STUDIES ON CELLULAR NUTRIENT RESPONSES AND PROTEIN DEGRADATION

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DEDICATION:

I dedicate this dissertation to my parents and grandparents, for inspiring me to pursue science, and for their unconditional love and support.

STUDIES ON CELLULAR NUTRIENT RESPONSES AND PROTEIN DEGRADATION

by

ANWESHA GHOSH
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I have worked on two projects. The first project investigates mechanisms involved in cellular responses to amino acids. Amino-acid abundance promotes protein synthesis and cell growth via activation of the protein kinase mTOR, while amino-acid deprivation promotes protein degradation by autophagy. The heterodimeric G protein coupled receptor (GPCR) T1R1-T1R3 can act as an extracellular sensor for amino acids, promoting mTOR activity while repressing autophagy in cells. Quantitative PCR analysis revealed that T1R3 depletion increases mRNA expression of amino acid transporters as a compensatory mechanism induced by perceived starvation. The arrestin proteins can bind GPCRs to mediate their internalization or to facilitate downstream signaling. I tested the hypothesis that β-arrestin 2

might participate in regulation of mTOR activity and autophagy by amino acids. siRNA-mediated β-arrestin 2 depletion decreased T1R1-T1R3 protein expression, reduced mTOR activity and increased autophagy in different cell types. β-arrestin 2 loss increased phosphorylation of the MAP kinase ERK1/2, which may play a role in promoting autophagy. Taken together, these findings demonstrate a role for β-arrestin 2 in promoting mTOR activity and suppressing autophagy.

The second project examined the role of different protein degradation pathways and an E3 ubiquitin ligase UBR5 in regulating the stability of the protein kinase WNK1, a key regulator of cellular ion homeostasis. Mutations that increase WNK1 protein expression cause familial hypertension, highlighting the importance of understanding the regulation of WNK1 protein expression. Cycloheximide chase experiments revealed that WNK1 degradation may be complex, as it does not follow simple exponential decay kinetics. Pharmacological inhibition of different protein degradation pathways showed that autophagy and the calpain system of non-lysosomal cysteine proteases, but not the proteasome, can promote WNK1 degradation. Inhibition of the protein chaperone Hsp90 increased WNK1 protein levels, possibly through stabilization of WNK1 by Hps70. Immunoprecipitation experiments demonstrated that UBR5 can associate with WNK1. siRNA-mediated silencing of UBR5 increased WNK1 stability, decreased the ubiquitination of an overexpressed N terminal fragment of WNK1, and reduced the levels of KLHL3, an adaptor protein that recruits WNK1 to the Cullin3-RBX1 E3 ligase complex for ubiquitination and degradation. Taken together, these findings identify degradation pathways and molecular players that regulate WNK1 stability.

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PRIOR PUBLICATIONS:

1. The G protein-coupled taste receptor T1R1/T1R3 regulates mTORC1 and autophagy. Wauson EM, Zaganjor E, Lee AY, Guerra ML, Ghosh AB, Bookout AL, Chambers CP, Jivan A, McGlynn K, Hutchison MR, Deberardinis RJ, Cobb MH. *Mol. Cell.* (2012) 47(6):851-62

2. G protein-coupled receptors and the regulation of autophagy. Wauson EM, Dbouk HA, Ghosh AB, Cobb MH. Trends Endocrinol. Metab. (2014) (5):274-82.

