primary

tumors suggest that the *HIF1α* locus is frequently lost along with the long arm of

chromosome 14 (14q) [25]. Secondly, while all VHL defective clear cell renal cell

carcinomas appear to overexpress HIF2α, about one third of these tumors appear to

lack HIF1α expression as well [26]. Finally, functional studies *in vitro* and *in vivo*

suggest that over expression of HIF1α in VHL wild type cells restrains tumor growth

while suppression of HIF1α in VHL deficient cells enhances tumor growth [18,25,27].

Together these studies show support for HIF1α as tumor suppressor gene in renal

cancer development and HIF2α as a key driver for renal cancer progression.

While there are a number of reasons to explain the contrasting properties of HIF1α

and HIF2α in ccRCC pathogenesis, one intriguing observation is that HIF1α and HIF2α

have opposing roles on the regulation of c-Myc activity. Specifically HIF1α acts to

suppress c-Myc activity while HIF2α promotes the transactivation or transrepression of

c-Myc specific target genes [26,28,29]. In keeping with this notion, ccRCC tumors that

exclusively express HIF2α have increased proliferation rates. Furthermore, intriguingly,

a subset of clear cell RCC tumors appear to have copy number amplification of 8q24,

where c-Myc resides [30,31].

1.2   Therapeutic options for advanced ccRCC

Unlike some cancers capable of being treated with radiation or chemotherapy,

advanced RCC unfortunately does not respond to such treatment regimens [8]. This

clinical observation has warranted efforts in selecting compounds capable of combating

advanced RCC. Prior to the past 10 years of targeted therapeutic advancement,

conventional treatment of advanced RCC was solely with immunotherapies, interferon

alfa (INF-α) and interleukin-2 (IL-2) [32]. Within the past 10 years, there have been

seven targeted therapeutics approved by the Food and Drug Administrations (FDA) for

treating advanced RCC. These seven drugs consist of small molecule receptor tyrosine

kinase (RTK) inhibitors and biologicals (i.e. monoclonal antibodies) targeting angiogenic

pathways, and targeted agents directed at inhibiting the mammalian target of rapamycin

(mTOR) (Table 1.2) [33].

Targetd Agents for Treating Advanced RCC			
Therapeutic	Class	Date Approved	Molecular Target
Sorafenib	TKI	2005	VEGFR, PDGFR, KIT, RAF
Suntinib	TKI	2006	VEGFR, PDGFR, KIT, FLT3, RET
Temsirolimus	Rapamycin Derivative	2007	mTOR
Everolimus	Rapamycin Derivative	2007	mTOR
Bevacizumab	Monoclonal Antibody	2009	VEGF
Pazopanib	TKI	2009	VEGFR, PDGFR, FGFR KIT
Axitinib	TKI	2012	VEGFR, PDGFR, KIT

Table 1.2: FDA approved targeted agents for treating advanced RCC.

Interestingly, patients treated with inhibitors targeting vascular endothelial growth

factor receptor (VEGF-R) appear to have the best response rates, with allosteric mTOR

inhibitors (everolimus and temsirolimus) having the most modest responses, yet still

prolonging overall survival [3]. The modest response rates seen with allosteric mTOR

inhibition warrants a better understanding of RCC biology in the context of allosteric

mTOR inhibition and/or inhibitors capable of more complete inhibition of mTOR.

However, there is still much needed understanding of the consequences of mTOR

inhibition in the context of RCC. Henceforth, advanced efforts to better understand the

biologic consequences of mTOR inhibition has elucidated potential targetable compensatory survival pathways capable of increasing efficacy and modest response rates.

1.3   mTOR signaling

mTOR is a serine/threonine kinase within the phophoinositide 3-kinase (PI3K)-related kinase family and is homologous to yeast TOR [34]. mTOR is one of the most widely studied nutritional sensors for cellular and organismal integrity. mTOR functions in two distinct complexes known as mTORC1 and mTORC2, and discoveries alluding to the realization that mTOR regulates signal transduction in two unique complexes was made possible by the observation that mTOR was a unique target of rapamycin, hence the acronym "TOR". Rapamycin inhibits mTORC1 in an allosteric manner binding to FK506-binding protein of 12 kDa (FKBP12-rapamycin) to bind to the FKBP12-rapamycin binding domain (FRB) of mTOR. A recent co-crystal structure of a truncated form of mTOR and mLST8 revealed that the FRB domain acts as a gatekeeper granting access of mTOR substrates to the catalytic-active site of mTOR. Of additional importance, the FRB domain of mTOR cannot be accessed by FKBP12-rapamycin resulting in ineffective inhibition of downstream mTORC2 signaling [35]. More detail of the effects of rapamycin on mTORC1 and mTORC2 signaling will be discussed in later sections of this document.

mTORC1 and mTORC2 both contain the catalytic subunit mTOR, tti1/tel2 complex, mammalian lethal with sec-13 protein 8 (mLST8), and DEP domain containing mTOR-interacting protein (DEPTOR). However, the two complexes differ in several protein components. The proline-rich AKT substrate 40 kDa (PRAS40) and regulatory-

associated protein of mammalian target of rapamycin (Raptor) are distinct to mTORC1, and mammalian stress-activated map kinase-interacting protein1 (mSin1), protein observed with Rictor1 and 2 (protor1/2), and rapamycin-insenstive companion of mTOR (Rictor) are specific to mTORC2 [36]. Importantly, Raptor and Rictor serve as essential components for mTORC1 and mTORC2 signaling, respectively [37,38].

At this time, regulation and function of mTORC1 is understood in more detail than mTORC2, most likely due to the use of rapamycin and its specificity to target the mTORC1 pathway. Specifically, mTORC1 has been described to act as signal transduction hub processing extracellular and intracellular cues such as hypoxia, energy levels, growth factors, and nutrients to regulate macromolecular biosynthesis, cell cycle progression, cellular growth, autophagy, and metabolism (Figure 1.3) [36].
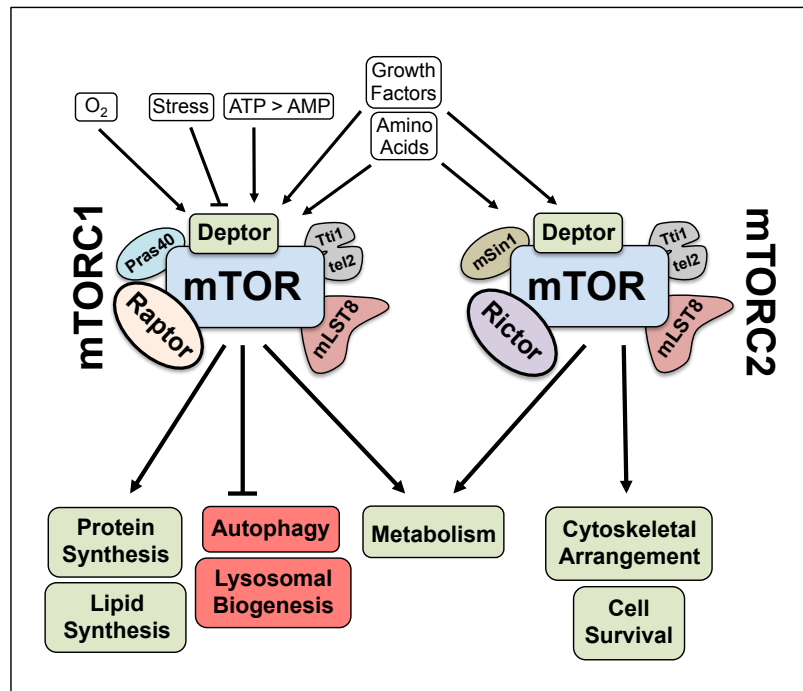
Figure 1.3: mTOR complexes – regulation and function.

The discovery that the tuberous sclerosis 1 (hamartin) and 2 (tuberin) complex (TSC1/2) functions upstream of mTORC1 to negatively regulate its activity is a hallmark

connecting augmented mTOR signaling to cancer [39]. Inactivation of TSC1/2 function correlates with the hereditary TSC syndrome which can manifest an array of different tumor types [40]. Hypoxic cellular conditions through regulated in development and DNA damage responses 1 protein (REDD1) and high AMP:ATP ratios through AMP-activated protein kinase (AMPK) directly activate TSC1/2 [41]. Active TSC1/2 negatively regulates mTORC1 through its GTPase-activating protein (GAP) activity to inhibit Ras homolog enriched in brain (Rheb) GTPase. In contrast, growth factors through stimulation of MEK/ERK and PI3K/AKT signaling cascades are capable of inhibiting TSC1/2 leading to a GTP bound activated Rheb to activate mTORC1 [36]. Interestingly, nutrient signaling (i.e. amino acids) dependent activation of mTORC1 does not appear to rely upon signaling through the TSC1/2 complex, and also appears to be the only essential extracellular signaling factor able to activate mTORC1 in isolation [42].

Activated mTORC1 is responsible for directly phosphorylating several effectors involved in anabolic processes such as protein synthesis, lipid biogenesis, and metabolism [36]. mTOR also negatively regulates autophagy and lysosome biogenesis. Interestingly, lysosomes and the enzymes found within their vesicles are essential components of the autophagic machinery.

The best understood effectors of mTORC1 are S6 kinase 1 (S6K1) a member of the protein kinase A/protein kinase G/protein kinase C (AGC) protein family, and eukaryotic translation initiation factor 4E (eiF4E)-binding protein 1 (4E-BP1), which are both involved in translational regulation [43]. It's important to note, that active mTORC1

is part of a negative feedback loop involving RTKs responsible for regulating PI3K/AKT

signaling (Figure 1.4) [44].
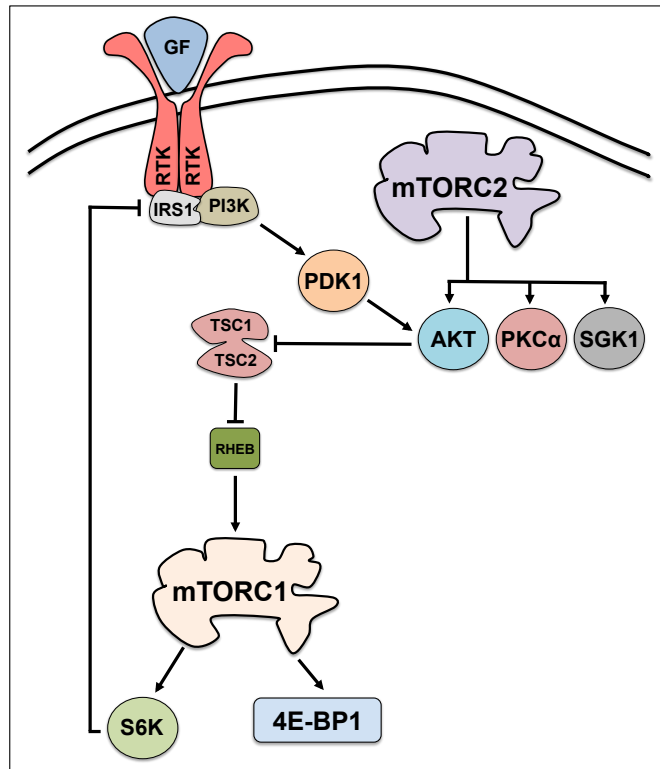
Figure 1.4: mTORC1 and mTORC2 signaling. Distinct signaling nodes specific to mTORC1 and mTORC2. mTORC1 phosphorylates S6K resulting in a negative feedback loop initiated by S6K mediated downregulation of IRS-1. Downregulation of IRS-1 regulates RTK mediated PI3K signaling. mTORC2 phosphorylates various AGC kinases: AKT, PKCα, SGK1. GF, growth factors; RTK, receptor tyrosine kinase.

One, well studied mechanism, is through insulin ligand signaling through insulin

receptor substrate 1 (IRS-1). Kobayashi and colleagues demonstrated that active

mTORC1 signaling through downstream effector, S6K1, can downregulate IRS-1 and

this may occur through S6K phosphorylation of IRS-1 and subsequent subcellular

relocation of IRS-1. The posttranslational phosphorylation of IRS-1 is thought to re-

localize IRS-1, targeting it for proteosomal degradation and leading to subsequent

decreases in PI3K/Akt signaling [45,46].

Aside from enhancing various anabolic processes, mTOR is also responsible for negatively inhibiting autophagy. Autophagy is a homeostatic process involving the catabolism of aggregated proteins and damaged mitochondria to essentially recycle necessary building blocks for cellular homeostasis [47]. Recently, mTORC1 has been shown to inhibit autophagy through the direct phosphorylation of an essential autophagy regulating protein (ATG) known as Unc-51 like autophagy activating kinase 1 (ULK1) at Ser 757 [48,49]. Autophagy is activated in conditions of low nutrients, hypoxia, low energy, and various cellular stresses and will be discussed in more detail in a later section of this document [50-52].

As mentioned before, mTOR is also the catalytic kinase within the mTORC2 complex, and this complex also appears to be sensitive to hormones (i.e. insulin), growth factors, and nutrients (amino acids) [53]. In conjunction with mTORC1, mTORC2 also regulates cellular metabolism, but autonomously regulates cytoskeletal organization and cell survival pathways [36]. Like mTORC1, mTORC2 is also capable of phosphorylating several members of the AGC protein family (Figure 1.4) [54]. mTORC2 is responsible for phosphorylating serum/glucocorticoid regulated kinase 1 (SGK1) and AKT within their hydrophobic motifs [55,56]. Furthermore, ablation of mTORC2 activity by knock-down of Rictor decreases expression of phosphorylated N-myc downstream regulated gene 1 protein (NDRG1) at Thr 346, a direct substrate of SGK1 [55,57]. It has also been shown that growth factors are dispensable for mTORC2 phosphorylation of AKT at Ser 473 and protein kinase C-alpha (PKCα) at Ser 657, two additional AGC protein kinases capable of regulating cell survival and cytoskeletal organization, respectively (Figure 1.4) [38,58].

Furthermore, work within the mTOR field has demonstrated that loss of the TSC1/2 complex attenuates mTORC2 signaling while elevating mTORC1 activity [58]. Needless to say there is not a clear mechanistic understanding of how mTORC2 is regulated, but it is important to note that the regulation of mTORC2 activity is complex and an area of much needed study. For example, mTORC2 activity was shown to be required for prostate cancer development in mice harboring a phosphatase and tensin homolog (PTEN) deletion arguing for the development of compounds specifically inhibiting mTORC2 activity [59].

As with the important discovery that mTOR is the functional target of rapamycin [34] and the dogma surrounding mTORC2's insensitivity to rapamycin [38], it is also important to note the contrasting observation that treatment with rapamycin over prolonged duration or within specific cellular context is capable of inhibition of mTORC2 [60,61].

One can see that the majority of research devoted to understanding mTOR signaling has been through the understanding of mTORC1, and this is due to the pharmacological tool, rapamycin. However, advances in understanding essential components of mTORC2 signaling has allowed researchers to design experiments to understand mTORC2 specific regulation and downstream signaling. Together, such advances and inevitably drug advancement to target mTORC2 aside from mTORC1 will further advance the mTOR field of research.

1.4   Autophagy

Autophagy, which literally means, "to eat ones self" is a conserved catabolic process where the cell engulfs cytoplasmic components to degrade and in a sense

recycle cellular building blocks into essential cellular pathways to maintain cellular

homeostasis in the context of various stresses such as low energy, low nutrients,

hypoxia, and pharmacological inhibitors such as mTOR inhibitors. Autophagy is also

involved in the regulation of gerosuppression (senecscnece) and molecular aging,

various aspects of cancer progression and suppression, metabolism,

neurodegenerative diseases, and development [62-66].

Autophagy can be thought of as a biological process that is in constant flux. Its

initiation is molecular regulated by several complexes, ULK1/ATG/FIP200 and

Vps34/Beclin1 [67]. Once initiated by cellular cues, sequestration of cellular cargo by a

double membrane vesicle called the phagophore will elongate to fully encompass all

cargo in a cellular structure termed the autophagosome. Cargo may include organelles

such as mitochondria and endoplasmic reticulum (ER), long lived aggregated proteins,

lipids, nucleic acids, and also some pathogens [68]. The elongation phase is regulated

by ATG5/ATG12 and ubiquitin-like protein LC3. LC3 is lipidated through its conjugation

to phosphatidylethanolamine (PE) to form LC3-II [69]. This lipidated form of LC3 is

inserted into the inner and outer autophagosomal membrane as this elongation phase

leads to closure. LC3-II is one of the few molecular markers utilized to measure mature

autophagosome formation [70]. Autophagosomes are termed mature autolysosomes

once fused to lysosomes. Fusion with lysosomes results in degradation of sequestered

cargo by lysosomal enzymes. Degradative end products of autophagy yield fatty acids,

amino acids, and nucleotides that are utilized for specific metabolic processes once

released back into the cytoplasm (Figure 1.5)[68] [68].
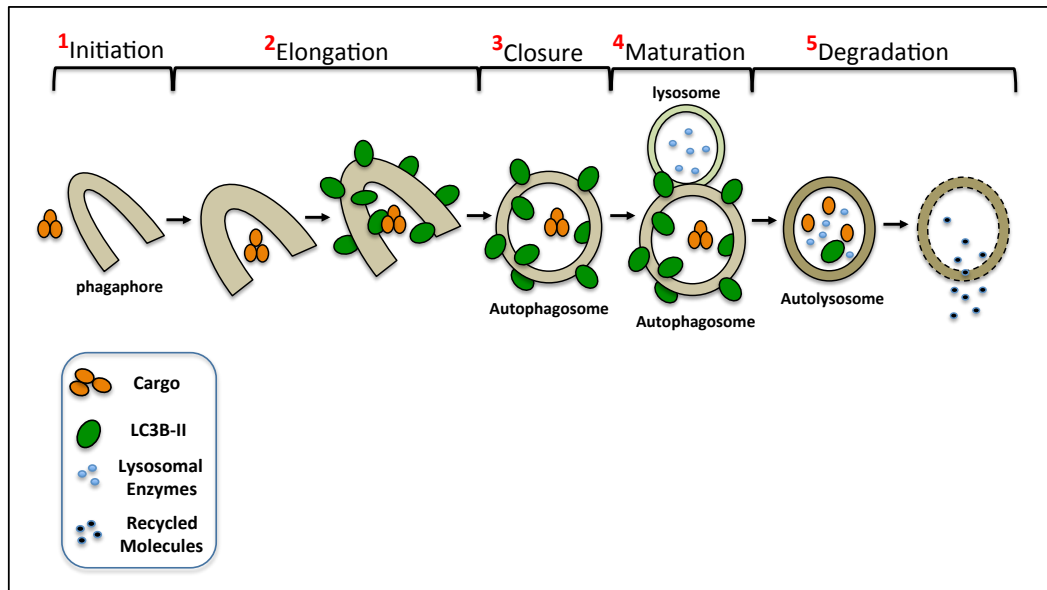
Figure 1.5: Autophagic flux pathway

Furthermore, completion of the autophagy pathway supports cellular homeostasis through provision of essential building blocks for critical cellular and metabolic processes such as energy production, lipid formation, and protein synthesis.

1.4.1   Autophagy and cancer

Recent genetic and molecular understandings have revealed support for autophagy as a pro-tumorigenic mechanism [71-74]. This paradigm is thought to be through autophagy supporting tumor maintenance in hypoxic regions or areas under metabolic stress [75]. However, opposing data also show that autophagy can act as a tumor suppressive cellular function [76-78]. Germline mutations in autophagy regulating gene, *Becn1* (Beclin1) have been observed ovarian, prostate, and breast cancer [79-81]. Additionally, autophagy's tumor suppressive function may be linked to tumors with defects in autophagy as well as apoptosis. Defects in both of these pathways can lead to necrotic cell death and a subsequent inflammatory response, inevitably attenuating tumor progression [64]. Importantly, the context at which these 2

paradigms establish themselves is unclear and involves complex cross-talk between both cellular processes, but may be linked to early stages of transformation [68].

Signal transduction pathways, in addition to mutations in specific autophagy regulating genes are also implemented in autophagy regulation of cancer development [62]. It has now been widely accepted that oncogenic and tumor suppressive pathways regulate various metabolic pathways. In addition to mTOR negatively regulating autophagy, AKT and epidermal growth factor receptor (EGFR) have been shown phosphorylate Beclin1 to negatively regulate autophagy through an mTOR independent mechanism [82,83]. As the field advances and elegant temporal and spatially controlled GEMMs are developed, a more clear understanding of ATGs, oncogenes, and tumor suppressors and their contribution to autophagy being tumor suppressive or tumor promoting will able to be addressed.

1.5   ccRCC and mTOR signaling

Previous studies and more recent TCGA (28% of core set samples) analyses has revealed alterations is various components responsible for increased mTOR signaling in patient ccRCC tumors [31,84]. Abnormal augmentation of oncogenic signaling pathways suggests possible addiction to the altered pathway. This "oncogenic addiction" reveals possible therapeutic potential and supports why patients receiving temsirolimus or everolimus for advanced RCC show progression free and overall survival benefit. However, not all patients respond and those that do present sensitivity eventually become refractory to treatment.

Treatment of ccRCC cell lines with rapamycin decreases HIF1α expression and modestly HIF2α expression, revealing distinct sensitivity of HIF1α and HIF2α to

rapamycin [85,86]. Furthermore, Thomas and colleagues demonstrated RCC cells harboring loss of pVHL show sensitivity to derivatives of rapamycin, and the efficacy observed may be due to attenuation of translational regulation of HIF by mTOR [86]. Additionally, Toschi and colleagues used RNAi to dissect out the contribution of mTORC1 and mTORC2 regulation of HIF1α and HIF2α. Their data revealed that RNAi directed to mTOR or Rictor+Raptor positively regulates HIF1α, while mTORC2 inhibition by Rictor KD alone is capable of inhibiting HIF2α [85]. Additionally, TCGA data analyzing ccRCC tumor samples reported mutations in mTOR and genomic alterations associated with activation of the PI3K/mTOR signaling cascade [31].

Such strategies as those above have revealed some insight into mTOR signaling amplification and subsequent regulation of relative oncogenic and tumor suppressive pathways in ccRCC. These observations shed light into why subpopulations of patients may respond better than others to current therapeutic options for inhibiting mTOR. Lastly, development of therapeutic inhibitors (i.e. catalytic mTOR inhibitors) capable of indirectly inhibiting not only HIF1α but also HIF2α may show increased efficacy against tumor supporting pathways in ccRCC [87,88].

1.6.1 Pharmacologic targeting of mTOR in ccRCC

Rapamycin is a macrolide constructed by *Streptomyces Hygroscopicus* bacteria with high specificity for inhibiting mTORC1 [89] and recent evidence for detecting aberrant mTOR signaling in a variety of cancers has yielded additional rapamycin derivatives (i.e. temsirolimus and everolimus) harboring more acceptable pharmacodynamics to be used in pre-clinical and clinical studies [44,90]. Specifically, intravenous rapamycin derivative, temsirolimus, was approved by the FDA in 2007 for

the treatment of advanced stage renal cell carcinoma [91], and in 2009 everolimus, an orally available analogue of rapamycin, was approved as a second therapeutic option for patients who's diseased progressed after tyrosine kinase inhibitor (TKI) treatment. However, patients typically acquire resistance to allosteric mTOR inhibitors resulting in progression of their disease [92]. This common clinical observation warrants new efforts to understand potential compensatory survival mechanisms capable of being targeted to improve clinical efficacy.

Advances in drug development has resulted in an array of various mTOR inhibitors capable of inhibiting the kinase activity of mTOR through binding and disruption of the interaction of ATP within the catalytic domain [59]. Disruption of mTOR kinase activity in RCC cells inhibits both mTORC1 and mTORC2 signaling cascades [93-96]. Specifically, catalytic mTOR inhibitors are capable of not only inhibiting S6K but also 4E-BP1 effectors downstream of mTORC1. Respective to mTORC2 inhibition, treatment with catalytic mTOR inhibitors inhibits AKT compensatory mTORC2 survival signaling induced in the context of allosteric mTOR inhibition. Secondary, to direct inhibition of mTORC1 and mTORC2, catalytic mTOR inhibitors are capable of decreasing HIF2α, an essential component shown to regulate tumor growth and progression and also poor prognosis factor in VHL deficient RCC cells and ccRCC patients [20,94]. Functionally, catalytic mTOR inhibitors are better at decreasing cell viability and tumor growth than allosteric mTOR inhibitors in the context of RCC [94,96]. Clinically, cases have been reported for patients that had above average response rates receiving a derivative of rapamycin after failing VEGF targeted therapy. Deep sequencing for genomic alterations within these patients' primary tumor and metastatic

sites revealed alterations supporting for activation of the mTOR pathway, illustrating why these select patients responded well to a therapeutic targeting mTOR [97].

Although rapaymcin derivatives have shown to be promising in treating advanced RCC [91,98] there can be activation of compensatory survival pathways [94,95]. For instance, compensatory activation of mTORC2 by rapamycin has been demonstrated in a variety of cancer types [99] and may also serve as a survival mechanism in RCC [100]. Although catalytic mTOR inhibitors have been shown to improve efficacy over allosteric mTOR inhibitors in RCC [94,96] there too are consequences of such inhibition. For instance, rapamycin and catalytic mTOR inhibitors have been shown to induce mitogen activated protein kinase (MAPK/ERK) signaling and autophagy in cancer cells [101-106]. Furthermore, treatment with catalytic inhibitors may increase activity of these pathways more than rapamycin [107,108]. Deciphering the regulation of mTORC1 and mTORC2 on these compensatory pathways in RCC has yet to be investigated more thoroughly and will be the main focus of Chapter 2 of this document.

1.6.2 Autophagy induction through mTOR inhibition

It is well understood that mTOR negatively regulates autophagy, and it has been demonstrated that treatment with mTOR inhibitors is capable of inducing autophagy in cancer cells [93,103,109-112]. Furthermore, several groups have revealed that catalytic mTOR inhibitors appear to augment autophagic flux more than allosteric mTOR inhibitors [107,108]. The mechanism behind these observations is not well understood, however several groups have shown decreased expression of $pULK1_{S757}$ with catalytic mTOR inhibitors and minimal to no reduction with rapamycin in cancer cells [107,112]. Phosphorylation of ULK1 at Ser757 by mTOR correlates with autophagy inhibition

[48,49,113]. Furthermore, only RNAi targeting mTOR was capable of decreasing expression of $pULK1_{S757}$ in ovarian cancer cells, again suggesting that direct inhibition of mTOR may be the reason for the increased autophagy observed with catalytic mTOR inhibitors when compared to allosteric mTOR inhibitors [107].

Additionally, activation of autophagy via mTOR inhibition has been shown to function as a compensatory survival mechanism that can be targeted to enhance the efficacy of mTOR inhibition on cancer cell proliferation and survival [93,103,111,114]. Interestingly, in glioblastoma [103], it appears that inhibition of autophagy with rapamycin does not yield the same effect of increasing apoptosis as catalytic PI3K/mTOR inhibition and autophagy inhibition. However, in RCC cells it appears that a derivative of rapamycin plus autophagy inhibition can induce necroptosis as a programmed cell-death signal [109]. Understanding the direct role of increasing autophagic flux by differing types of mTOR inhibitors may require a better understanding of not only mTORC1, but also mTORC2's regulation of autophagy. Chapter 3 will investigate autophagic flux response to allosteric and catalytic mTOR inhibition to determine if autophagic induction is indeed targetable in RCC cells treated with various mTOR inhibitors.

1.6.3 mTOR inhibition and MEK/MAPK signaling crosstalk

It is well understood that oncogenic and tumor suppressive signal transduction pathways are not linear, but exhibit complex cross-talk between pathways [115]. This paradigm has been clearly observed within the PI3K-mTOR and Ras-ERK signaling pathways [115]. A hallmark of cancer is its ability to acquire resistance to targetable therapies resulting in a reduction of tumor responsiveness. PI3K-mTOR pathway

activation was found in 28% of the TCGA ccRCC samples and has been linked to several noted genetic lesions in a cohort of ccRCC patient tumors [31] supporting possible addiction to this pathway and respectable therapeutic targets. Unfortunately, patients with advanced RCC treated with mTOR inhibitors typically result in cytostatic responses followed by an acquired resistance to the current therapeutic regimen. Observations such as these imply the importance to understand potential resistance mechanisms to generate high-order combinatorial approaches prolong survival.

Specifically, compensatory targetable MAPK survival and proliferation signals have been observed in the context of mTOR inhibition [101,102,105,106,116]. Interestingly, it has been noted that allosteric and catalytic mTOR inhibitors have varying effects on MAPK signaling induction [102], but the molecular mechanisms deciphering the sensitivity to this induction are not well understood. A mTORC1-MAPK feedback loop dependent upon PI3K activation has been described through studies with rapamycin as a potential mechanism for the compensatory MAPK activation in the presence of mTOR inhibition [101].   Additionally, activation of p90RSK and the subsequent inhibition of downstream pro-apoptotic effectors are also plausible mechanisms of resistance to allosteric mTOR inhibition [116]. The importance of mTOR inhibition and compensatory modulation of MAPK apoptotic signaling pathways in RCC has not been established. Therefore, understanding the genetic and molecular underpinnings of MAPK compensatory survival and proliferation signals will aid clinicians in selecting plausible therapeutic combinations in conjunction with current FDA approved rapamycin derivatives and possible future catalytic mTOR inhibitors when treating advanced RCC.

CHAPTER 2

MTOR INHIBITION INDUCES COMPENSATORY, THERAPEUTICALLY TARGETABLE MEK ACTIVATION IN RENAL CELL CARCINOMA[2]

2.1   Overview

Rapamycin derivatives allosterically targeting mTOR are currently approved by the FDA to treat advanced renal cell carcinoma (RCC), and catalytic inhibitors of mTOR/PI3K are now in clinical trials for treating various solid tumors. We sought to investigate the relative efficacy of allosteric versus catalytic mTOR inhibition, evaluate the crosstalk between the mTOR and MEK/ERK pathways, as well as the therapeutic potential of dual mTOR and MEK inhibition in RCC. Pharmacologic (rapamycin and BEZ235) and genetic manipulation of the mTOR pathway were evaluated by *in vitro* assays as monotherapy as well as in combination with MEK inhibition (GSK1120212). Catalytic mTOR inhibition with BEZ235 decreased proliferation and increased apoptosis better than allosteric mTOR inhibition with rapamycin. While mTOR inhibition upregulated MEK/ERK signaling, concurrent inhibition of both pathways had enhanced therapeutic efficacy. Finally, primary RCC tumors could be classified into subgroups [(I) MEK activated, (II) Dual MEK and mTOR activated, (III) Not activated, and (IV) mTOR activated] based on their relative activation of the PI3K/mTOR and MEK pathways.

---

[2]Chapter 2 is from Sean T. Bailey, Bing Zhou, Jeffrey S. Damrauer, Bhavani Krishnan, Harper L. Wilson, Aleisha M. Smith, Mingqing Li, Jen Jen Yeh, and William Y. Kim. *mTOR inhibition induces compensatory, therapeutically, targetable MEK activation in renal cell carcinoma*. PLoS 2014 (in press)

Patients with mTOR only activated tumors had the worst prognosis. In summary, dual targeting of the mTOR and MEK pathways in RCC can enhance therapeutic efficacy and primary RCC can be subclassified based on their relative levels of mTOR and MEK activation with potential therapeutic implications.

2.2   Introduction

Recent statistics suggest that there are predicted to be roughly 65,000 new cases and 14,000 deaths in 2013 from renal cell carcinoma (RCC) [1,3]. Clear cell renal cell carcinoma (ccRCC) is the most common histologic subtype of RCC and the vast majority of sporadic ccRCC have inactivation of the von Hippel-Lindau tumor suppressor protein (pVHL). Patients with VHL disease have inherited mutations of *VHL* and renal cyst and/or tumors develop when these individuals undergo somatic inactivation or loss of the remaining wild-type *VHL* allele [6,117]. pVHL's most well understood function is to negatively regulate the hypoxia-inducible factor alpha (HIFα) family of transcription factors (HIF1α, HIF2α, HIF3α) in an oxygen dependent manner via its E3 ubiquitin ligase activity [12,89]. Importantly, pVHL's tumor suppressor function is dependent upon the downregulation of HIFα subunits and in particular HIF2α [20-22].

Stabilization of HIFα, either as a consequence of hypoxia or pVHL inactivation leads to transcriptional activation of numerous genes associated with adaptation to a hypoxic environment as well as a favorable tumor microenvironment [3,89,118]. The development of FDA approved therapies for combating ccRCC has been heavily influenced by an understanding of the molecular underpinnings of VHL disease. Specifically, small-molecule tyrosine kinase inhibitors (e.g. sunitinib and pazopanib)

have been developed to inhibit vascular endothelial growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR) [117,119]. Additionally, temsirolimus and everolimus, derivatives of rapamycin, are approved to treat advanced RCC [89]. While significant tumor responses are seen in the setting of VEGFR inhibition they are much less common upon mTOR inhibition suggesting potential compensatory survival and proliferative mechanisms that can be co-targeted [91,98].

Rapamycin and its derivatives are allosteric inhibitors of the serine/threonine kinase mechanistic target of rapamycin (mTOR) that require rapamycin's association with cytosolic protein, FKBP12 [35,89]. mTOR integrates extracellular growth signals with cellular responses such as proliferation, autophagy, metabolism, cell growth and survival [44]. The mTOR protein kinase interacts with several proteins to form two distinct complexes, mTORC1 and mTORC2. Both mTORC1 and mTORC2 are composed of the common subunits: DEP domain containing mTOR-interacting protein (DEPTOR), mammalian lethal with sec-13 protein 8 (mLST8), and tti1/tel2 complex. However, they differ in composition by several additional proteins. Regulatory-associated protein of mammalian target of rapamycin (Raptor) and proline-rich AKT substrate 40 KDa (PRAS40) are distinct to the mTORC1 signaling complex while rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated map kinase-interacting protein1 (mSin1), and protein observed with Rictor 1 and 2 (protor1/2) are associated with mTORC2 [36]. Notably, the mTORC2 complex is thought to be relatively insensitive to rapamycin [38]. Furthermore, treatment with rapamycin and it's derivatives causes a release of negative feedback on the PI3K/AKT signaling pathway [45,99]. Therefore, the inability of rapamycin to inhibit all signaling nodes of mTOR has

warranted efforts to develop catalytic mTOR inhibitors capable of perturbing mTOR's kinase activity and therefore blocking both mTORC1 and mTORC2 complexes [90].

However, recent reports have demonstrated that inhibitors of mTOR are capable of increasing MEK/ERK activation and its associated proliferation and survival signaling in cancer cells [101,105,106,120-123]. Interestingly, several groups have observed that catalytic mTOR inhibition increases compensatory MEK/ERK signaling greater than allosteric mTOR inhibition [102,105]. This particular observation has resulted in pre-clinical and clinical studies utilizing mTOR inhibition in combination with MEK inhibition for treating several cancer types [106,124-126].

Here, we investigate, through both a pharmacologic and genetic approach, the compensatory proliferation and survival pathways observed in the context of allosteric and catalytic mTOR inhibition. The studies conducted here support that catalytic mTOR inhibition may be better than allosteric inhibition at restraining cellular proliferation and increasing apoptosis. However, we also observe that catalytic mTOR inhibition is more robust at initiating compensatory MEK/ERK signaling in RCC. We address these compensatory cross-talk pathways through pharmacologic inhibition and demonstrate that the selected combinatorial approaches reveal an enhanced effect at attenuating cellular proliferation and augmenting the apoptotic response in RCC cells.

2.3   Results

2.3.1   Novel renal cell carcinoma cell lines lack VHL and overexpress HIFα

In order to aid our studies, we generated two novel ccRCC cell lines (hereafter called UNC-R1 and UNC-R2) from primary patient-derived xenografts (PDX). H&E staining of a portion of the PDX tumor demonstrated clear cell histology (Figure 2.1A).

Cell morphology of the cell lines remained consistent over time. To characterize the novel cell lines VHL, HIF1α, and HIF2α expression of the cell lines were determined by western blot (Figure 2.1B). RCC4 2-1 (VHL null) and RCC4 3-14 (VHL wt) were used as controls to validate current findings. Both the UNC-R1 and the UNC-R2 cell lines lacked appreciable expression of VHL. While both cells lines expressed HIF2α, only UNC-R1 expressed HIF1α (Figure 2.1B), suggesting that UNC-R2 cells have lost HIF1α expression as is seen in a proportion of ccRCC cell lines and primary tumors and consistent with the notion that HIF1α is potentially a tumor suppressor gene [25].
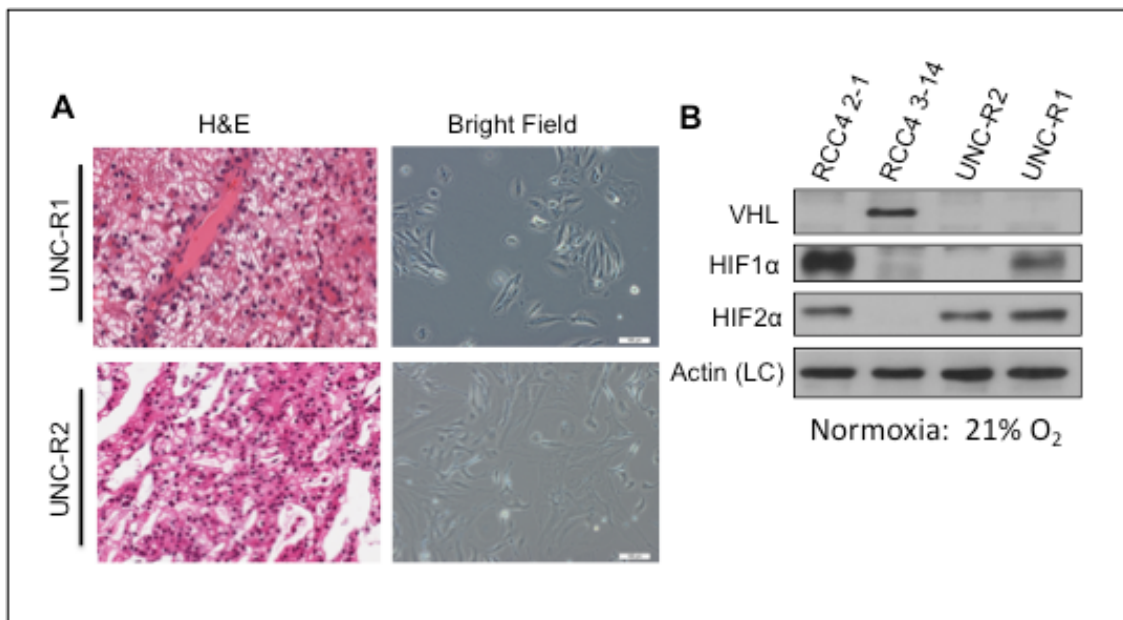
Figure 2.1: Novel renal cell carcinoma cell lines lack VHL and overexpress HIF
(A) Photomicrographs of H&E stains (left panels) and bright field images (right panels) of UNC-R1 and UNC-R2 PDX derived cell lines. **(B)** Whole cell extracts from UNC-R1 and UNC-R2s were immunoblotted with the indicated antibodies. RCC4 2-1 (VHL null) and RCC4 3-14 (VHL positive) were included as controls.

2.3.2 Catalytic mTOR inhibitors block mTORC1 signaling more fully than allosteric mTOR inhibition

Previous studies have demonstrated that the dual catalytic PI3K/mTOR inhibitor, BEZ235, inhibits mTORC1 signaling better than allosteric mTOR inhibition with rapamycin or other rapalogs [94,104,111,127,128]. We wished to see whether these results could be replicated in our hands. Dose titrations of rapamycin and BEZ235 in a panel of human RCC cell lines showed that 200nM and 1uM respectively were required to inhibit mTORC1 and/or mTORC2 signaling (Supplemental Figure 2.1A and 2.1B). These doses of rapamycin and BEZ235 were therefore used to treat a panel of RCC cell lines. As expected, while both compounds inhibited the phosphorylation of S6, only BEZ235 inhibited phosphorylation of 4EBP1 and AKT Ser 473 (Figure 2.2). Moreover, as previously described, allosteric mTOR inhibition with rapamycin resulted in increased $pAKT_{S473}$ expression in 786-0, RCC4, and UNC-R2 cells (Figure 2.2) presumably as a result of release of S6K and IRS1 dependent negative feedback inhibition of PI3K/AKT signaling [99].
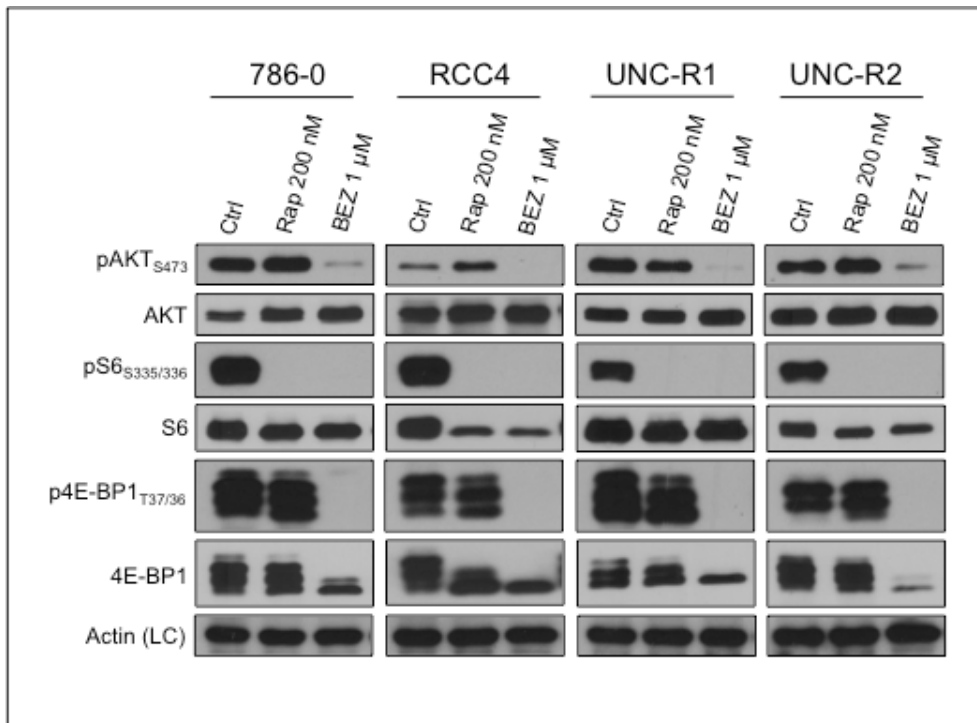
Figure 2.2: Catalytic mTOR inhibitors block mTORC1 signaling more fully than allosteric mTOR inhibition. The indicated cell lines were treated with the allosteric and catalytic mTOR inhibitors (rapamycin and BEZ235 respectively) at the indicated concentrations for 24 hrs. Whole cell extracts were then immunoblotted with the indicated antibodies.

2.3.3 Catalytic mTOR inhibition is superior to allosteric mTOR inhibition at attenuating cellular proliferation and inducing apoptosis

Previous groups have demonstrated that BEZ235 is better than rapamycin at decreasing cellular proliferation in RCC [94]. We utilized CellTiter-Glo® to measure alterations in cellular viability over the course of 4 days. Consistent with previous results, our data show that BEZ235 inhibits cellular proliferation better than rapamycin (Figure 2.3A, Supplemental Figure 2.2). Interestingly, both primary cell lines (UNC-R1 and UNC-R2), but especially UNC-R2, seemed exquisitely sensitive to BEZ235 as evidenced by significantly fewer cells present at day 4 than to day 0 (Figure 2.3A).