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CHAPTER 1: Introduction

PROJECT 1: UNDERSTANDING CELLULAR NUTRIENT RESPONSES AND AUTOPHAGY

A. Amino acids, mTOR signaling, and autophagy

Cells constantly monitor intracellular and extracellular nutrient concentrations to co-ordinate anabolic and catabolic processes with nutritional status. This information on nutrient abundance is integrated with information on extracellular growth factor concentrations as well as intracellular ATP availability to determine cellular behavior. In particular, cells must survey amino acid availability for homeostatic control of protein synthesis, protein degradation, and cell growth. Amino acid-rich conditions promote the anabolic processes of protein synthesis and cell growth. A central regulator of these processes is the protein-serine threonine kinase mechanistic target of rapamycin (mTOR). mTOR exists in two complexes in cells, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [1]. While both of these complexes contain mTOR and mLST8 (mammalian lethal with SEC13 protein 8), mTORC1 is characterized by the presence of Raptor (Regulatory-associated protein of mTOR1), while mTORC2 is characterized by the presence of Rictor (Rapamycin-insensitive companion of mTOR) and Sin1 (target of rapamycin complex subunit MAPKAP1). mTORC1 is activated by amino acids, which facilitate correct localization of the complex, and by growth factors, which stimulate kinase activity via the small GTPase Rheb (RAS homolog enriched in brain) [2]. Rheb is inhibited by the actions of TSC1/2 (tuberous sclerosis complex 1/2), which acts as a GTPase activating protein [3]. Growth factors activate the protein kinases AKT and the MAP kinase extracellular signal regulated kinases 1/2 (ERK1/2), which results in the inhibition of TSC1/2, thereby relieving repression of Rheb [4].

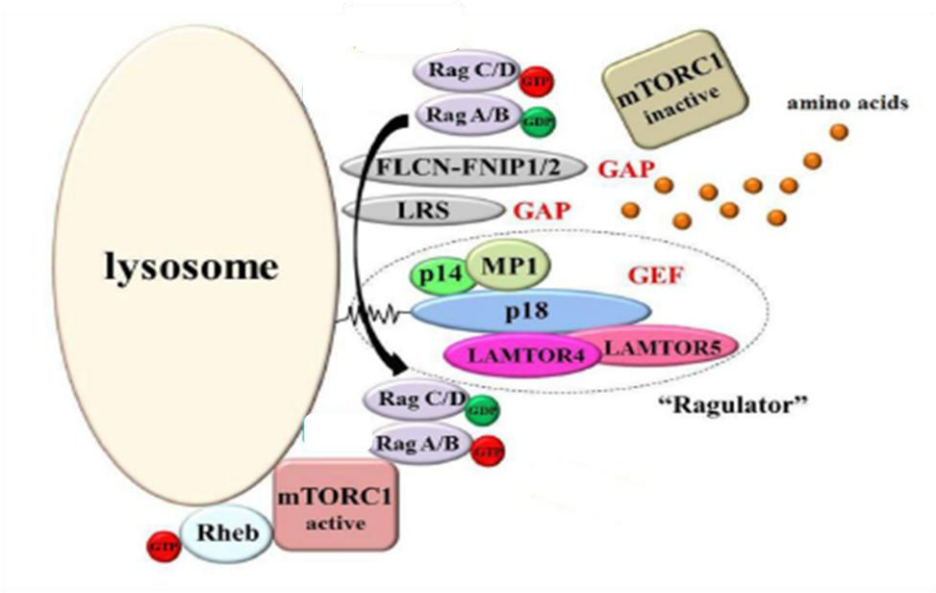
Because Rheb resides on the surface of late endosomal/lysosomal membranes, translocation of mTOR to these membranes is critical for its activation, and this process has been

a focus of intense research. It has been demonstrated that lysosomal localization of mTOR is governed by Rag GTPases (Ras-related GTPases) and by a hetero-pentameric protein complex called the Ragulator (Fig 1A) [5-7]. Amino acids promote the binding of Raptor to Rag GTPases [8]. GTPase activating proteins (GAPs) such as the GATOR1 and GATOR2 complexes and other negative regulators such as SH3BP4 (SH3-domain binding protein 4) have been identified for the Rags [9-11] and the Ragulator complex can function as a guanine nucleotide exchange factor (GEF) for Rag GTPases [12]. An ATP dependent complex called the TTT-RUVBL1/2 complex has been demonstrated to be required for the interaction of mTORC1 with the Rag GTPases [13]. Little is known about how Ragulator components are regulated. The predominant concept in the field of mTOR activation has been that amino acids stimulate mTOR activity in a Rag GTPase and Ragulator –dependent manner. However, recent studies have demonstrated that different amino acids activate mTOR differently, with leucine activating mTOR in a Rag-dependent manner and glutamine inducing lysosomal localization and activation of mTOR in a Rag-independent manner [14].