While they prolong overall survival, allosteric mTOR inhibitors such as everolimus and temsirolimus have displayed little cytotoxic effects in patients (i.e. they lead to few objective responses) [91]. Catalytic mTOR inhibitors have shown increased efficacy in generating an apoptotic response in preclinical studies, likely as a result of decreasing AKT mediated survival signals [111,127]. Treatment of RCC4, 786-0, and RCC10 cells with BEZ235 resulted in increased apoptosis as evidenced by the increased expression of the apoptotic marker, cleaved PARP (poly ADP ribose polymerase) (Figure 2.3B). Moreover, BEZ235 also increased expression of another apoptotic marker, cleaved-caspase 3, in RCC4 and RCC10 cells (Supplemental Figure 2.3). Interestingly, 786-0 and RCC4 cells showed a decrease in cleaved-PARP expression when treated with rapamycin (Figure 2.3B) likely as a consequence of the increased survival signaling from AKT (Figure 2.2). Additionally, assessment of

45

apoptosis by flow cytometry (Annexin V+/PI- fraction) also showed that RCC cells treated with BEZ235 had increased apoptosis respective to rapamycin treated cells (Figure 2.3C). Therefore, catalytic mTOR inhibition is superior to allosteric mTOR inhibition at attenuating cellular proliferation and inducing apoptosis.
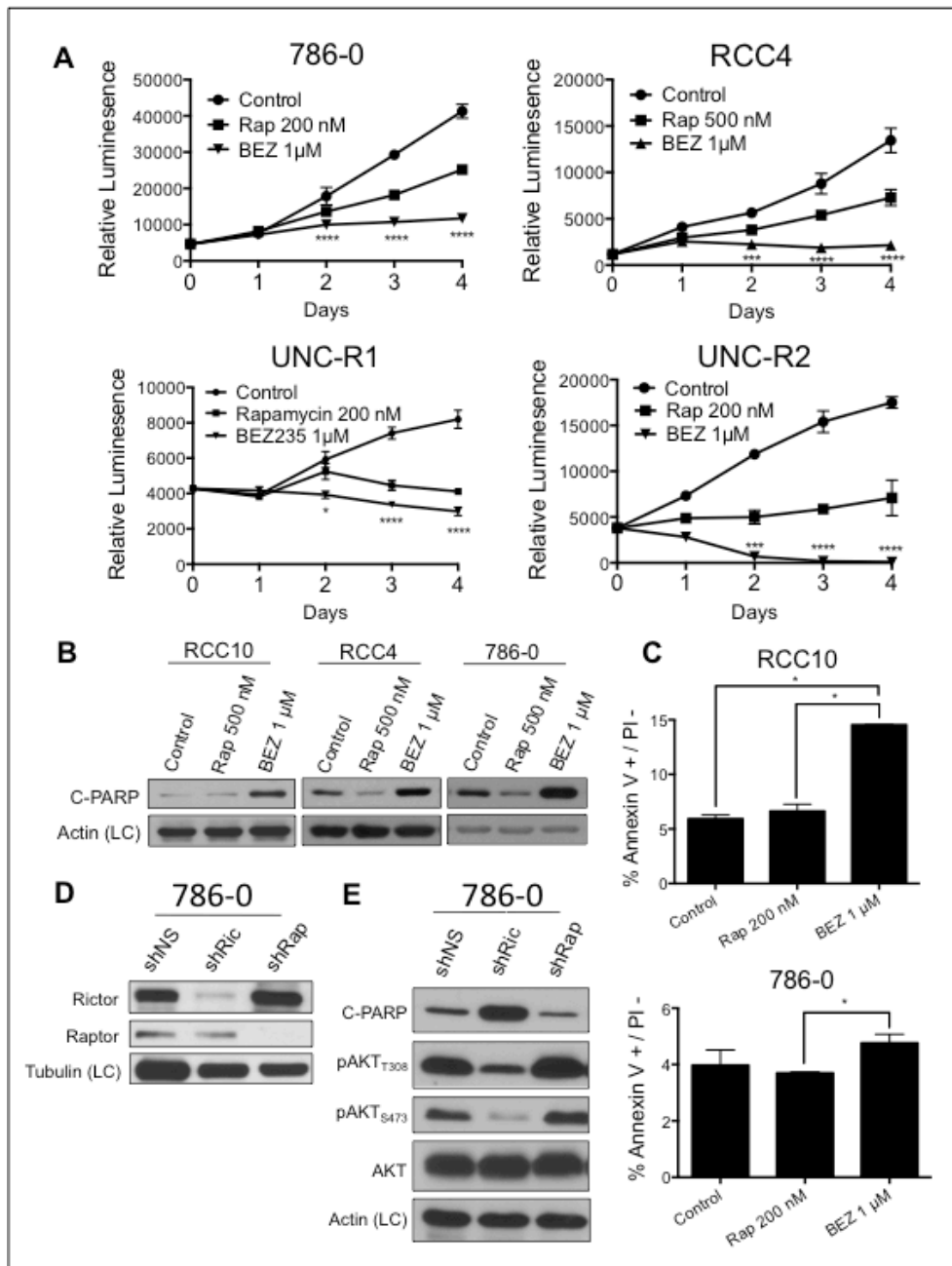
Figure 2.3: Catalytic mTOR inhibition attenuates proliferation and induces apoptosis better than allosteric mTOR inhibition. (A) The indicated cell lines were

assessed for viability on the indicated days using CellTiter-Glo®. Statistical significance was determined by comparing rapamycin and BEZ235 treated groups. **(B)** The indicated cell lines were treated with rapamycin and BEZ235 for 48 hours and immunoblotted with the indicated antibodies. **(C)** The indicated cell lines were treated with rapamycin and BEZ235 for 48 hours and assessed for apoptosis by flow cytometry analysis of the Annexin V+/PI – fraction. **(D)** 786-0 cells were stably infected with shRNAs targeting Raptor (mTORC1) or Rictor (mTORC2) and confirmed for knock-down by western blot. **(E)** Whole cell extracts from 786-0 shNS, shRaptor, and shRictor cells were immunoblotted with the indicated antibodies.

2.3.4   mTORC2 activity negatively regulates the apoptotic response through phosphorylation of AKT

We wanted to determine whether the enhanced apoptosis seen with BEZ235

treatment (relative to rapamycin) was due to its ability to inhibit mTORC2 and

subsequent downregulation of AKT dependent survival signaling. Since there are no

pharmacologic inhibitors capable of specifically inhibiting mTORC2, we silenced Rictor

expression, which is required for mTORC2 activity (Figure 2.3D). Knock-down of Rictor

significantly decreased expression of $pAKT_{S473}$ and $pAKT_{T308}$ as well as increased

cleaved-PARP (Figure 2.3E). In contrast, knock-down of Raptor, which is required for

mTORC1 activity, appeared to slightly decrease cleaved-PARP expression while mildly

increasing $pAKT_{S473}$ or $pAKT_{T308}$ expression. These results support the notion that the

increased apoptosis seen with BEZ235 relative to rapamycin treatment are a result of

BEZ235's inhibition of mTORC2 activity.

2.3.5   mTOR inhibition induces compensatory activation of MEK/ERK signaling

Recent reports have demonstrated cross-talk between the mTOR and MEK/ERK

signaling pathways [115]. To see whether this interplay was present in the context of

RCC we examined the response of ERK and a canonical ERK substrate, p90RSK, to

rapamycin or BEZ235. Both pERK and p-p90RSK were induced by allosteric and

catalytic mTOR inhibition (Figure 2.4A). While there was a sense that BEZ235 treatment

resulted in a slightly larger increase in p-p90RSK in a subset of the cell lines, this was

not accompanied by the same amount of induction of pERK. This could reflect

enhanced ERK activity that is not appreciable by pERK western blotting or mTOR

inhibition induced p-p90RSK that is ERK independent. However, ERK is the only

described kinase to phosphorylate p90RSK on the S380 site [129]. Overall, these

results suggest that mTOR inhibition of RCC cells upregulates MEK/ERK signaling and

that catalytic mTOR inhibition may do so in a more robust manner than allosteric
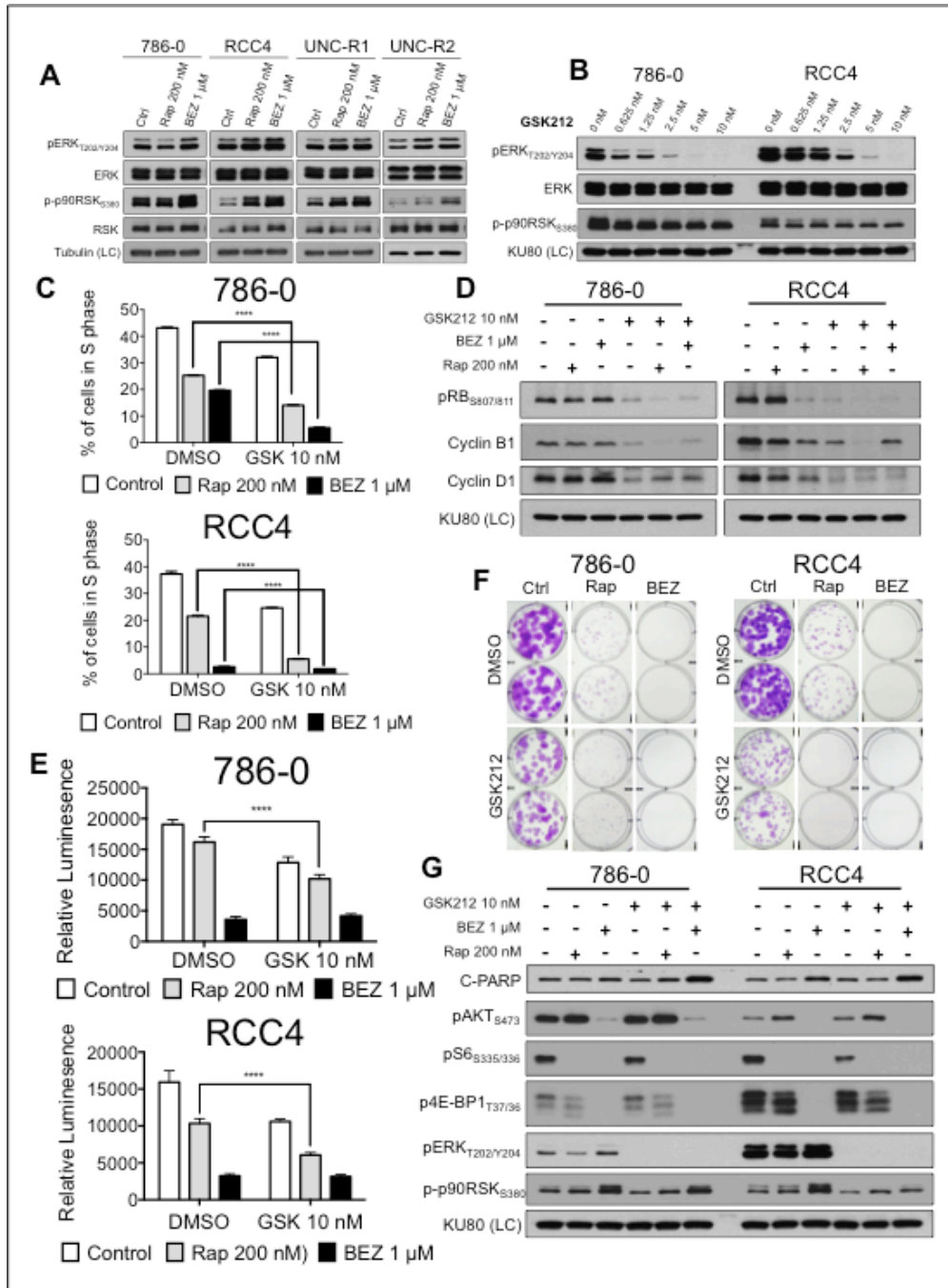
inhibition.

Figure 2.4: Combined mTOR and MEK inhibition attenuates cellular proliferation and increases the apoptotic response. (A) The indicated cells were treated for 24 hrs. with rapamycin or BEZ235 and immunoblotted with the indicated antibodies. **(B)** 786-0 and RCC4 cells were treated increasing doses of GSK212 for 24 hrs. and immunoblotted with the indicated antibodies. **(C)** 786-0 and RCC4 cells were treated for 24 hrs with rapamycin and BEZ235 in the presence of Edu. Edu incorporation was assessed by flow cytometry. **(D)** 786-0 and RCC4 cells were treated with the indicated compounds for 24 hrs. Whole cell extracts were immunoblotted for the cell cycle related proteins indicated. **(E)** 786-0 and RCC4 cells were treated with indicated drugs and

assessed for viability on day 4 using CellTiter-Glo® 4. **(F)** 786-0 and RCC4 cells were plated, allowed to attach, and treated with the indicated drug(s). Photographs of wells containing 786-0 (day 11) and RCC4 (day 17) cells fixed with 4% PFA and stained with 0.1% crystal violet. **(G)** 786-0 and RCC4 cells were treated with the indicated compounds for 24 hrs. Whole cell extracts were immunoblotted with the indicated antibodies.

2.3.6   Combination of mTOR and MEK inhibition attenuates cellular proliferation and increases the apoptotic response

The observation of increased MEK/ERK signaling in the context of mTOR inhibition led us to hypothesize that attenuation of this compensatory signal may decrease cellular proliferation and induce apoptosis. We saw that 10nM of the MEK inhibitor GSK1120212 (hereafter called GSK212) was sufficient to fully inhibit MEK activity as assessed by pERK $_{T202/Y204}$ in several RCC cell lines (Figure 2.4B). Treatment of RCC cell lines with rapamycin or BEZ235 led to a decrease in the percentage of cells in S phase as determined by Edu incorporation (Figure 2.4C). The combination of MEK inhibition with mTOR inhibition led to a potent reduction in S phase fraction, particularly when GSK212 was combined with BEZ235. As expected, MEK inhibition led to hypophosphorylation of Rb as well as downregulation of cyclin B1 and cyclin D1 consistent with increased cell cycle arrest. However, the addition of mTOR inhibition did not further change levels of these proteins (Figure 2.4D).

Despite the fact that the combination of BEZ235 and GSK212 potently inhibited cell cycle progression, there did not appear to be an additive effect on proliferation or colony formation (Figure 2.4E and 2.4F). We hypothesized that this lack of additivity was secondary to the high level of inhibition of proliferation and colony formation by 1 μM BEZ235 alone. Therefore, we determined the IC50 for BEZ235 in several RCC cell lines (Supplemental Figure 4A), confirmed that the determined IC50 was capable of

inducing activation of MEK/ERK signaling (Supplemental Figure 2.4B), and examined its

effects on proliferation on colony formation. The combination of 2 nM of BEZ235 with

GSK212 resulted in significant decreases in proliferation (Supplemental Figure 2.5A)

and colony formation (Supplemental Figure 2.5B) over either single agent alone.

Furthermore, the combination of mTOR inhibition with MEK inhibition augmented the

apoptotic response as evidence of increased C-PARP expression in 786-0 and RCC4

cells treated with the combination (Figure 2.4G). Together, these data support the

notion that combined mTOR and MEK inhibition might be an effective therapy in RCC

2.3.7   Subclasses of RCC can be defined by MEK and mTOR pathway activation

To assess the potential relevance of MEK and /or mTOR inhibition in ccRCC we

examined reverse phase protein array data (RPPA) from the TCGA clear cell kidney

cancer project (KIRC) to determine the relative activation state of these pathways in

human RCC [31]. Reverse phase protein arrays are a highly validated technique

allowing the assessment of protein expression across hundreds of proteins

simultaneously and because of the multiplatform nature of the TCGA allows for

correlations to other genomic aspects of a tumor. To this end, TCGA KIRC tumors were

hierarchically clustered using log2 transformed, median centered, RPPA expression

data of canonical phosphoproteins that represent activation of the MEK ($pERK_{T202/Y204}$),

PI3K ($pAkt_{T308}$), mTORC1 ($p4E-BP1_{T70}$, $p4E-BP1_{T37}$, $pS6_{S235/236}$, $pS6_{S240/244}$,

$p70S6K_{T389}$) and mTORC2 ($pAkt_{S473}$) pathways (Figure 2.5A). There were 4 well-

defined clusters of tumors that appeared to represent differential patterns of MEK and

mTOR activation: 1) MEK activation [I: black]. 2) dual MEK and mTOR activation [II:

red]. 3) no activation [III: green]. and 4) mTOR activation [IV: blue]. These subgroups

could be also visualized using a plot that graphed the relative expression of the canonical markers of MEK activation ($pERK_{T202/Y204}$) and mTORC1 activation ($pS6_{S235}$) (Figure 2.5B).

Finally, we wanted to see if our MEK/mTOR subgroups held prognostic value and thus assessed their patterns of overall survival. We found that patients with mTOR activated tumors (IV: blue) had the worse overall survival while patients with high MEK activation, regardless of mTOR status, had the best survival (I: black and II: red) (Figure 2.5C). Patients with RCC tumors without activation (III: green) had an intermediate overall survival. Therefore, subclasses of RCC tumors can be identified based on their relative activation of the MEK and mTOR pathways and the subclasses correlate with prognosis.
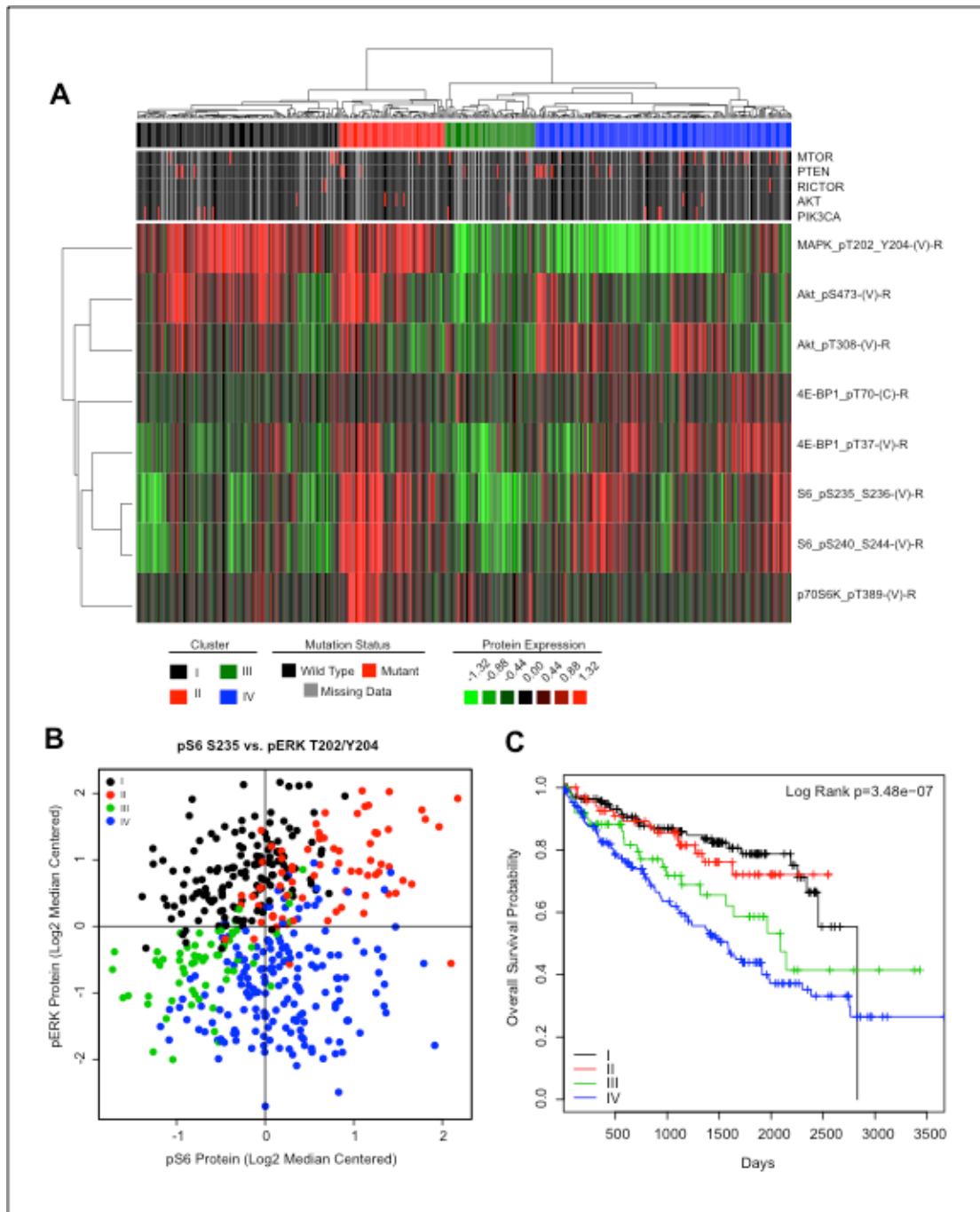
Figure 2.5: Subclasses of RCC can be defined by MEK and mTOR pathway activation. (A) TCGA KIRC RPPA data was log2 transformed, median centered. Tumors were then hierarchically clustered and the indicated subgroups were determined based on expression patterns of the indicated phosphoproteins. Mutational data for mTOR pathway related genes were annotated in the upper tracks. **(B)** Scatter plot of TCGA KIRC tumors based on expression of pS6 and pERK. Each dot indicates a tumor. The MEK-PI3K/mTOR subclasses defined in **(A)** are indicated by color. **(C)** Patients harboring tumors within each MEK-PI3K/mTOR subclass were evaluated for

differences in overall survival by the Log Rank test and shown as a Kaplan-Meier plot of overall survival.

2.4 Discussion

Our studies investigate the relative efficacy of allosteric versus catalytic mTOR inhibition in RCC through both pharmacologic and genetic approaches. We show that as monotherapy, catalytic mTOR inhibition is better at decreasing cellular proliferation and inducing apoptosis than allosteric mTOR inhibition consistent with previous studies in RCC [94]. However, despite these potentially therapeutically beneficial characteristics, we show that catalytic mTOR inhibition also induces a more robust induction of compensatory MEK/ERK signaling. Nonetheless, the compensatory upregulation in MEK/ERK signaling can be targeted with small molecule kinase inhibition, resulting in enhanced therapeutic efficacy. Finally, we demonstrate that primary RCC tumors can be classified based on their relative activation of the MEK and mTOR pathways and that these different MEK/mTOR subtypes are associated with differences in overall survival.

Dual inhibition of the MEK and PI3K/mTOR pathways has shown preclinical promise as a therapeutic strategy in a variety of tumors [106,122-124,130-132] and has entered into phase 1 trials in humans [125]. Inhibition of the MEK/ERK and PI3K/mTOR pathways is a rational strategy based on the extensive crosstalk between the two pathways and the well documented compensatory signaling that occurs in the face of MEK or mTOR inhibition [115]. Nonetheless, neither dual inhibition nor the compensatory cross-talk between the MEK and PI3K/mTOR pathways has been explored specifically in the context of RCC where it is highly clinically relevant given the approval of the allosteric mTOR inhibitors everolimus and temsirolimus for patients with

advanced disease [91]. Therefore, our studies are the first to investigate this crosstalk and its potential clinical relevance in RCC.

Our studies showed that mTOR inhibition in RCC cell lines resulted in increased MAPK signaling in the context of both allosteric and catalytic mTOR inhibition (Figure 4A). Moreover, we noted that catalytic mTOR inhibition enhanced ERK phosphorylation, as well as phosphorylation of the ERK substrate, p90RSK more than robustly than allosteric mTOR inhibition (Figure 4A and G). Precisely how mTOR inhibition in RCC results in increased MEK/ERK signaling remains to be determined. Further investigation into this is warranted but overall our results are consistent with the notion that kinase inhibition results in upregulation of compensatory pathways and kinome reprogramming [133].

Examination of the RPPA data from the TCGA-Kidney Renal Clear Cell Carcinomas (KIRC) project allowed us to assess the possibility that RCC could be divided into subclasses based on the relative activation of the MEK and mTOR pathways as well as evaluate their potential therapeutic significance [31]. We have named these groups, MEK activated, mTOR activated, dual MEK and mTOR activated, and not activated. We propose that rational targeted therapy for the MEK/mTOR subgroups might include: MEK activated – MEK inhibitor, mTOR activated – allosteric or catalytic mTOR inhibitor, dual MEK and mTOR activated – combination MEK and mTOR inhibitor, and not activated – VEGFR tyrosine kinase inhibitor.

Patients with mTOR activated tumors had the worse overall survival relative to the other subgroups and are also the only subgroup that would be predicted to benefit from single agent, allosteric mTOR inhibition. Intriguingly, temsirolimus has been

shown in a phase III randomized trial to prolong the overall survival of patients with "poor prognosis" as defined by the MSKCC criteria [91,134]. While we cannot be sure that our mTOR activated group corresponds to the "poor prognosis" patients defined by the MSKCC criteria, if they do correlate, our data provides a biological explanation for this interesting clinical observation.

In summary, our studies demonstrate that catalytic mTOR inhibition is more effective than allosteric, but that catalytic mTOR inhibition appears to more robustly induce alternative compensatory pathways (i.e. MEK/ERK). Nonetheless, compensatory upregulation of MEK/ERK signaling can be co-targeted with enhanced therapeutic effectiveness. Furthermore, we describe distinct subclasses of RCC that can be defined by the activation of the MEK and mTOR pathways, have clinically distinct prognosis, and would be predicted to have differential responses to MEK and mTOR kinase inhibition. In aggregate, our data suggests that catalytic mTOR inhibition should be investigated in RCC and that the compensatory upregulation of MEK/ERK signaling may actually be a potential synthetic vulnerability in RCC

2.5   Materials and methods

Patient-derived xenograft cell isolation

Xenografts were excised and washed in a solution of Pen-Strep, 1XPBS solution (1:1). In sterile conditions, xenografts were then cut into small 2x2mm fragments and dissociated in gentleMACS^{TM} C-Tube (Miltenyi Biotec) using the gentleMACs Dissociator (program: m_imp Tumor_02) in 5 mL of complete DMEM. Then 100 μL of collagenase D/dispase II (Roche: 40 mg/mL) was added to the tumor fragments and continuously inverted for 30 min at 37°C. Fragments were then subjected to another

round of dissociation using the gentleMACS™ Dissociator (program: m_imp

Tumor_03). 5 mL of protein extraction buffer (PEB: buffer 0.5% FBS, 2mM EDTA in

PBS) was added to the dissociated fragments and resuspended by pipetting. The cell

suspension was transferred to a 50 mL conical tube through a 40 μm nylon mesh sterile

cell strainer (Fisher). An additional 20 mL of PEB buffer was added to the cell

suspension and it was then centrifuged at 300 g's for 5 min. Supernatant was removed

and cell pellet was resuspended in 6 mL of complete DMEM and placed in a 6 cm

sterile cell culture plate. De-identified tumor tissue was obtained from the University of

North Carolina Institutional Review Board (IRB) approved tissue procurement facility

after University of North Carolina IRB approval. The animal work was approved by the

University of North Carolina Institutional Animal Care and Use Committee.

Cell lines and culture conditions

RCC10, 786-0, RCC4, UNC-R1, UNC-R2 were cultured in complete DMEM

(CORNING-Cellgro #10-013-CV) supplemented with 10%FBS, 1X Penn/Strep at 37°C,

5% CO_2, 21% O_2. 786-0 cells were obtained from ATCC. RCC4 cells were obtained

from Dr. Kimryn Rathmell [11] and RCC10 cells were obtained from Dr. Michael Ohh

[135]. RCC tumor tissue from de-identified patients were obtained from the University

of North Carolina Institutional Review Board (IRB) approved tissue procurement facility

after IRB approval. UNC-R1 and UNC-R2 cell lines were generated as above from the

renal patient derived xenografts. BEZ235 (Center for Integrative Chemical Biology &

Drug Discovery, UNC Eshelman School of Pharmacy), GSK1120212

(GlaxoSmithKline), Rapamycin (LC Laboratories) were dissolved in DMSO.

Immunoblot conditions

Cells were lysed in RIPA buffer complemented with Set I and Set II phosphatase inhibitors at 1X (Calbiochem), and protease inhibitors at 1X (Roche). Whole cell lysate concentration was determined with Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad). Proteins were resolved on SDS-PAGE gels and electrotransferred to nitrocellulose membranes, 0.2 μm (Bio-Rad). Primary antibodies $pS6_{S235/236}$, S6, $pAKT_{S473}$, AKT, $p4E\text{-}BP1_{T37/46}$, 4E-BP1, Cleaved PARP, $pAkt_{T308}$, p62, Rictor, Raptor, HIF-1α, HIF-2α, $pERK1/2_{T202/Y204}$ (mouse), ERK, $p\text{-}p90RSK_{S380}$, RSK1/2/3, $pBAD_{S112}$, $pBAD_{S136}$, $pEGFR_{Y1068}$, cleaved-caspase3 were from Cell Signaling Technologies[®]. VHL (Santa Cruz #FL-181). mTOR primary antibody was from Millipore. Primary antibody dilutions were to manufactures' specifications (See Supplemental Table 1). Tubulin (Sigma #T5168), KU-80 (GeneTex #GTX70485) and Actin-HRP (Santa Cruz #C-11) primary antibodies served as loading controls (LC) where noted. Secondary anti-Rabbit and ant-mouse antibodies were from (Fisher) and diluted in 5% milk, 1X TBS-T solution. ECL Western Blotting Detection reagents (GE Healthcare) were used for developing blots onto autoradiography film. For difficult to detect proteins SuperSignal[®] West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used in combination with ECL.

Cell viability assay

To determine cell viability in the context of the various culture conditions we used a CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega) per manufacture's

protocol. Cells were counted and plated in quadruplicate in a 96 well opaque side/clear bottom cell culture plates (Corning) in culture medium containing the noted concentration. Luminescence measurements were captured using a Biotek® Synergy 2 plate reader. 2-way ANOVA analysis was used to determine statistical significance.

Cell cycle analysis by flow cytometry

Cells were plated in triplicate and treated for 24 hrs with indicated drug. Cell cycle analysis was performed by EdU incorporation using Click-iT EdU Alexa Fluor 647 flow assay kit (Invitrogen, catalog number C-10424) according to the manufacturer's instructions. After treatment cells were exposed to 10 µM EdU for 2h. Cells were then dissociated with 0.05% Trypsin/EDTA and fixed immediately, with 4% PFA, for incorporated EdU detection. Total DNA content was stained with propidium iodide (PI) at 10 µg/ml after RNase A treatment. Flow cytometry was performed on a CyAnTM ADP flow cytometer (Dako, Glostrup, Denmark) and data analysis was performed using FlowJo software (Tree Star, Inc.). Statistical significance was measured by Student's T-Test.

Apoptosis analysis by flow cytometry

Cells were plated in duplicate and treated with the indicated drug for 48 hrs. Percentage of apoptotic cells were determined by staining with Annexin V Alexa Fluor® 488 & PI (Dead Cell Apoptosis Kit, Invitrogen, cat# V13241) according to the manufacturer's instructions. Flow was performed on a Dako CyAn ADP and data were

analyzed using FlowJo software. Statistical significance was measured by Student's T-Test.

RNAi experiments

pLKO.1 shRNA plasmids were obtained form the UNC Viral Vector Core, packaged and infected per manufacture's protocols. Addgene catalogue numbers: shNS (#1864), shRictor (#1853), shRaptor (#1857), shmTOR (#1853). Cells were incubated with viral media over-night, and replaced with fresh complete media. Selection with 1 μg/mL puromycin was started 48 hrs later.

Colony formation assay

RCC cells were plated at low-density in a 6 well plate (786-0: 50 cell/well and RCC4: 100 cells/well). Cells were allowed to attach and treated with indicated drug(s). Treatment conditions were changed every 72 hrs. Cells were fixed with 4% PFA and stained with crystal violet.

TCGA data analysis

TCGA KIRC RPPA protein data was log2 transformed and median centered. Tumors samples (n=454) and proteins relating to ERK and mTOR signaling were hierarchically clustered by centroid linkage using Cluster 3.0 and protein clusters were determined based on tumors sharing a common node. Mutations in mTOR related genes were annotated and superimposed as tracks above the heatmap for visualization.

Scatter and Kaplan-Meier plots were generated in R (http://cran.r-project.org). Survival

differences were determined by log-rank test.

2.6   Supplemental Figures
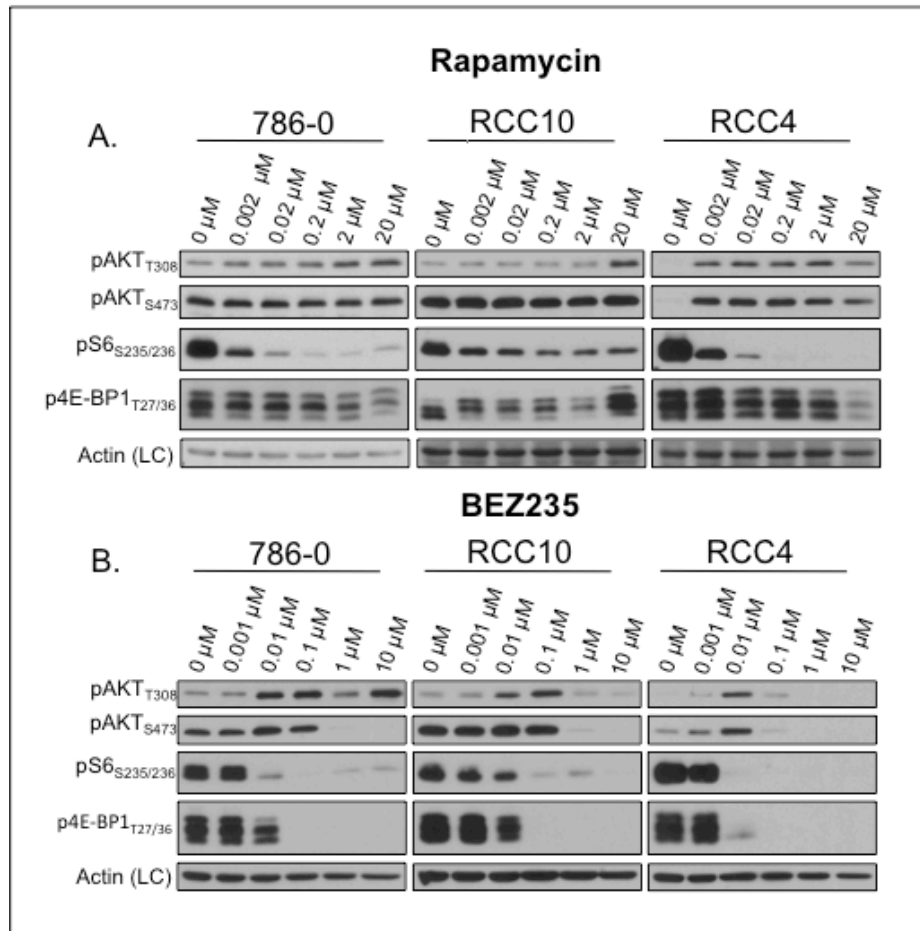
Supplemental Figure 2.1: The indicated cell lines were treated with increasing doses of rapamycin **(A)** or BEZ235 **(B)** for 24 hours. Whole cell extracts were immunoblotted with the indicated antibodies to evaluate changes in mTORC1 and mTORC2 signaling.
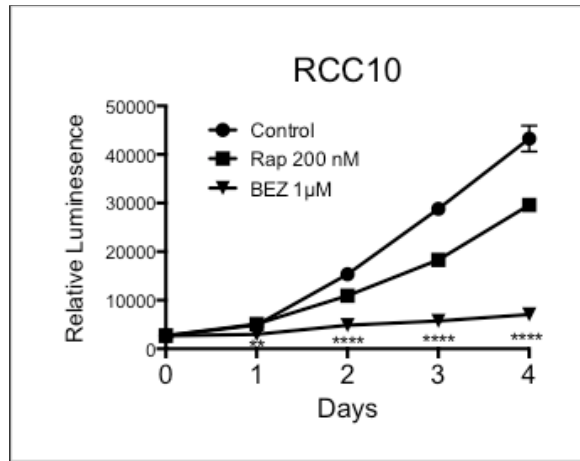
Supplemental Figure 2.2: RCC10 cells were treated over the course of 4 days with the indicated drugs and assessed for viability using CellTiter-Glo®.
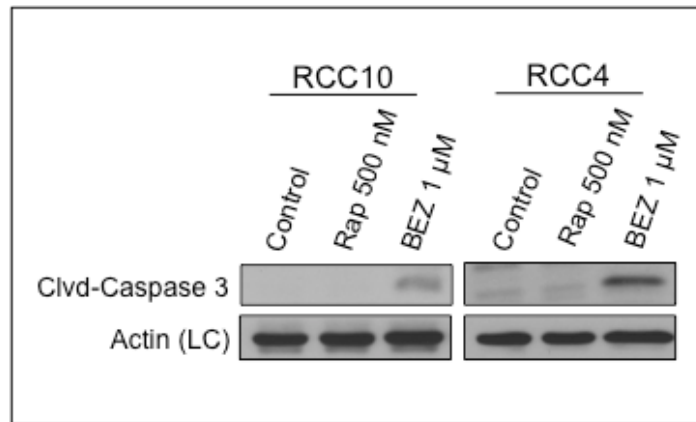
Supplemental Figure 2.3: RCC10 and RCC4 cells treated with rapamycin or BEZ235 for 24 hrs and analyzed by western blot for apoptotic marker cleaved-caspase 3.
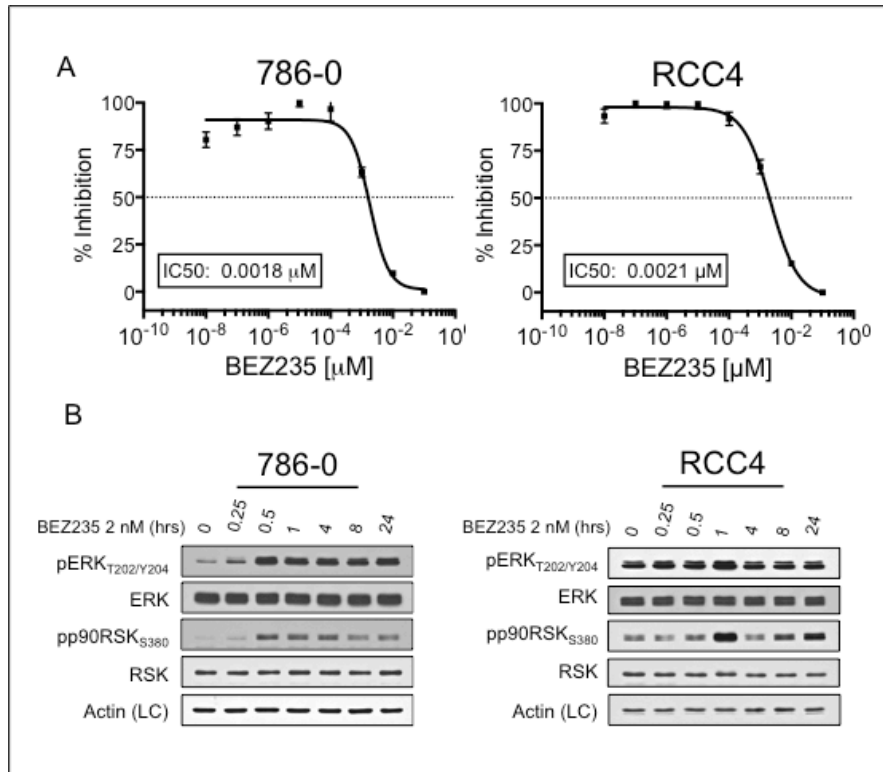
Supplemental Figure 2.4: **(A)** 786-0 and RCC4 cells were plated and treated with a dose titration of BEZ235 and IC50 value determined using CellTiter-Glo® cell viability reagent. **(B)** 786-0 and RCC4 cells were treated with 2 nM BEZ235 over a 24 hr. time course and immunobloted for protein expression of mTORC1, mTORC2, and MEK/ERK signaling proteins.
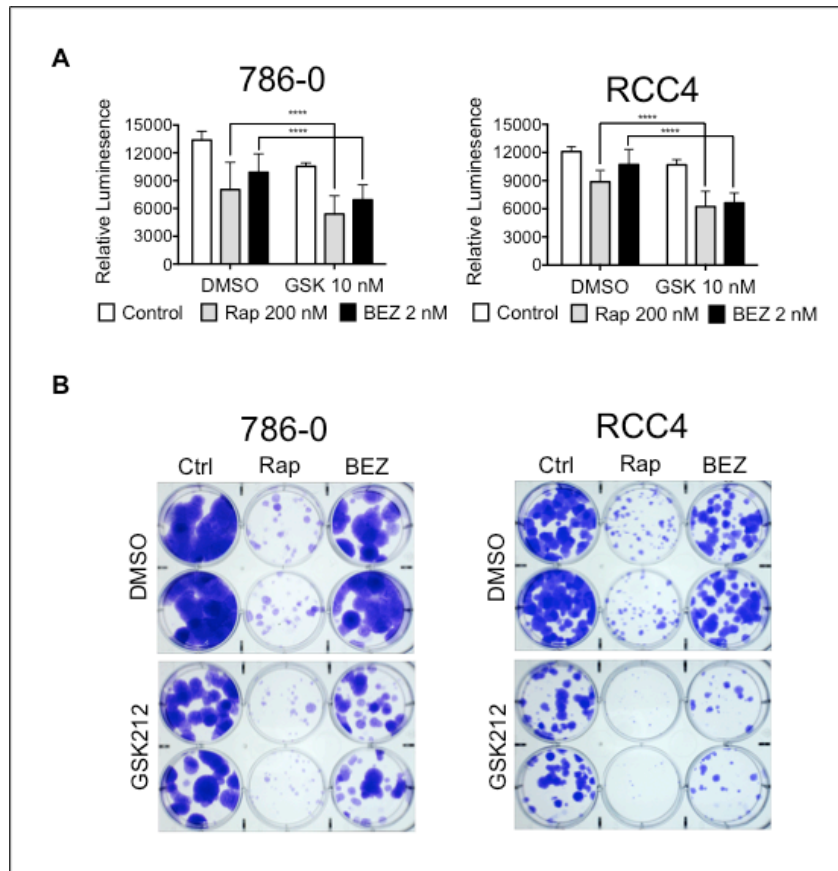
Supplemental Figure 2.5: **(A)** 786-0 and RCC4 cells were treated with indicated drugs and assessed for viability on day 4 using CellTiter-Glo®. Statistical significance was determined by comparing rapamycin and BEZ235 treated groups. **(B)** 786-0 and RCC4 cells were plated, allowed to attach, and treated with 200 nM rapamycin, 2 nM BEZ235, 10 nM GSK212. Photographs of wells containing 786-0 and RCC4 cells fixed with 4% PFA and stained with 0.1% crystal violet.

Antibody	Company	Catalogue #
phospho-RB Ser807/811	CST	9308
phospho-MAPK(Erk1/2) Thr202/Tyr204	CST	9106
phospho-AKT Thr308	CST	2965
phospho-AKT Ser473	CST	4060
phospho-S6 Ser235/236	CST	4858
phospho-4E-BP1	CST	9459
phospho-p90RSK Ser380	CST	9335
S6	CST	2217
4E-BP1	CST	9452
AKT	CST	9272
RSK1/2/3	CST	9355
Cyclin D1	CST	2922
Cyclin B1	CST	4138
Raptor	CST	2280
Rictor	CST	9476
HIF-1α	CST	3716
HIF-2α	CST	7096
Cleaved-PARP	CST	5625
Caspase-3	CST	9662
VHL	SC	sc-5575
Ku80	GT	GTX70485
Tubulin	S	T5168
Actin	SC	C-11
MAPK(Erk1/2)	CST	9102

CST = Cell Signaling Technology
SC = Santa Cruz
AB = Abcam
M = Millpore
GT = GeneTex
S = Sigma

Supplemental Table 2: Antibodies used in Chapter 2 experiments

CHAPTER 3

AUTOPHAGY IS A TARGETABLE COMPENSATORY SURVIVAL PATHWAY, INCREASED IN THE CONTEXT OF MTOR INHIBITION IN RENAL CELL CARCINOMA

3.1   Overview

The mechanistic target of rapamycin (mTOR), which exists in two independent complexes: TORC1 and TORC2 has been shown to be dysregulated in renal cell carcinoma (RCC). mTOR inhibition results in compensatory activation of PI3K/Akt survival signaling as well as autophagy. Two allosteric mTOR inhibitors are FDA approved for treating advanced RCC and catalytic, active site inhibitors are in clinical development. We interrogated the effects of mTOR inhibition on autophagic flux in RCC cell lines and compared the differential effects of allosteric versus catalytic mTOR inhibition. While both allosteric and catalytic inhibition increase autophagic flux, catalytic mTOR inhibition did so more extensively. Furthermore, assessment of cell lines with stable knock-down of Raptor or Rictor suggest that inhibition of both TORC1 and TORC2 contribute to the increased autophagic flux induced by catalytic mTOR inhibition. Finally, we show that catalytic mTOR inhibition in combination with autophagy inhibition increases apoptosis and reduces cellular proliferation. In summary, our studies demonstrate that catalytic mTOR inhibition induces autophagic flux greater than allosteric mTOR inhibition and suggest that the combinatorial targeting of mTOR and autophagy may have therapeutic benefit in RCC.

3.2   Introduction

In 2013 renal cell carcinoma accounted for approximately 14,000 deaths and 65,000 new cases [1]. Renal cell carcinoma (RCC) can be classified into several distinct histological subtypes, each associated with unique genetic alterations [9]. Clear cell renal cell carcinoma (ccRCC) is the most common of these histological classifications encompassing roughly 75% of all cases of kidney cancer [6]. Clear cell renal cell carcinoma has been tightly linked to inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene and patients with VHL disease are more susceptible to developing hereditary ccRCC and a variety of other cancers [6]. However, sporadic ccRCC cases can occur and are associated with biallelic inactivation of the VHL gene. The VHL protein (pVHL) negatively regulates the alpha subunit of the hypoxia-inducible factor (HIFα) family of transcription factors (HIF1α, HIF2α, HIF3α) in a oxygen dependent manner via its E3 ubiquitin ligase activity [12]. Importantly, pVHL's tumor suppressor function is dependent upon the downregulation of HIFα subunits and in particular HIF2α [20-23].

Stabilization of HIFα, either as a consequence of hypoxia or pVHL inactivation leads to transcriptional activation of numerous genes associated with tumor progression and tumor adaptation to a hypoxic tumor microenvironment [118] and HIFα has been shown to play important roles in invasion and metastases in genetically engineered murine models [136-138]. Currently, the drugs approved by the FDA for treating advanced RCC target signaling networks activated upon disruption of VHL (i.e. HIFα activation) [118]. Specifically, small-molecule tyrosine kinase inhibitors (e.g. sunitinib and pazopanib) have been established to inhibit the vascular endothelial growth factor receptor (VEGFR) and platelet derived growth factor receptor (PDGFR) [118].

Additionally, temsirolimus and everolimus, derivatives of rapamycin, have been approved to treat advanced RCC [89]. Unfortunately, while mTOR inhibitors clearly prolong overall survival of patients with advanced RCC, actual tumor responses are limited [91,98]. Regardless of the selected therapeutic regimen, a high frequency of patients become refractory to therapy and eventually succumb to progression of disease, indicating potential compensatory survival and proliferative mechanisms.

Rapamycin and its derivatives are allosteric inhibitors of the serine/threonine kinase, mechanistic target of rapamycin (mTOR) [35,89]. mTOR exists within two distinct complexes, mTORC1 and mTORC2. Several proteins are common to both mTORC1 and mTORC2, such as DEP domain containing mTOR-interacting protein (DEPTOR), mammalian lethal with sec-13 protein 8 (mLST8), and tti1/tel2 complex. However, mTORC1 and mTORC2 differ in composition by several proteins. Regulatory-associated protein of mammalian target of rapamycin (Raptor) and proline-rich AKT substrate 40 KDa (PRAS40) are distinct to the mTORC1 signaling complex while rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated map kinase-interacting protein1 (mSin1), and protein observed with Rictor 1 and 2 (protor1/2) are associated with mTORC2 [36]. Notably, the mTORC2 complex and distinct signaling nodes of mTORC1 are thought to be relatively insensitive to rapamycin [38]. The inability of rapamycin and its derivatives to inhibit all signaling nodes of mTOR has resulted in development of catalytic mTOR inhibitors capable of attenuating mTOR's kinase activity resulting in perturbation all signaling nodes of mTORC1 and mTORC2 complexes [90].

mTOR serves as a key negative regulator of autophagy, an evolutionarily conserved physiological process utilized by cells to recycle damaged protein aggregates and faulty organelles [139,140], but also implemented in response to a variety of cellular stresses (low nutrient availability, low energy, and hypoxia) [47,141]. mTOR negatively regulates autophagy induction through mTORC1's ability to phosphorylate autophagy regulating protein 1 (ULK1) [48,49,107]. RCC cells have been stated to have high levels of autophagy [109]. While it appears that enhanced autophagy can function to promote cell survival in established tumors, there is also evidence that cancer cells can undergo autophagic cell death when autophagic flux is dysregulated [62,68,142,143]. Therefore, autophagy's role in tumor progression is complex and likely context dependent.

Here, we investigate, through both pharmacologic and genetic approaches, the effect of allosteric and catalytic mTOR inhibitors in RCC. We show evidence that catalytic mTOR inhibitors may induce autophagy greater than allosteric mTOR inhibitors. Furthermore, we show that attenuation of this compensatory survival signal may increase therapeutic efficacy in RCC through attenuation of cellular proliferation and augmentation of cell death signals.

3.3   Results

3.3.1   Catalytic mTOR inhibition induces autophagic flux greater than allosteric mTOR inhibition in RCC

Several groups have shown that either allosteric or catalytic mTOR inhibition increase autophagic flux of cancer cells [93,103,104,109,110,144]. We wished to investigate whether allosteric and catalytic mTOR inhibition have differing effects on autophagy in the context of RCC. Autophagy induction results in lipidation of LC3B-I

through the addition of a phosphatidylethanolamine (PE) moiety generating LC3B-II.

Therefore, processing of LC3B-I to LC3B-II serves as a marker for early

autophagosome to mature autolysosome formation [145]. We assessed the processing

of LC3B-I to LC3B-II in RCC cell lines when treated with doses of allosteric (rapamycin)

or catalytic (BEZ235 and AZD8055) mTOR inhibitors shown to inhibit mTOR signaling

(Supplemental Figure 3.1). To determine if Catalytic mTOR inhibition with AZD8055 or

BEZ235 increased flux of autophagy relative to rapamycin treated cells we

immunoblotted for LC3B-II (Figure 3.1A and Supplemental Figure 3.2). To establish

whether this increase in LC3B-II accumulation was secondary to increased

autophagosome formation (so called "autophagic flux") or reduced autophagosome

degradation, we assessed the accumulation of LC3B-II in cells treated with AZD8055

and chloroquine (CQ) or BEZ235 and CQ or relative to cells treated with rapamycin and

CQ, suggesting that catalytic mTOR inhibition more robustly increases autophagy

(Figure 3.1A and Supplemental Figure 3.2) [70,145]. Treatment with both AZD8055 and

CQ or BEZ235 and CQ increased the LC3B-II to LC3B-I ratio relative to cells treated

with rapamycin and CQ. Therefore, catalytic mTOR inhibition increases autophagic flux

to a greater degree than allosteric mTOR inhibition.

To assess the effects of rapamycin and BEZ235 on autophagic flux through an

orthogonal assay we generated 786-0 RCC cells stably expressing a tandem mCherry-

GFP-LC3 autophagy reporter, which emits dual mCherry/GFP fluorescence in early

autophagosomes and because of pH-dependent quenching of GFP fluorescence emits

mCherry only in mature autolysosomes. The mCherry-GFP tandem reporter is

therefore a faithful assessment of autophagic flux and also allows the dissection of

differing stages of autophagolysosome maturation. CQ treated 786-0 cells showed

modest induction of autophagosome formation (yellow punctae on merged images)

while rapamycin and in particular BEZ235 treatment led to robust induction of both

autophagosome (yellow punctae) and progression to autolysosome (red / orange

punctae) formation, corroborating that mTOR inhibition and in particular catalytic

inhibition by BEZ235 induces autophagic flux (Figure 3.1B).

Sequestosome 1(SQSTM1), also known as p62, is a protein capable of binding

to LC3 and can serve as selective substrate capable of directing long-lived aggregated

proteins marked with ubiquitin polymers targeting them for autophagic degradation.

Hence, decreases in p62 protein expression can serve as a marker of functional

autophagic flux [47,146]. In conjunction with the above findings; when RCC cells were

treated with rapamycin or BEZ235 we observed decreases in p62 expression (Figure

3.1C). These data in aggregate further support that allosteric and catalytic mTOR

inhibition induce autophagy

mTORC1 signaling is well documented to negatively regulate autophagic flux via

its phosphorylation of ULK1 at Ser757 [48,49] and rapamycin has been shown to

decrease $pULK1_{S757}$ in primary mouse embryonic fibroblasts (MEFs) [48]. In keeping

with this notion, we observed a decrease in $pULK1_{S757}$ in RCC cells treated with

BEZ235 or AZD8055 (Figure 3.1D). In contrast and unexpectedly, we saw no change in

$pULK1_{S757}$ in two cell lines (RCC10 and UNC-R1 cells) and interestingly an increase in

two other cell lines (786-0 and UNC-R2) when they were treated with rapamycin (Figure

3.1D). Similar findings in $pULK1_{S757}$ have been seen in transformed cancer cells treated

with rapamycin [107,112] raising the possibility that rapamycin dependent autophagy

induction is regulated through ULK1 in primary cells but through an alternative

mechanism in cancer cells or that mTORC1's regulation of $pULK1_{S757}$ may be less

sensitive to allosteric mTOR inhibition than catalytic mTOR inhibition, similar to the

differences seen in the inhibition of S6K and 4E-BP1 phosphorylation.
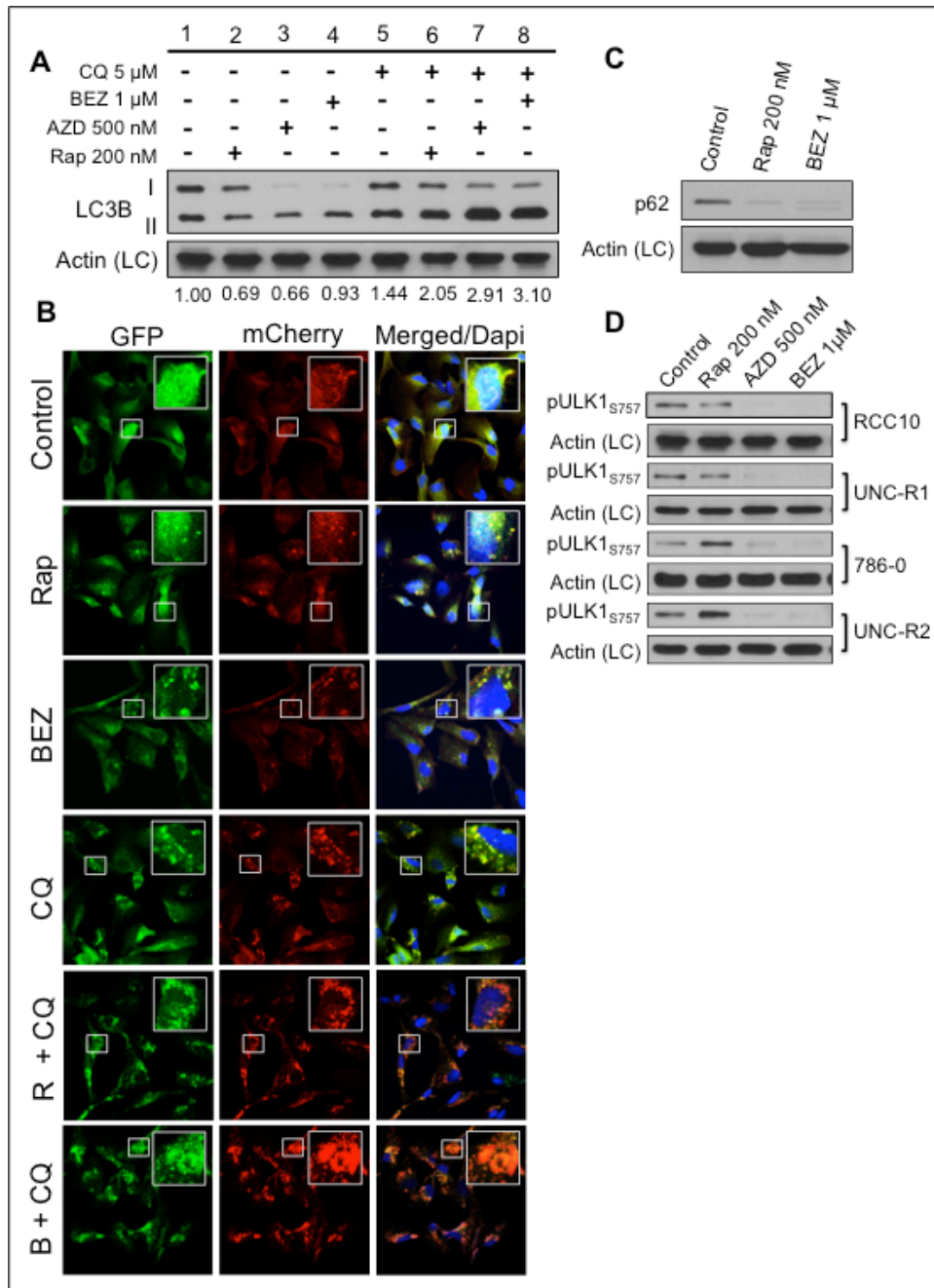
Figure 3.1: Catalytic mTOR inhibition induces autophagic flux greater than allosteric mTOR inhibition. (A) RCC10 cells were treated with Rapamycin, BEZ235,

AZD8055 and CQ for 24 hrs and whole cell lysates were immunoblotted for mature autophagosome marker, LC3B-II (quantification represents LC3B-II expression normalized to Actin as a ratio of LC3B-II/Actin respective to control). **(B)** 786-0 RCC cells transfected with mCh.GFP.LC3B were treated for 24 hrs. with 200 nM rapamycin, 1 μM BEZ235, and 20 μM chloroquine and subjected to immunofluorescence analysis for LC3B processing evident by puncta formation (orange represents increased mature autolysosomes) **(C)** RCC cells were treated with Rapamycin, AZD8055, or BEZ235 for 24 hrs. and analyzed by western blot for $pULK1_{S757}$ (expression of $pULK1_{S757}$ is a marker for mTOR regulated inhibition of autophagy induction). **(D)** UNC-R2 RCC cells were treated with rapamycin, BEZ235, and CQ for 24 hrs and immmunobloted for p62 protein expression (decreases in p62 correlates with increased autophagy). (quantification represents p62 expression normalized to Actin as a ratio of p62/Actin respective to control)

3.3.2   Both mTORC1 and mTORC2 restrain autophagic flux

The more prominent induction of autophagic flux seen by BEZ235 treatment, relative to rapamycin treatment could either be because of BEZ235's ability to better inhibit mTORC1 signaling or because of its ability to inhibit mTORC2. To dissect the specific contributions of TORC1 and TORC2 on regulation of autophagy we stably knocked-down essential components, Raptor and Rictor, for mTORC1 and mTORC2 signaling, respectively (Figure 3.2A and Supplemental Figure 3.3A). As expected, given the well documented role of mTORC1 in negatively regulating autophagy, we noted that stable knock-down of Raptor resulted in increased accumulation of LC3B-II in the presence of CQ relative to shNS CQ treated cells (Figure 3.2B). However, we also observed that knock-down of Rictor in the presence of CQ increased the levels of LC3B-II accumulation relative to CQ treated cells expressing shRNAs to a non-specific sequence. Furthermore, we observed a decrease in p62 protein expression in cells stably expressing shRNA for Raptor and to a lesser extent Rictor (Figure 3.2C). Finally, our data revealed a decrease in phosphorylation of ULK1 at Ser757 in cells expressing shRNA for Raptor and minimally for cells with shRNA for Rictor (Figure 3.2D). These results, in aggregate with our pharmacologic data, suggest that both mTORC1 and

mTORC2 work to restrain autophagy and that the enhanced induction of autophagic flux

seen with catalytic mTOR inhibition may in part may be mediated through its ability to

inhibit mTORC2. Nonetheless, to fully recapitulate catalytic mTOR inhibition we

transfected RCC cells with siRNAs for Rictor, Raptor, mTOR, or Rictor+Raptor and

assessed processing of LC3B-I to LC3B-II. We noted that as before, knock-down of

Raptor or Rictor induced autophagy (Figure 3.2F: lanes 2 and 3). In addition, knock-

down of mTOR or combined knock-down of Raptor and Rictor appeared to more

robustly induce autophagy (lanes 4 and 5) and autophagic flux (lanes 9 and 10) (Figure

3.2F). Together, these data suggest a potential utility not only for mTORC1 but also

mTORC2 in contributing to regulation of autophagic flux and lend insight into why

catalytic mTOR inhibitors augment autophagy more than allosteric mTOR inhibitors.
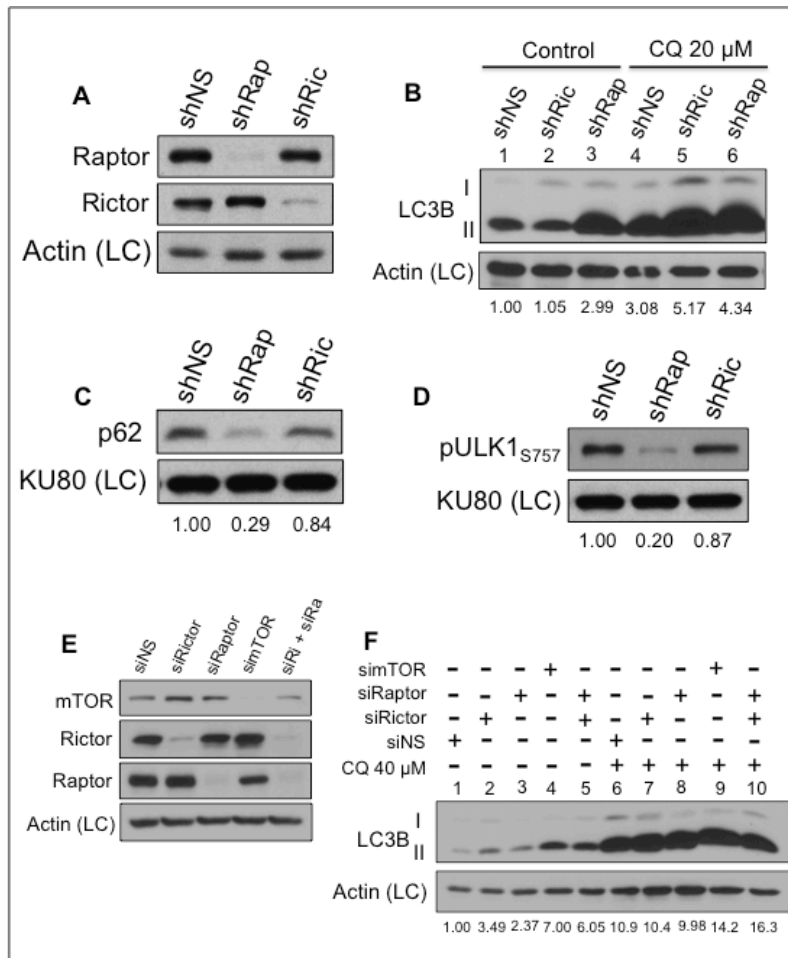
Figure 3.2: Both mTORC1 and mTORC2 regulate autophagy. (A) Knock-down confirmation for 786-0 RCC cell line expressing stable shRNA for Rictor and Raptor. **(B)** Protein expression of mature autophagosome marker, LC3B-II by immunoblot, in cells from Figure 2A treated with late autophagy inhibitor CQ for 24 hrs. **(C)** p62 protein expression by immunoblot for RCC4 RCC cells established from Supplemental Figure 3. (quantification represents p62 expression normalized to Actin as a ratio of p62/Actin respective to control) **(D)** pULK1$_{S757}$ expression by immunoblot for RCC4 cells established in Supplemental Figure 3 (quantification represents pULK1$_{S757}$ expression normalized to Actin as a ratio of pULK1$_{S757}$/Actin respective to control). **(E)** Knock-down confirmation for 786-0 RCC cells transfected with siRNA for Rictor, Raptor, mTOR, or Rictor+Raptor. **(F)** Protein expression of mature autophagosome marker, LC3B-II by immunoblot, in cells from Figure 2E (quantification represents LC3B-II expression normalized to Actin as a ratio of LC3B-II/Actin respective to control). (NS: Non-Specific, Rap: Raptor, Ric: Rictor)

3.3.3 Autophagy inhibition enhances the anti-proliferative effects of mTOR inhibition in RCC

To determine the functional role of increased autophagic flux in the context of

mTOR inhibition, we treated cells with rapamycin and BEZ235 in the presence or

absence of the late autophagy inhibitor, CQ, and assayed their effects on cellular proliferation. Proliferation as assessed by CellTiter-Glo® showed that both allosteric and catalytic mTOR inhibition decreased cellular proliferation and that the effects were further enhanced when combined with late autophagy inhibition (Figure 3.3A). Additionally, bright field images of several RCC cell lines displayed a greater reduction in cell number after 48 hrs. of treatment when comparing combinatorial treatment (BEZ235 + CQ) to either single agent alone (Figure 3.3B). Furthermore, we also saw a significant decrease in the percentage of cells in S-phase, when BEZ235 was combined with CQ in an Edu cell cycle incorporation assay (Figure 3.3C). Finally, to dissect out the individual roles of mTORC1 and mTORC2 in promoting proliferation of RCC cells, we assessed the anti-proliferative effects of Rictor and Raptor shRNA in 786-0 cells and noted a significant decrease in proliferation when combined with CQ (Figure 3.3D) suggesting that both mTORC1 and mTORC2 contribute to the proliferation of RCC cells.
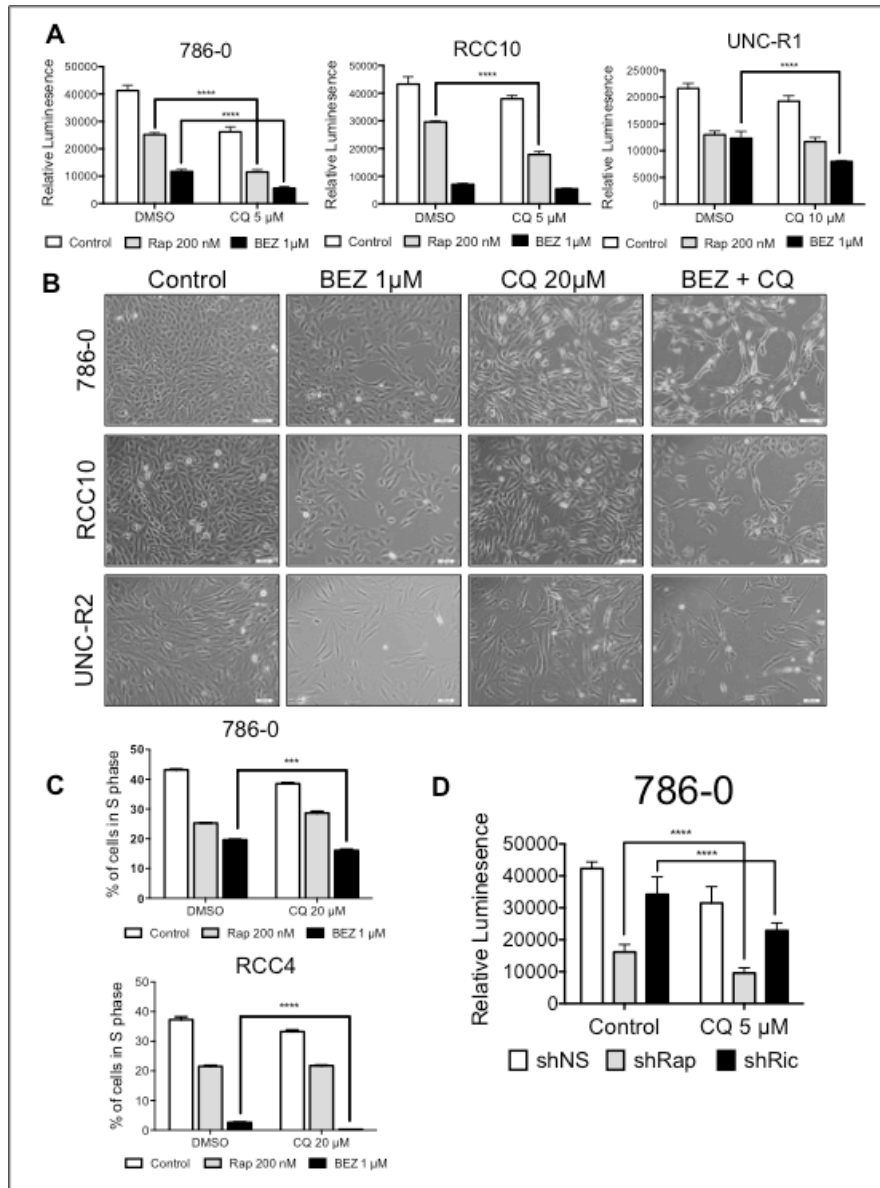
Figure 3.3: Autophagy inhibition enhances the anti-proliferative effects of mTOR inhibition. (A) Day 4 data shown for Cell-titer glo® cellular proliferation assay of RCC cells treated with Rapamycin, BEZ235, and chloroquine. **(B)** Bright field images of RCC cells treated for 48 hrs with the noted treatments. **(C)** RCC cells were treated for 24 hrs, stained for Edu and subjected to flow cytometry for percentage of cells in S-Phase. **(D)** Day 4 data shown for Cell-Titer glo® cellular proliferation assay for RCC cells from Figure 2A.

3.3.4   Autophagy inhibition enhances the apoptotic effects of mTOR inhibition in RCC

We next wanted to determine if the decrease in cell number was solely due to a

decrease in proliferation, but also an increase in cell death. While independent groups

have demonstrated that cell death is enhanced when allosteric [109] or catalytic [93] mTOR inhibition is combined with autophagy inhibition (CQ) their relative efficacy has not been directly compared, in RCC. We observed that autophagy inhibition enhanced the apoptotic effects of allosteric mTOR inhibition as defined by Annexin V+/ PI- cells, but that the effect was more robust when autophagy inhibition was combined with catalytic mTOR inhibition (Figure 3.4A). Moreover, immunoblotting for the apoptotic marker cleaved-caspase 3 supported that the combination of catalytic mTOR inhibition with CQ is better at inducing apoptosis than allosteric mTOR inhibition with CQ (Figure 3.4B).

Finally, To examine the contributions of mTORC1 and mTORC2 to the observed apoptotic response, we examined the effects of RNAi to Rictor, Raptor, mTOR, or Rictor+Raptor in the presence or absence of CQ and observed that RNAi for mTOR or Rictor+Raptor were able to induce cleaved PARP and that the apoptotic response to these siRNAs was greater when combined with CQ (Figure 3.4C). These data support the notion that both mTOR complexes contribute to pro-survival and proliferative signals and that the increased therapeutic effect observed when catalytic mTOR inhibitors are combined with autophagy inhibition may in part be due to mTORC2's regulation of autophagy.
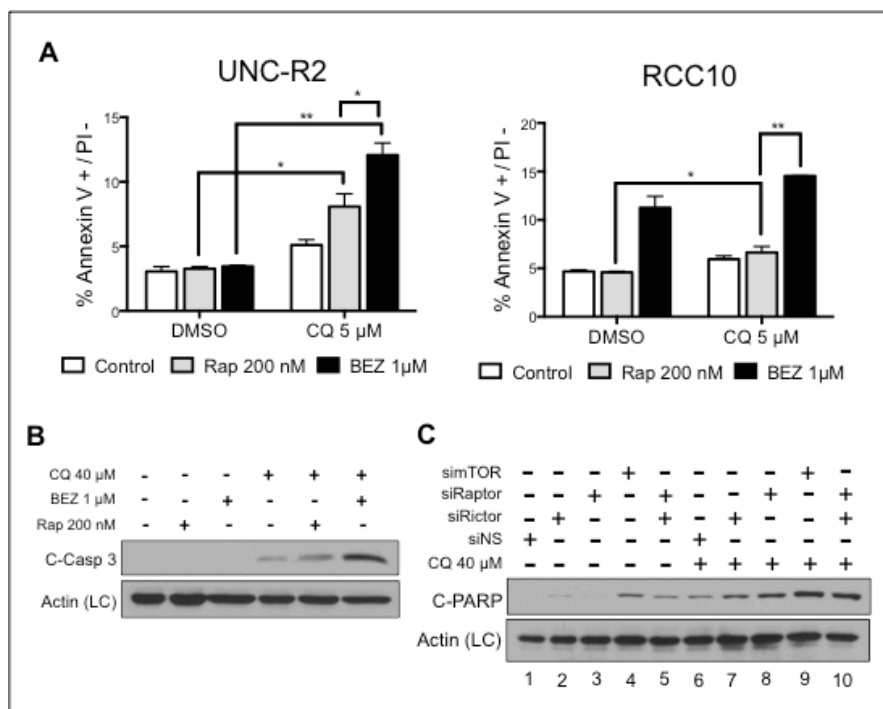
Figure 3.4: Autophagy inhibition increases the apoptotic response in conjunction with mTOR inhibition. (A) RCC cells treated for 48 hrs. and analyzed by flow cytometry to distinguish the apoptotic fraction (Annexin V+/ PI-). **(B)** UNC-R2 RCC cells were treated for 48 hrs. and analyzed by western blot for apoptotic marker, cleaved-caspase-3. **(C)** 786-0 RCC cells from Figure 2E were treated for 48 hrs. with the indicated treatments and analyzed by western blot for apoptotic marker cleaved-PARP.

3.4   Discussion

Several groups [93,109] have examined the effects of combined allosteric or catalytic mTOR inhibition in combination with inhibition of autophagy in the context of RCC. Our studies however directly compare the effects of allosteric and catalytic mTOR inhibition on autophagy and assess their relative efficacy when combined with CQ. We show that mTOR inhibition in conjunction with autophagy inhibition decreases cellular proliferation and increases apoptosis. Additionally, we show that catalytic mTOR inhibition augments the autophagic process more than allosteric mTOR inhibition and offer this as a potential reason as to why catalytic mTOR inhibitors appear to more potently synergize with CQ.

Phosphorylation of ULK1 by AMPK is required for autophagy induction [49]. It is well understood that mTORC1 is a critical negative regulator of autophagy [139] and directly phosphorylates ULK1 at Ser757 [49,140]. mTOR mediated phosphorylation of ULK1 at Ser757 disrupts the necessary interaction between ULK1 and AMPK resulting in inhibition of autophagy induction [48]. Rapamycin would therefore be expected to inhibit $ULK1_{S757}$ phosphorylation [48]. Interestingly, while previous literature has shown that rapamycin inhibits $pULK1_{S757}$ expression in non-transformed MEFs [48], in our hands rapamycin either did not change, or in several cell lines actually increased $ULK1_{S757}$ phosphorylation (Figure 3.1C). In contrast, catalytic mTOR inhibition with BEZ235 or AZD8055 robustly inhibited expression of $pULK1_{S757}$ (Figure 3.1C). Interestingly, similar findings have been observed in other cancer types where allosteric mTOR inhibition did not alter $pULK1_{S757}$ levels in ovarian and hepatocellular cancer, respectiveily [107,112]. The responsiveness of $pULK1_{S757}$ to BEZ235 and AZD8055 but not rapamycin may be due to their superior ability to inhibit mTORC1 kinase activity (as evidenced by their ability to decrease p4E-BP1), their ability to inhibit mTORC2, or off target effects [90]. Nevertheless, our data suggest that at least in transformed RCC cell lines, rapamycin can induce autophagy without clearly decreasing the phosphorylation status of the mTOR responsive residue of ULK1.

It is well understood that mTOR inhibition can negatively impact oxidative respiration and glycolysis [147-149]. However, comparison of mechanistically different mTOR inhibitors (i.e. rapalogs or catalytic mTOR inhibitors) and their effects on oxidative respiration and glycolysis has not been addressed. One possible explanation for the apparent ability of rapamycin to induce autophagy without attenuating mTOR's

phosphorylation of ULK1 is that allosteric mTOR inhibition has a greater affect on energetic stress (ie. decreased levels of ATP) than does catalytic mTOR inhibition. This more profound decrease in ATP might stimulate AMPK activity leading to mTOR independent activation of ULK1 and subsequent autophagy induction [49]. Additional studies assessing energy metabolism and autophagy induction in the context of mechanistically different types of mTOR inhibitors will help in understanding our observed results.

Knocking down essential mTORC1 and mTORC2 signaling components, Rictor and Raptor, respectively, led to increased autophagic flux in conjuction with decreased p62 levels (Figure 3.2C) and decreased $pULK1_{S757}$ expression (Figure 3.2D). This observation is interesting because it supports that mTORC2 may also negatively regulate autophagy (Figure 3.2C). These results may explain in part why catalytic mTOR inhibition may increase autophagic flux greater than allosteric mTOR inhibition. Nonetheless, the observed decrease in $pULK1_{S757}$ and p62 expression by catalytic mTOR inhibition correlates with the increase in LC3B-II expression observed in response to BEZ235 or AZD8055 relative to rapamycin.

In summary, our studies suggest a model that catalytic mTOR inhibition increases autophagic flux more than allosteric mTOR inhibition because of mTORC2's ability to restrain autophagic flux. Additionally, this compensatory activation may function as a pro-survival and pro-proliferative signal limiting the efficacy of various classes of mTOR inhibition. Herein, we demonstrate that combining mTOR inhibition and autophagy inhibition decreases cellular proliferation and increases cell death. Moreover, our data suggests that catalytic mTOR inhibition should be investigated in

RCC and that the compensatory upregulation of autophagy may actually be a potential synthetic vulnerability.

3.5   Materials and Methods

Cell lines and culture conditions

RCC10, 786-0, RCC4, UNC-R1, UNC-R2 were cultured in complete DMEM (CORNING-Cellgro #10-013-CV) supplemented with 10%FBS, 1X Penn/Strep at 37°C, 5% CO_2, 21% O_2. 786-0 cells were obtained from ATCC and RCC4 cells were a gift of Dr. Kimryn Rathmell [11] and RCC10 cells were obtained from Dr. Michael Ohh [135]. UNC-R1 and UNC-R2 cell lines were generated as described in (Chapter 2: Bailey, PloS One "in press").   AZD8055 (Selleckchem), BEZ235 (Center for Integrative Chemical Biology & Drug Discovery, UNC Eshelman School of Pharmacy), Rapamycin (LC Laboratories) were dissolved in DMSO. Chloroquine (Sigma) was dissolved into sterile PBS.

Immunoblot conditions

Cells were lysed in RIPA buffer complemented with Set I and Set II phosphatase inhibitors at 1X (Calbiochem), and protease inhibitors at 1X (Roche). Whole cell lysate concentration was determined with Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad). Proteins were resolved on SDS-PAGE gels and electrotransferred to nitrocellulose membranes, 0.2 μm (Bio-Rad) were used to transfer protein from SDS-PAGE gel. Primary antibodies $pS6_{S235/236}$, $pAKT_{S473}$, $pAkt_{T308}$, $p4E\text{-}BP1_{T37/46}$, Cleaved PARP, LC3B, $pULK1_{S757}$, p62, Rictor, Raptor, cleaved-caspase3 were from Cell Signaling Technologies®. KU-80 (GeneTex #GTX70485) and Actin-HRP (Santa Cruz

#C-11) primary antibodies served as loading controls (LC) where noted. Secondary anti-Rabbit and anti-mouse antibodies were from (Fisher) and diluted in 5% milk, 1X TBS-T solution. ECL Western Blotting Detection reagents (GE Healthcare) were used for developing blots onto autoradiography film. For difficult to detect proteins SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used in combination with ECL.

Quantification of immunoblots

Quantification of protein expression was analyzed with ImageJ software. All expressed values were normalized to loading controls. Fold change was expressed as a ratio of the treatment condition divided by the control condition (DMSO or shNS were noted).

Measuring autophagosomes by confocal microscopy

The mCherry-GFP-LC3B cDNA was PCR'd from Addgene #22418 plasmid using primers containing EcoRI and BamHI sites. The PCR product was TOPO cloned into pCR®-Blunt II-TOPO (Invitrogen). The EcoRI and BamHI product was then cut and put into pCDH EF1 IRES puro into the EcoRI and BamHI sites. RCC cells were stably transfected with this lentivirial vector and selected with 1 μg/mL puromycin. Stably transfected RCC cells were treated for 24 hrs. then fixed with 4% PFA. Cells were then stained with Phalloidin 647: #A22287 from life technologies 1:40 dilution of stock, DAPI: Invitrogen molecular probes #D3571 1:10,000 of 10mg/ml stock for 20 min at room temperature and mounted with fluorosave (Millpore #345789). Cells were

imaged using the Zeiss 710 confocal microscope at the Microscopy Services Laboratory at UNC-CH.

Cell viability assay

To determine cell viability in the context of the various culture conditions we used a CellTiter-Glo® Luminescent Cell Viability Assay (Promega) per manufacture's protocol. Cells were counted and plated in a 96 well opaque side/clear bottom cell culture plates (Corning) in culture medium containing the noted concentration. Measurements were taken using a Biotek® Synergy 2 plate reader. 2-way ANOVA analysis was used to determine statistical significance.

Cell cycle analysis by flow cytometry

Cells were plated in triplicate and treated for 24 hrs with indicated drug. Cell cycle analysis was performed as in [150].

Apoptosis analysis by flow cytometry

Cells were plated in duplicate and treated with the indicated drug for 48 hrs. Percentage of apoptotic cells were determined by staining with Annexin V Alexa Fluor® 488 & PI (Dead Cell Apoptosis Kit, Invitrogen, cat# V13241) according to the manufacturer's instructions. Flow was performed on a Dako CyAn ADP and data were analyzed using FlowJo software. Statistical significance was measured by Student T-Test.

RNAi experiments

siNS (#4390844) simTOR (#4390824 – ID:s603) siRictor (#4392420 – ID:s226001),

Raptor (#4392420 – ID:s33215) were all purchased from Ambion®, and RCC cells

were transfected with X-tremeGENE 9 DNA Transfection Reagent (#06365787001)

from Roche as per manufactures protocol. pLKO.1 shRNA plasmids were obtained

form the UNC Viral Vector Core, packaged and infected per manufacture's protocols.

Addgene catalogue numbers: shNS (#1864), shRictor (#1853), shRaptor (#1857),

shmTOR (#1853). Cells were incubated with viral media over-night, and replaced with

fresh complete media. Selection with 1 μg/mL puromycin was started 48 hrs later.

3.6   Supplemental Figures
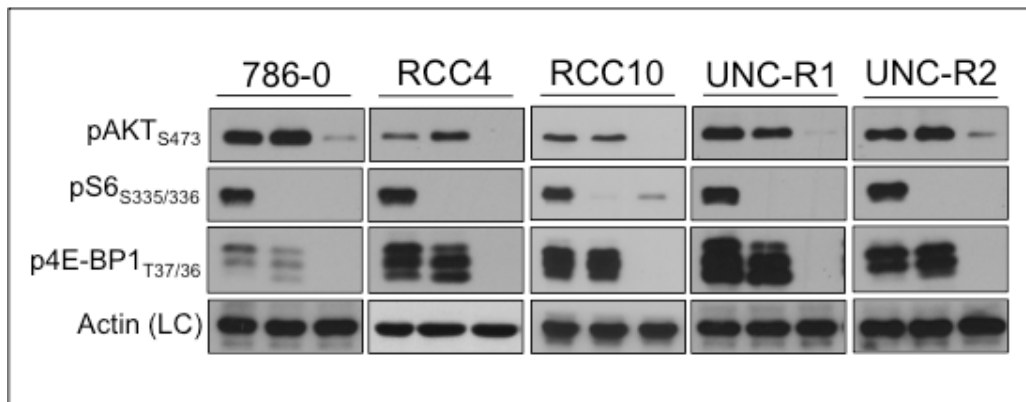
Supplemental Figure 3.1: RCC cells were treated for 24 hrs with DMSO (control), rapamycin 200 nM, BEZ235 1 μM, AZD8055 500 nM and whole cell lysate was immunblotted for mTORC1 and mTORC2 signaling inhibition

	1	2	3	4	5	6	7	8
CQ 5 µM	-	-	-	-	+	+	+	+
BEZ 1 µM	-	-	-	+	-	-	-	+
AZD 500 nM	-	-	+	-	-	-	+	-
Rap 200 nM	-	+	-	-	-	+	-	-

LC3B I / II

Actin (LC)

1.00 2.19 2.35 1.93 4.35 4.62 4.55 5.38
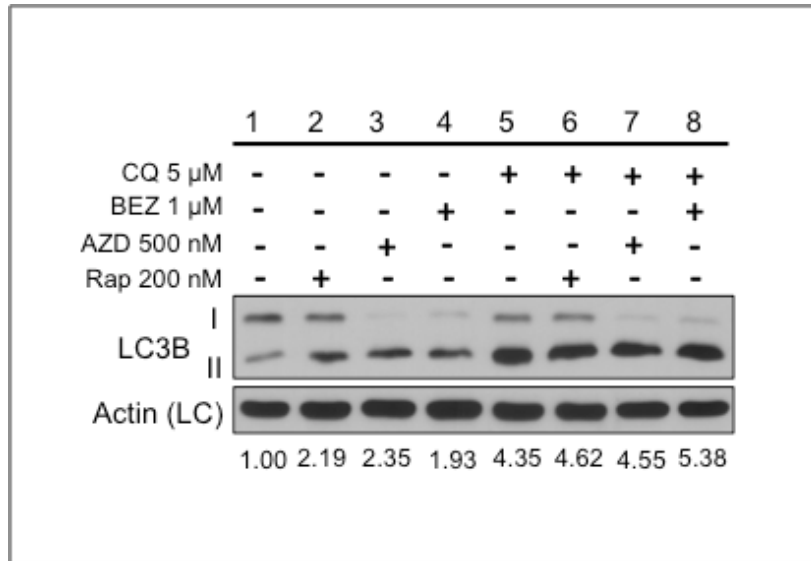
Supplemental Figure 3.2: UNC-R2 RCC cells were treated for 24 hrs. with the indicated drugs and whole cell lysate was immunoblotted for LC3B-II protein expression (quantification represents LC3B-II expression normalized to Actin as a ratio of LC3B-II/Actin respective to control).
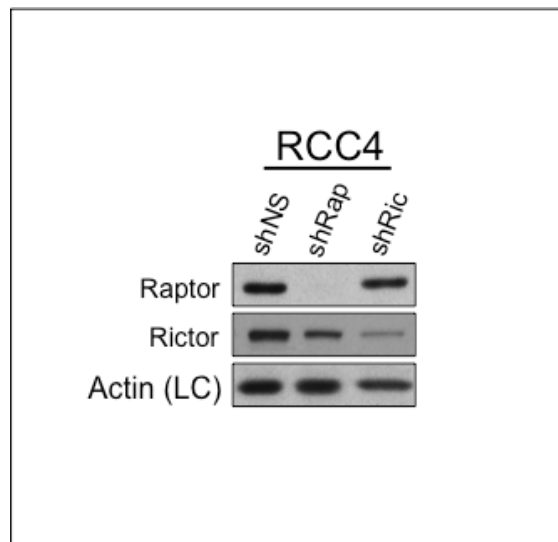
Supplemental Figure 3.3: Confirmed KD for RCC4 cells expressing stable shRNA for Rictor, Raptor, or NS (Non-Specific).

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Compensatory pathway activation and potential molecular mechanisms induced by mTOR inhibition in RCC

In the context of mTOR inhibition we observed compensatory activation of both MEK/ERK and autophagy signaling pathways. Activation of these pathways supported the notion that combinatorial-targeted inhibition may increase efficacy of mTOR inhibition. Not only did we show that combining mTOR inhibition with either MEK or autophagy inhibition could increase efficacy over mTOR inhibition alone; we additionally showed that allosteric and catalytic mTOR inhibition had varying effects on activation of these pathways.

Our observation of increased MEK activation in the context of mTOR inhibition has been observed in other cancer types [102,105,116,122,123,131,151,152]. Early studies by Carrecedo et al. demonstrated that rapamycin inhibition lead to compensatory activation of MEK/ERK signaling, through a S6K/PI3K-dependent mechanism [101]. However, emphasis on additional mTORC1 signaling node regulating phosphorylation of 4E-BP1 was not investigated by Carrecedo et al.

Several groups have observed increases in MEK/ERK signaling in the context of catalytic mTOR inhibitors [102,105,106]. Inhibition of 4E-BP1 phosphorylation by catalytic mTOR inhibitors serves as a potential mechanism for compensatory MEK/ERK signaling by catalytic inhibition of mTOR [105]. Notably allosteric mTOR inhibitors do not

affect 4E-BP1 phosphorylation. We have presented RNAi data supporting that inhibition of mTORC1, through knock-down of Raptor is capable of increasing MEK signaling as shown by increases in phosphorylation of ERK and downstream effector, p90RSK (data not shown).

In our hands, we have observed that mTOR inhibitors increase phosphorylation of ERK effector and downstream effector p90RSK. p90RSK is an AGC kinase that is capable of phosphorylating BCL2-associated agonist of cell death (BAD). Hyperphosphorylated BAD is recognized by 14-3-3 proteins resulting in sequestration of BAD in the cytosol and away from pro-survival BCL2 family proteins. Treatment of lung cancer cells with rapamycin has been shown to induce phosphorylation of ERK and BAD and this phosphorylation serves as a resistance mechanism to rapamycin treatment [116]. Hypophosphorylated BAD functions as a pro-apoptotic protein through disruption of pro-survival BCL2 family proteins with BAX and BAK [115]. This disruption results in BAX and BAK being able to localize to the mitochondria to induce an apoptotic cascade.

In RCC cells, we have demonstrated that mTOR inhibition induces compensatory, therapeutically targetable activation of MEK/ERK signaling. Combining MEK inhibitor, GSK212, with rapamycin or BEZ235 increases therapeutic efficacy through attenuated cellular proliferation and increases in an apoptotic response. We have preliminary data supporting that the increase in apoptosis may be through GSK212's ability to decrease phosphorylation of BAD at ser 112 (data not shown). Unfortunately, BAD is a substrate of other AGC kinases. Specifically, AKT and S6K are both capable of contributing to the hypersphorylated state of BAD, through

phosphorylation of BAD at ser 136 [115]. It is unclear the exact phosphorylation status of BAD that is required for its role in regulating apoptosis, and would be an area of needed investigation in deciphering a more clear mechanism behind the efficacy observed with mTOR and MEK inhibition in RCC.

Pharmacologically, we observed that treatment with catalytic mTOR inhibition increased autophagic flux greater than allosteric mTOR inhibition. While this finding may be a result of more potent inhibition of mTORC1 activity by catalytic mTOR inhibition we also investigated whether catalytic mTOR inhibition more robustly induced autophagy secondary to its ability to inhibit mTORC2. Specifically, we demonstrated genetically that mTORC2 plays a direct role in regulating autophagy. Nonetheless, a detailed mechanism as to how catalytic mTOR inhibition causes increased autophagic flux greater than allosteric mTOR inhibition needs to be investigated. For example, mTORC2's regulation/phosphorylation of essential autophagy inducing genes (ULK1) may be a plausible component within this proposed mechanism.

Autophagy and proteosomal degradation are tightly linked degradative processes for post-translational regulation of proteins [153]. HIF2α levels are primarily regulated in a pVHL and proteasome dependent manner. More recently, HIF2α has been shown to be targeted for autophagic degradation but only in the context of intact pVHL [154]. However, while Liu et al. show data indicating that autophagy may have a tumor suppressive role in ccRCC, they do not show a direct relation of autophagy promoting ccRCC tumorogenesis in RCC cells lacking functional *VHL*. Furthermore, our data indicates the increased autophagy observed with mTOR inhibition is targetable and increases the efficacy observed with various types of mTOR inhibitors.

It is well understood that mitochondria contribute to ROS production and the number of mitochondria and levels of reactive oxygen species (ROS) is tightly regulated by the cell [155]. However, damaged mitochondria or increases in mitochondrial number can increase ROS production resulting in increases in autophagy [156]. The increase in autophagy is thought to eliminate damaged mitochondria and possibly oxidized proteins to help reduce ROS levels in effort to regulate cellular homeostasis. Interestingly, in RCC cells, it has been shown that allosteric mTOR inhibitor, CCI-779 is capable of increasing ROS and that treatment with chloroquine further augments ROS accumulation [109]. Bray and colleagues also demonstrated that the combination of CCI-779 with chloroquine increased cytotoxicity which is in part due to the increase in ROS species and a cell death program involving necroptosis [109]. The effect of ROS production by catalytic mTOR inhibition has not been thoroughly investigated in RCC, however we have observed an increase in heme oxygenase decycling1 (HMOX-1), an enzyme that correlates with increases in oxidative stress [157], also known as HO-1 in RCC cells treated with BEZ235 (data not shown). We also noticed further increases in HO-1 expression when BEZ235 was combined with chloroquine (data not shown) supporting the observation by Bray et al. that combining autophagy inhibition in combination with mTOR inhibition increases ROS levels. While Bray et al. did not observe induction of apoptosis or autophagic cell death as the cell death program induced by combined mTOR and autophagy inhibition, others have described compounds that increase autophagy in VHL null RCC cell lines leading to autophagic cell death [143]. Moreover, when combining BEZ235 and CQ we observed increases in

an apoptotic response, but it is important to note that we did not investigate necroptosis as a potential mechanism of cell death in our context.

Most importantly, autophagic, necroptotic, and apoptotic programmed cell death may be interlinked [64,158,159], and most likely share molecular pathways resulting in troublesome determination of which pathway oversees cell death as the final outcome. Therefore, it is important to understand, in which cellular context, which cell death pathway(s) are capable of being activated with the selected therapeutic combination.

In summary, the data presented shows genetic and pharmacological evidence for compensatory survival and proliferation pathway activation by mTOR inhibition. Specifically, we addressed AKT, MEK/ERK, and autophagy pathway activation as targetable compensatory pathways in the context various classes of mTOR inhibitors. We demonstrated that co-targeting mTOR and the noted compensatory pathways yielded increased efficacy at inhibiting cellular proliferation and/or augmenting apoptosis. Together, recent KIRC_TCGA RPPA data and our new understanding of the molecular and functional consequences of various types of mTOR inhibition in treating advanced ccRCC will help aid clinicians in stratifying patients who may benefit best due to potential oncogenic pathways addiction.

REFERENCES

1.   Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA Cancer J Clin 63: 11–30. doi:10.3322/caac.21166.

2.   Linehan WM, Walther MM, Zbar B (2003) The genetic basis of cancer of the kidney. J Urol 170: 2163–2172. doi:10.1097/01.ju.0000096060.92397.ed.

3.   Jonasch E, Futreal PA, Davis IJ, Bailey ST, Kim WY, et al. (2012) State of the Science: An Update on Renal Cell Carcinoma. Molecular Cancer Research. doi:10.1158/1541-7786.MCR-12-0117.

4.   Ericsson JL, Seljelid R, Orrenius S (1966) Comparative light and electron microscopic observations of the cytoplasmic matrix in renal carcinomas. Virchows Arch Pathol Anat Physiol Klin Med 341: 204–223.

5.   Sundelin JP, Ståhlman M, Lundqvist A, Levin M, Parini P, et al. (2012) Increased Expression of the Very Low-Density Lipoprotein Receptor Mediates Lipid Accumulation in Clear-Cell Renal Cell Carcinoma. PLoS ONE 7: e48694. doi:10.1371/journal.pone.0048694.s004.

6.   Kim WY, Kaelin WG (2004) Role of VHL gene mutation in human cancer. J Clin Oncol 22: 4991–5004. doi:10.1200/JCO.2004.05.061.

7.   Kaelin WG (2004) The Von Hippel-Lindau Tumor Suppressor Gene and Kidney Cancer. Clinical Cancer Research 10: 6290S–6295S. doi:10.1158/1078-0432.CCR-sup-040025.

8.   Cohen HT, McGovern FJ (2005) Renal-cell carcinoma. N Engl J Med 353: 2477–2490. doi:10.1056/NEJMra043172.

9.   Linehan WM, Srinivasan R, Schmidt LS (2010) The genetic basis of kidney cancer: a metabolic disease. Nature Publishing Group 7: 277–285. doi:10.1038/nrurol.2010.47.

10.   Richards FM, Schofield PN, Fleming S, Maher ER (1996) Expression of the von Hippel-Lindau disease tumour suppressor gene during human embryogenesis. Human Molecular Genetics 5: 639–644.

11.   Maxwell PH (1999) The Tumour Suppressor Protein VHL Targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature: 1–5.

12.   Kaelin WG (2008) The von Hippel-Lindau tumour suppressor protein: O2

sensing and cancer. Nat Rev Cancer 8: 865–873. doi:10.1038/nrc2502.

13. Kaelin WG, Ratcliffe PJ (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Molecular Cell 30: 393–402. doi:10.1016/j.molcel.2008.04.009.

14. Jaakkola P, Mole DR, Tian Y-M, Wilson MI, Gielbert J, et al. (2001) Targeting of HIF-alpha to the von Hippel-Lindau Ubiquitylation Complex by O2-Regulated Prolyl Hydroxylation. Science 292: 468–472. doi:10.1126/science.1059796.

15. Semenza GL (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3: 721–732. doi:10.1038/nrc1187.

16. Gordan JD, Simon MC (2007) Hypoxia-inducible factors: central regulators of the tumor phenotype. Curr Opin Genet Dev 17: 71–77. doi:10.1016/j.gde.2006.12.006.

17. Semenza GL (2010) HIF-1: upstream and downstream of cancer metabolism. Curr Opin Genet Dev 20: 51–56. doi:10.1016/j.gde.2009.10.009.

18. Raval RR, Lau KW, Tran MGB, Sowter HM, Mandriota SJ, et al. (2005) Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol Cell Biol 25: 5675–5686. doi:10.1128/MCB.25.13.5675-5686.2005.

19. Cheong H, Lu C, Lindsten T, Thompson CB (2012) Therapeutic targets in cancer cell metabolism and autophagy. Nat Biotechnol: 1–8. doi:10.1038/nbt.2285.

20. Kondo K, Kim WY, Lechpammer M, Kaelin WG (2003) Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. PLoS Biol 1: E83. doi:10.1371/journal.pbio.0000083.

21. Kondo K, Klco J, Nakamura E, Lechpammer M, Kaelin WG (2002) Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein. Cancer Cell 1: 237–246.

22. Zimmer M, Doucette D, Siddiqui N, Iliopoulos O (2004) Inhibition of hypoxia-inducible factor is sufficient for growth suppression of VHL-/- tumors. Mol Cancer Res 2: 89–95.

23. Maranchie JK, Vasselli JR, Riss J, Bonifacino JS, Linehan WM, et al. (2002) The contribution of VHL substrate binding and HIF1-alpha to the phenotype of VHL loss in renal cell carcinoma. Cancer Cell 1: 247–255.

24. Dalgliesh GL, Furge K, Greenman C, Chen L, Bignell G, et al. (2010) Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. Nature 463: 360–363. doi:10.1038/nature08672.

25. Shen C, Beroukhim R, Schumacher SE, Zhou J, Chang M, et al. (2011) Genetic and Functional Studies Implicate HIF1 as a 14q Kidney Cancer Suppressor Gene. Cancer Discovery 1: 222–235. doi:10.1158/2159-8290.CD-11-0098.

26. Gordan JD, Lal P, Dondeti VR, Letrero R, Parekh KN, et al. (2008) HIF-α Effects on c-Myc Distinguish Two Subtypes of Sporadic VHL-Deficient Clear Cell Renal Carcinoma. Cancer Cell 14: 435–446. doi:doi:10.1016/j.ccr.2008.10.016.

27. Biswas S, Troy H, Leek R, Chung Y-L, Li J-L, et al. (2010) Effects of HIF-1α and HIF2α on Growth and Metabolism of Clear-Cell Renal Cell Carcinoma 786-0 Xenografts. Journal of Oncology 2010: 1–14. doi:10.1155/2010/757908.

28. Gordan JD, Bertout JA, Hu C-J, Diehl JA, Simon MC (2007) HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. Cancer Cell 11: 335–347. doi:10.1016/j.ccr.2007.02.006.

29. Gordan JD, Thompson CB, Simon MC (2007) HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. Cancer Cell 12: 108–113. doi:10.1016/j.ccr.2007.07.006.

30. Beroukhim R, Brunet J-P, Di Napoli A, Mertz KD, Seeley A, et al. (2009) Patterns of Gene Expression and Copy-Number Alterations in von-Hippel Lindau Disease-Associated and Sporadic Clear Cell Carcinoma of the Kidney. Cancer Res 69: 4674–4681. doi:10.1158/0008-5472.CAN-09-0146.

31. Creighton CJ, Morgan M, Gunaratne PH, Wheeler DA, Gibbs RA, et al. (2013) Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 499: 43–49. doi:10.1038/nature12222.

32. Janowitz T, Welsh SJ, Zaki K, Mulders P, Eisen T (2013) Adjuvant Therapy in Renal Cell Carcinoma—Past, Present, and Future. Semin Oncol 40: 482–491. doi:10.1053/j.seminoncol.2013.05.004.

33. Dutcher JP (2013) Recent developments in the treatment of renal cell carcinoma. Ther Adv Urol 5: 338–353. doi:10.1177/1756287213505672.

34. Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH (1994) RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell 78: 35–43.

35. Yang H, Rudge DG, Koos JD, Vaidialingam B, Yang HJ, et al. (2014) mTOR kinase structure, mechanismand regulation. Nature 497: 217–223. doi:10.1038/nature12122.

36. Laplante M, Sabatini DM (2012) mTOR Signaling in Growth Control and Disease. Cell 149: 274–293. doi:10.1016/j.cell.2012.03.017.

37. Hara K, Maruki Y, Long X, Yoshino K-I, Oshiro N, et al. (2002) Raptor, a binding

partner of target of rapamycin (TOR), mediates TOR action. Cell 110: 177–189.

38. Sarbassov DD, Ali SM, Kim D-H, Guertin DA, Latek RR, et al. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol 14: 1296–1302. doi:10.1016/j.cub.2004.06.054.

39. Sabatini DM (2006) mTOR and cancer: insights into a complex relationship. Nat Rev Cancer 6: 729–734. doi:10.1038/nrc1974.

40. Jones AC, Shyamsundar MM, Thomas MW, Maynard J, Idziaszczyk S, et al. (1999) Comprehensive mutation analysis of TSC1 and TSC2-and phenotypic correlations in 150 families with tuberous sclerosis. Am J Hum Genet 64: 1305–1315. doi:10.1086/302381.

41. Sengupta S, Peterson TR, Sabatini DM (2010) Regulation of the mTOR Complex 1 Pathway by Nutrients, Growth Factors, and Stress. Molecular Cell 40: 310–322. doi:10.1016/j.molcel.2010.09.026.

42. Smith EM, Finn SG, Tee AR, Browne GJ, Proud CG (2005) The Tuberous Sclerosis Protein TSC2 Is Not Required for the Regulation of the Mammalian Target of Rapamycin by Amino Acids and Certain Cellular Stresses. Journal of Biological Chemistry 280: 18717–18727. doi:10.1074/jbc.M414499200.

43. Efeyan A, Sabatini DM (2010) mTOR and cancer: many loops in one pathway. Curr Opin Cell Biol 22: 169–176. doi:10.1016/j.ceb.2009.10.007.

44. Guertin DA, Sabatini DM (2007) Defining the Role of mTOR in Cancer. Cancer Cell 12: 9–22. doi:10.1016/j.ccr.2007.05.008.

45. Haruta T, Uno T, Kawahara J, Takano A, Egawa K, et al. (2000) A rapamycin-sensitive pathway down-regulates insulin signaling via phosphorylation and proteasomal degradation of insulin receptor substrate-1. Mol Endocrinol 14: 783–794.

46. Takano A, Usui I, Haruta T, Kawahara J, Uno T, et al. (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. doi:10.1128/MCB.21.15.5050-5062.2001.

47. Mizushima N (2007) Autophagy: process and function. Genes & Development 21: 2861–2873. doi:10.1101/gad.1599207.

48. Kim J, Kundu M, Viollet B, Guan K-L (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nature Publishing Group 13: 132–141. doi:10.1038/ncb2152.

49.   Egan DF, Shackelford DB, Mihaylova MM, Gelino S, Kohnz RA, et al. (2011) Phosphorylation of ULK1 (hATG1) by AMP-Activated Protein Kinase Connects Energy Sensing to Mitophagy. Science 331: 456–461. doi:10.1126/science.1196371.

50.   Mazure NM, Pouyssã Gur J (2010) Hypoxia-induced autophagy: cell death or cell survival? Curr Opin Cell Biol 22: 177–180. doi:10.1016/j.ceb.2009.11.015.

51.   Meijer AJ, Codogno P (2011) Autophagy: Regulation by Energy Sensing. Curr Biol 21: R227–R229. doi:10.1016/j.cub.2011.02.007.

52.   Rabinowitz JD, White E (2010) Autophagy and Metabolism. Science 330: 1344–1348. doi:10.1126/science.1193497.

53.   Tato I, Bartrons R, Ventura F, Rosa JL (2011) Amino Acids Activate Mammalian Target of Rapamycin Complex 2 (mTORC2) via PI3K/Akt Signaling. Journal of Biological Chemistry 286: 6128–6142. doi:10.1074/jbc.M110.166991.

54.   Pearce LR, Komander D, Alessi DR (2010) The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol 11: 9–22. doi:10.1038/nrm2822.

55.   García-Martínez JM, Alessi DR (2008) mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem J 416: 375. doi:10.1042/BJ20081668.

56.   Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science 307: 1098–1101. doi:10.1126/science.1106148.

57.   Tanaka K, Babic I, Nathanson D, Akhavan D, Guo D, et al. (2011) Oncogenic EGFR Signaling Activates an mTORC2-NF- B Pathway That Promotes Chemotherapy Resistance. Cancer Discovery 1: 524–538. doi:10.1158/2159-8290.CD-11-0124.

58.   Huang J, Wu S, Wu C-L, Manning BD (2009) Signaling events downstream of mammalian target of rapamycin complex 2 are attenuated in cells and tumors deficient for the tuberous sclerosis complex tumor suppressors. Cancer Res 69: 6107–6114. doi:10.1158/0008-5472.CAN-09-0975.

59.   Guertin DA, Stevens DM, Saitoh M, Kinkel S, Crosby K, et al. (2009) mTOR complex 2 is required for the development of prostate cancer induced by Pten loss in mice. Cancer Cell 15: 148–159. doi:10.1016/j.ccr.2008.12.017.

60.   Sarbassov DD, Ali SM, Sengupta S, Sheen J-H, Hsu PP, et al. (2006) Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Molecular Cell 22: 159–168. doi:10.1016/j.molcel.2006.03.029.

61.   Barilli A, Visigalli R, Sala R, Gazzola GC, Parolari A, et al. (2008) In human endothelial cells rapamycin causes mTORC2 inhibition and impairs cell viability and function. Cardiovascular Research 78: 563–571. doi:10.1093/cvr/cvn024.

62.   White EJ, Martin V, Liu J-L, Klein SR, Piya S, et al. (2011) Autophagy regulation in cancer development and therapy. Am J Cancer Res 1: 362–372.

63.   Mathew R, White E (2011) Autophagy in tumorigenesis and energy metabolism: friend by day, foe by night. Curr Opin Genet Dev 21: 113–119. doi:10.1016/j.gde.2010.12.008.

64.   Mathew R, Karantza-Wadsworth V, White E (2007) Role of autophagy in cancer. Nat Rev Cancer 7: 961–967. doi:10.1038/nrc2254.

65.   Levine B, Kroemer G (2008) Autophagy in the Pathogenesis of Disease. Cell 132: 27–42. doi:10.1016/j.cell.2007.12.018.

66.   Blagosklonny MV (2013) Hypoxia, MTOR and autophagy: Converging on senescence or quiescence. Autophagy 9: 14–13. doi:10.4161/auto.22783.

67.   Ganley IG, Lam DH, Wang J, Ding X, Chen S, et al. (2009) ULK1{middle dot}ATG13{middle dot}FIP200 Complex Mediates mTOR Signaling and Is Essential for Autophagy. J Biol Chem 284: 12297–12305. doi:10.1074/jbc.M900573200.

68.   Kimmelman AC (2011) The dynamic nature of autophagy in cancer. Genes & Development 25: 1999–2010. doi:10.1101/gad.17558811.

69.   Tanida I, Ueno T, Kominami E (2008) LC3 and Autophagy. Methods Mol Biol 445: 77–88. doi:10.1007/978-1-59745-157-4_4.

70.   Mizushima N, Yoshimori T (2007) How to interpret LC3 immunoblotting. Autophagy 3: 542–545.

71.   Yang S, Wang X, Contino G, Liesa M, Sahin E, et al. (2011) Pancreatic cancers require autophagy for tumor growth. Genes & Development. doi:10.1101/gad.2016111.

72.   Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, et al. (2011) Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes & Development 25: 460–470. doi:10.1101/gad.2016311.

73.   Strohecker AM, Guo JY, Karsli-Uzunbas G, Price SM, Chen GJ, et al. (2013) Autophagy Sustains Mitochondrial Glutamine Metabolism and Growth of BRAFV600E-Driven Lung Tumors. Cancer Discovery. doi:10.1158/2159-8290.CD-13-0397.

74.   Gong C, Bauvy C, Tonelli G, Yue W, Deloménie C, et al. (2013) Beclin 1 and

autophagy are required for the tumorigenicity of breast cancer stem-like/progenitor cells. Oncogene 32: 2261–72–2272e.1–11. doi:10.1038/onc.2012.252.

75.   Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, et al. (2006) Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10: 51–64. doi:10.1016/j.ccr.2006.06.001.

76.   Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, et al. (2009) Autophagy Suppresses Tumorigenesis through Elimination of p62. Cell 137: 1062–1075. doi:10.1016/j.cell.2009.03.048.

77.   Yue Z, Jin S, Yang C, Levine AJ, Heintz N (2003) Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci USA 100: 15077–15082. doi:10.1073/pnas.2436255100.

78.   Kuo W-L, Sharifi MN, Lingen MW, Ahmed O, Liu J, et al. (2014) p62/SQSTM1 Accumulation in Squamous Cell Carcinoma of Head and Neck Predicts Sensitivity to Phosphatidylinositol 3-Kinase Pathway Inhibitors. PLoS ONE 9: e90171. doi:10.1371/journal.pone.0090171.s001.

79.   Li Z, Chen B, Wu Y, Jin F, Xia Y, et al. (2010) Genetic and epigenetic silencing of the beclin 1 gene in sporadic breast tumors. BMC Cancer 10: 98. doi:10.1186/1471-2407-10-98.

80.   Gao X, Zacharek A, Salkowski A, Grignon DJ, Sakr W, et al. (1995) Loss of heterozygosity of the BRCA1 and other loci on chromosome 17q in human prostate cancer. Cancer Res 55: 1002–1005.

81.   Saito H, Inazawa J, Saito S, Kasumi F, Koi S, et al. (1993) Detailed deletion mapping of chromosome 17q in ovarian and breast cancers: 2-cM region on 17q21.3 often and commonly deleted in tumors. Cancer Res 53: 3382–3385.

82.   Wang RC, Wei Y, An Z, Zou Z, Xiao G, et al. (2012) Akt-Mediated Regulation of Autophagy and Tumorigenesis Through Beclin 1 Phosphorylation. Science. doi:10.1126/science.1225967.

83.   Wei Y, Zou Z, Becker N, Anderson M, Sumpter R, et al. (2013) EGFR-Mediated Beclin 1 Phosphorylation in Autophagy Suppression, Tumor Progression, and Tumor Chemoresistance. Cell 154: 1269–1284. doi:10.1016/j.cell.2013.08.015.

84.   Pantuck AJ, Seligson DB, Klatte T, Yu H, Leppert JT, et al. (2007) Prognostic relevance of the mTOR pathway in renal cell carcinoma: implications for molecular patient selection for targeted therapy. Cancer 109: 2257–2267. doi:10.1002/cncr.22677.

85.   Toschi A, Lee E, Gadir N, Ohh M, Foster DA (2008) Differential Dependence of

Hypoxia-inducible Factors 1 and 2 on mTORC1 and mTORC2. Journal of Biological Chemistry 283: 34495–34499. doi:10.1074/jbc.C800170200.

86.   Thomas GV, Tran C, Mellinghoff IK, Welsbie DS, Chan E, et al. (2006) Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer. Nat Med 12: 122–127. doi:10.1038/nm1337.

87.   Zhang T, Niu X, Liao L, Cho E-A, Yang H (2013) The Contributions of HIF-Target Genes to Tumor Growth in RCC. PLoS ONE 8: e80544. doi:10.1371/journal.pone.0080544.s002.

88.   Chow W-H, Dong LM, Devesa SS (2010) Epidemiology and risk factors for kidney cancer. Nature Publishing Group 7: 245–257. doi:10.1038/nrurol.2010.46.

89.   Hanna SC, Heathcote SA, Kim WY (2008) mTOR pathway in renal cell carcinoma. Expert Rev Anticancer Ther 8: 283–292. doi:10.1586/14737140.8.2.283.

90.   Guertin DA, Sabatini DM (2009) The Pharmacology of mTOR Inhibition. Sci Signal 2: pe24. doi:10.1126/scisignal.267pe24.

91.   Hudes G, Carducci M, Tomczak P, Dutcher J, Figlin R, et al. (2007) Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. N Engl J Med 356: 2271–2281. doi:10.1056/NEJMoa066838.

92.   Garcia JA, Cowey CL, Godley PA (2009) Renal cell carcinoma. Curr Opin Oncol 21: 266–271. doi:10.1097/CCO.0b013e32832a05c8.

93.   Li H, Jin X, Zhang Z, Xing Y, Kong X (2012) Inhibition of autophagy enhances apoptosis induced by the PI3K/AKT/mTor inhibitor NVP-BEZ235 in renal cell carcinoma cells. Cell Biochem Funct: n/a–n/a. doi:10.1002/cbf.2917.

94.   Cho DC, Cohen MB, Panka DJ, Collins M, Ghebremichael M, et al. (2010) The Efficacy of the Novel Dual PI3-Kinase/mTOR Inhibitor NVP-BEZ235 Compared with Rapamycin in Renal Cell Carcinoma. Clinical Cancer Research: 1–11. doi:10.1158/1078-0432.CCR-09-3022.

95.   Zhang H, Berel D, Wang Y, Li P, Bhowmick NA, et al. (2013) A Comparison of Ku0063794, a Dual mTORC1 and mTORC2 Inhibitor, and Temsirolimus in Preclinical Renal Cell Carcinoma Models. PLoS ONE 8: e54918. doi:10.1371/journal.pone.0054918.s008.

96.   Yu K, Shi C, Toral-Barza L, Lucas J, Shor B, et al. (2010) Beyond Rapalog Therapy: Preclinical Pharmacology and Antitumor Activity of WYE-125132, an ATP-Competitive and Specific Inhibitor of mTORC1 and mTORC2. Cancer Res 70: 621–631. doi:10.1158/0008-5472.CAN-09-2340.

97. Voss MH, Hakimi AA, Pham CG, Brannon AR, Chen Y-B, et al. (2014) Tumor genetic analyses of patients with metastatic renal cell carcinoma and extended benefit from mTOR inhibitor therapy. Clin Cancer Res 20: 1955–1964. doi:10.1158/1078-0432.CCR-13-2345.

98. Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, et al. (2008) Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. Lancet 372: 449–456. doi:10.1016/S0140-6736(08)61039-9.

99. O'Reilly KE, Rojo F, She Q-B, Solit D, Mills GB, et al. (2006) mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res 66: 1500–1508. doi:10.1158/0008-5472.CAN-05-2925.

100. Harada K, Miyake H, Kumano M, Fujisawa M (2013) Acquired resistance to temsirolimus in human renal cell carcinoma cells is mediated by the constitutive activation of signal transduction pathways through mTORC2: 1–7. doi:10.1038/bjc.2013.602.

101. Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, et al. (2008) Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J Clin Invest. doi:10.1172/JCI34739DS1.

102. Soares HP, Ni Y, Kisfalvi K, Sinnett-Smith J, Rozengurt E (2013) Different Patterns of Akt and ERK Feedback Activation in Response to Rapamycin, Active-Site mTOR Inhibitors and Metformin in Pancreatic Cancer Cells. PLoS ONE 8: e57289. doi:10.1371/journal.pone.0057289.s001.

103. Fan Q-W, Cheng C, Hackett C, Feldman M, Houseman BT, et al. (2010) Akt and autophagy cooperate to promote survival of drug-resistant glioma. Sci Signal 3: ra81. doi:10.1126/scisignal.2001017.

104. Chresta CM, Davies BR, Hickson I, Harding T, Cosulich S, et al. (2010) AZD8055 Is a Potent, Selective, and Orally Bioavailable ATP-Competitive Mammalian Target of Rapamycin Kinase Inhibitor with In vitro and In vivo Antitumor Activity. Cancer Res 70: 288–298. doi:10.1158/0008-5472.CAN-09-1751.

105. Hoang B, Hoang B, Benavides A, Benavides A, Shi Y, et al. (2012) The PP242 Mammalian Target of Rapamycin (mTOR) Inhibitor Activates Extracellular Signal-regulated Kinase (ERK) in Multiple Myeloma Cells via a Target of Rapamycin Complex 1 (TORC1)/ Eukaryotic Translation Initiation Factor 4E (eIF-4E)/RAF Pathway and Activation Is a Mechanism of Resistance. J Biol Chem 287: 21796–21805. doi:10.1074/jbc.M111.304626.

106. Roberts PJ, Usary JE, Darr DB, Dillon PM, Pfefferle AD, et al. (2012) Combined PI3K/mTOR and MEK Inhibition Provides Broad Antitumor Activity in Faithful

Murine Cancer Models. Clinical Cancer Research 18: 5290–5303. doi:10.1158/1078-0432.CCR-12-0563.

107.   Huang S, Yang ZJ, Yu C, Sinicrope FA (2011) Inhibition of mTOR Kinase by AZD8055 Can Antagonize Chemotherapy-induced Cell Death through Autophagy Induction and Down-regulation of p62/Sequestosome 1. J Biol Chem 286: 40002–40012. doi:10.1074/jbc.M111.297432.

108.   Carayol N, Vakana E, Sassano A, Kaur S, Goussetis DJ, et al. (2010) Critical roles for mTORC2- and rapamycin-insensitive mTORC1-complexes in growth and survival of BCR-ABL-expressing leukemic cells. Proc Natl Acad Sci USA 107: 12469–12474. doi:10.1073/pnas.1005114107.

109.   Bray K, Mathew R, Lau A, Kamphorst JJ, Fan J, et al. (2012) Autophagy Suppresses RIP Kinase-Dependent Necrosis Enabling Survival to mTOR Inhibition. PLoS ONE 7: e41831. doi:10.1371/journal.pone.0041831.

110.   Xie X, White EP, Mehnert JM (2013) Coordinate Autophagy and mTOR Pathway Inhibition Enhances Cell Death in Melanoma. PLoS ONE 8: e55096. doi:10.1371/journal.pone.0055096.s002.

111.   Xu C-X, Zhao L, Yue P, Fang G, Tao H, et al. (2011) Augmentation of NVP-BEZ235's anticancer activity against human lung cancer cells by blockage of autophagy. Cancer Biol Ther 12: 549–555. doi:10.4161/cbt.12.6.16397.

112.   Kirstein MM, Boukouris AE, Pothiraju D, Buitrago-Molina LE, Marhenke S, et al. (2013) Activity of the mTOR inhibitor RAD001, the dual mTOR and PI3-kinase inhibitor BEZ235 and the PI3-kinase inhibitor BKM120 in hepatocellular carcinoma. Liver Int 33: 780–793. doi:10.1111/liv.12126.

113.   Mihaylova MM, Shaw RJ (2011) The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nature Publishing Group 13: 1016–1023. doi:10.1038/ncb2329.

114.   Ghadimi MP, Lopez G, Torres KE, Belousov R, Young ED, et al. (2012) Targeting the PI3K/mTOR Axis, Alone and in Combination with Autophagy Blockade, for the Treatment of Malignant Peripheral Nerve Sheath Tumors. Molecular Cancer Therapeutics 11: 1758–1769. doi:10.1158/1535-7163.MCT-12-0015.

115.   Mendoza MC, Er EE, Blenis J (2011) The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. Trends in Biochemical Sciences 36: 320–328. doi:10.1016/j.tibs.2011.03.006.

116.   Liu Y, Sun S-Y, Owonikoko TK, Sica GL, Curran WJ, et al. (2012) Rapamycin induces Bad phosphorylation in association with its resistance to human lung cancer cells. Molecular Cancer Therapeutics 11: 45–56. doi:10.1158/1535-7163.MCT-11-0578.

117. Conti A, Santoni M, Amantini C, Burattini L, Berardi R, et al. (2013) Progress of Molecular Targeted Therapies for Advanced Renal Cell Carcinoma. BioMed Research International 2013: 1–9. doi:10.1158/0008-5472.CAN-07-5723.

118. Kim WY, Kaelin WG (2006) Molecular pathways in renal cell carcinoma--rationale for targeted treatment. Semin Oncol 33: 588–595. doi:10.1053/j.seminoncol.2006.06.001.

119. Kim J-W, Dang CV (2006) Cancer's molecular sweet tooth and the Warburg effect. Cancer Res 66: 8927–8930. doi:10.1158/0008-5472.CAN-06-1501.

120. Wang X, Wang X, Hawk N, Hawk N, Yue P, et al. (2008) Overcoming mTOR inhibition-induced paradoxical activation of survival signaling pathways enhances mTOR inhibitors' anticancer efficacy. Cancer Biol Ther 7: 1952–1958. doi:10.4161/cbt.7.12.6944.

121. Zhong H, Sanchez C, Spitrzer D, Plambeck-Suess S, Gibbs J, et al. (2013) Synergistic Effects of Concurrent Blockade of PI3K and MEK Pathways in Pancreatic Cancer Preclinical Models. PLoS ONE 8: e77243. doi:10.1371/journal.pone.0077243.

122. Kinross KM, Kinross KM, Brown DV, Brown DV, Kleinschmidt M, et al. (2011) In Vivo Activity of Combined PI3K/mTOR and MEK Inhibition in a KrasG12D;Pten Deletion Mouse Model of Ovarian Cancer. Molecular Cancer Therapeutics 10: 1440–1449. doi:10.1158/1535-7163.MCT-11-0240.

123. Sheppard KE, Cullinane C, Hannan KM, Wall M, Chan J, et al. (2013) Synergistic inhibition of ovarian cancer cell growth by combining selective PI3K/mTOR and RAS/ERK pathway inhibitors. Eur J Cancer 49: 3936–3944. doi:10.1016/j.ejca.2013.08.007.

124. Renshaw J, Renshaw J, Taylor KR, Taylor KR, Bishop R, et al. (2013) Dual blockade of the PI3K/AKT/mTOR (AZD8055) and RAS/MEK/ERK (AZD6244) pathways synergistically inhibits rhabdomyosarcoma cell growth in vitro and in vivo. Clin Cancer Res 19: 5940–5951. doi:10.1158/1078-0432.CCR-13-0850.

125. Shimizu T, Tolcher AW, Papadopoulos KP, Beeram M, Rasco DW, et al. (2012) The clinical effect of the dual-targeting strategy involving PI3K/AKT/mTOR and RAS/MEK/ERK pathways in patients with advanced cancer. Clin Cancer Res 18: 2316–2325. doi:10.1158/1078-0432.CCR-11-2381.

126. Corcoran RB, Rothenberg SM, Hata AN, Faber AC, Piris A, et al. (2013) TORC1 Suppression Predicts Responsiveness to RAF and MEK Inhibition in BRAF-Mutant Melanoma. Science Translational Medicine 5: 196ra98–196ra98. doi:10.1126/scitranslmed.3005753.

127. Yu K, Toral-Barza L, Shi C, Zhang WG, Lucas J, et al. (2009) Biochemical, Cellular, and In vivo Activity of Novel ATP-Competitive and Selective Inhibitors

of the Mammalian Target of Rapamycin. Cancer Res 69: 6232–6240. doi:10.1158/0008-5472.CAN-09-0299.

128.   Feldman ME, Apsel B, Uotila A, Loewith R, Knight ZA, et al. (2009) Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol 7: e38. doi:10.1371/journal.pbio.1000038.

129.   Dalby KN, Morrice N, Caudwell FB, Avruch J, Cohen P (1998) Identification of regulatory phosphorylation sites in mitogen-activated protein kinase (MAPK)-activated protein kinase-1a/p90rsk that are inducible by MAPK. J Biol Chem 273: 1496–1505.

130.   Migliardi G, Migliardi G, Sassi F, Sassi F, Torti D, et al. (2012) Inhibition of MEK and PI3K/mTOR Suppresses Tumor Growth but Does Not Cause Tumor Regression in Patient-Derived Xenografts of RAS-Mutant Colorectal Carcinomas. Clinical Cancer Research 18: 2515–2525. doi:10.1158/1078-0432.CCR-11-2683.

131.   Haagensen EJ, Kyle S, Beale GS, Maxwell RJ, Newell DR (2012) The synergistic interaction of MEK and PI3K inhibitors is modulated by mTOR inhibition. Br J Cancer 106: 1386–1394. doi:10.1038/bjc.2012.70.

132.   Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, et al. (2008) Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nat Med 14: 1351–1356. doi:10.1038/nm.1890.

133.   Duncan JS, Whittle MC, Nakamura K, Abell AN, Midland AA, et al. (2012) Dynamic Reprogramming of the Kinome in Response to Targeted MEK Inhibition in Triple-Negative Breast Cancer. Cell 149: 307–321. doi:10.1016/j.cell.2012.02.053.

134.   Motzer RJ, Mazumdar M, Bacik J, Berg W, Amsterdam A, et al. (1999) Survival and prognostic stratification of 670 patients with advanced renal cell carcinoma. J Clin Oncol 17: 2530–2540.

135.   Krieg M, Haas R, Brauch H, Plate K (2000) Up-Regulation of hypoxia-inducible factors HIF1/2 under normoxic conditions in renal carcinoma cells by VHL suppressor gene loss of function: 1–9.

136.   Kim WY (2009) HIF2 cooperates with Ras to promote lung tumorigenesis in mice: 1–11.

137.   Mazumdar J, Hickey MM, Pant DK, Durham AC, Sweet-Cordero A, et al. (2010) HIF-2alpha deletion promotes Kras-driven lung tumor development. Proceedings of the National Academy of Sciences 107: 14182–14187. doi:10.1073/pnas.1001296107.

138.   Hanna SC, Krishnan B, Bailey ST, Moschos SJ, Kuan P-F, et al. (2013) HIF1α

and HIF2α independently activate SRC to promote melanoma metastases. J Clin Invest 123: 2078–2093. doi:10.1172/JCI66715DS1.

139. Mizushima N, Noda T, Yoshimori T, Tanaka Y, Ishii T, et al. (1998) A protein conjugation system essential for autophagy. Nature 395: 395–398. doi:10.1038/26506.

140. Meijer AJ, Codogno P (2004) Regulation and role of autophagy in mammalian cells. Int J Biochem Cell Biol 36: 2445–2462. doi:10.1016/j.biocel.2004.02.002.

141. Papandreou I, Lim AL, Laderoute K, Denko NC (2008) Hypoxia signals autophagy in tumor cells via AMPK activity, independent of HIF-1, BNIP3, and BNIP3L. Cell Death Differ 15: 1572–1581. doi:10.1038/cdd.2008.84.

142. Debnath J, Baehrecke EH, Kroemer G (2005) Does autophagy contribute to cell death? Autophagy 1: 66–74.

143. Turcotte S, Chan DA, Sutphin PD, Hay MP, Denny WA, et al. (2008) A molecule targeting VHL-deficient renal cell carcinoma that induces autophagy. Cancer Cell 14: 90–102. doi:10.1016/j.ccr.2008.06.004.

144. Gini B, Zanca C, Guo D, Matsutani T, Masui K, et al. (2013) The mTOR Kinase Inhibitors, CC214-1 and CC214-2, Preferentially Block the Growth of EGFRvIII-Activated Glioblastomas. Clinical Cancer Research 19: 5722–5732. doi:10.1158/1078-0432.CCR-13-0527.

145. Barth S, Glick D, Macleod KF (2010) Autophagy: assays and artifacts. J Pathol 221: 117–124. doi:10.1002/path.2694.

146. Pankiv S, Clausen TH, Lamark T, Brech A, Bruun J-A, et al. (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282: 24131–24145. doi:10.1074/jbc.M702824200.

147. Li L, Chen Y, Gibson SB (2012) Starvation-induced autophagy is regulated by mitochondrial reactive oxygen species leading to AMPK activation. Cellular Signalling: 1–16. doi:10.1016/j.cellsig.2012.09.020.

148. Schieke SM, Phillips D, McCoy JP, Aponte AM, Shen R-F, et al. (2006) The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. J Biol Chem 281: 27643–27652. doi:10.1074/jbc.M603536200.

149. Edinger AL, Linardic CM, Chiang GG, Thompson CB, Abraham RT (2003) Differential effects of rapamycin on mammalian target of rapamycin signaling functions in mammalian cells. Cancer Res 63: 8451–8460.

150. Zhou B, Damrauer JS, Bailey ST, Hadzic T, Jeong Y, et al. (2014) Erythropoietin

promotes breast tumorigenesis through tumor-initiating cell self-renewal. J Clin Invest 124: 553–563. doi:10.1172/JCI69804DS1.

151.   Cirone P, Andresen CJ, Eswaraka JR, Lappin PB, Bagi CM (2014) Patient-derived xenografts reveal limits to PI3K/mTOR- and MEK-mediated inhibition of bladder cancer. Cancer Chemother Pharmacol. doi:10.1007/s00280-014-2376-1.

152.   Watson AL, Anderson LK, Greeley AD, Keng VW, Rahrmann EP, et al. (2014) Co-targeting the MAPK and PI3K/AKT/mTOR pathways in two genetically engineered mouse models of schwann cell tumors reduces tumor grade and multiplicity. Oncotarget.

153.   Hao R, Nanduri P, Rao Y, Panichelli RS, Ito A, et al. (2013) Proteasomes activate aggresome disassembly and clearance by producing unanchored ubiquitin chains. Molecular Cell 51: 819–828. doi:10.1016/j.molcel.2013.08.016.

154.   Liu X-D, Yao J, Tripathi DN, Ding Z, Xu Y, et al. (2014) Autophagy mediates HIF2: 1–11. doi:10.1038/onc.2014.199.

155.   Scherz-Shouval R, Elazar Z (2011) Regulation of autophagy by ROS: physiology and pathology. Trends in Biochemical Sciences 36: 30–38. doi:10.1016/j.tibs.2010.07.007.

156.   Huang J, Lam GY, Brumell JH (2011) Autophagy Signaling Through Reactive Oxygen Species. Antioxidants & Redox Signaling 14: 2215–2231. doi:10.1089/ars.2010.3554.

157.   Le WD, Xie WJ, Appel SH (1999) Protective role of heme oxygenase-1 in oxidative stress-induced neuronal injury. J Neurosci Res 56: 652–658.

158.   Galluzzi L, Kroemer G (2008) Necroptosis: A Specialized Pathway of Programmed Necrosis. Cell 135: 1161–1163. doi:10.1016/j.cell.2008.12.004.

159.   Rikiishi H (2012) Novel Insights into the Interplay between Apoptosis and Autophagy. International Journal of Cell Biology 2012: 317645. doi:10.1155/2012/317645.