Once activated, mTORC1 promotes protein synthesis via phosphorylation of eIF4E binding protein 1 (4EBP1) and p70 S6 kinase (Fig 1B) [15, 16]. Phosphorylation of 4EBP-1 releases it from eukaryotic translation initiation factor 4E (eIF4E), which enhances recruitment of other initiation factors and the 40S ribosomal subunit to the 7-methylGTP cap at the 5’ end of mRNA [17]. Upon activation by mTOR, p70 S6 kinase phosphorylates the 40s ribosomal protein S6. Although the consequence of S6 phosphorylation is not clear, it is nonetheless a valuable readout of mTOR activity.

Amino acid scarcity promotes protein degradation by a catabolic process called autophagy in which proteins, organelles, and other cytoplasmic contents are engulfed by double-membrane vesicles called autophagosomes and delivered to lysosomes, where they are degraded (Fig 2)

A



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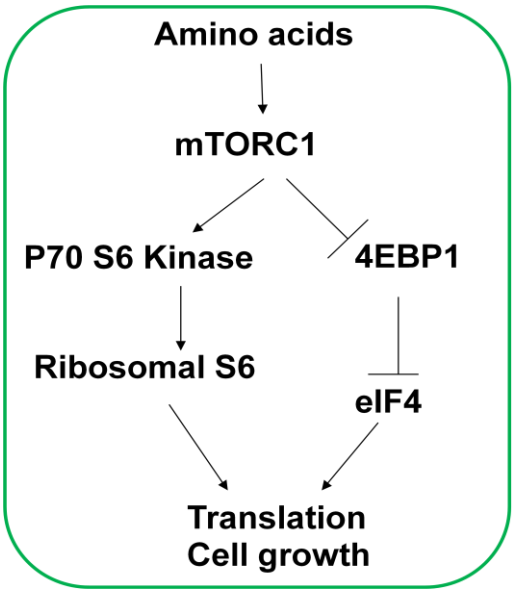


Fig 1: mTOR localization and signaling in response to amino acids. (A) The Ragulator-Rag model of mTORC1 localization. Amino acids promote the binding of mTORC1 to the Rag GTPases. Lysosomal targetting of mTORC1 brings it in proximity to its activator Rheb. The pentameric Ragulator complex is tethered to the lysosomal membrane via myristoylated p18 and acts as a guanine nucleotide exchange factor (GEF) for the Rag GTPases. FLCN-FNIP1/2 and LRS have been identified as GTPase activating proteins (GAPs) for the Rag GTPases. (B) Once activated, mTORC1 promotes protein synthesis and cell growth via phosphorylation and activation

of p70 ribosomal S6 kinase as well as phosphorylation and inhibition of the translation suppressor 4EBP1.

[18-20]. In this way, autophagy can provide nutrients to promote survival under stress. Under nutrient replete conditions, mTORC1 inhibits autophagy by phosphorylating and inhibiting a key autophagy kinase called ULK-1 (UNC51-like kinase 1) [21]. Nutrient deprivation and other stresses inhibit mTORC1, resulting in increased activity of ULK1/2, which then binds to other regulatory proteins to initiate autophagy. Another key activator of autophagy is AMP-activated protein kinase (AMPK), which phosphorylates and activates ULK1 [21]. Formation of the pre-autophagosome or phagophore requires the generation of phosphatidylinositol-3-phosphate (PI3P) by the type III phosphoinositide-3 kinase (PI3K) VPS34 [22]. During expansion of the phagophore into the autophagosome, the ubiquitin-like protein LC3 (microtubule-associated protein 1A/1B-light chain 3) undergoes proteolytic cleavage and conjugation to phosphatidylethanolamine to form LC3-II [23]. LC3-II is incorporated into the inner and outer membranes of autophagosomes, and is thought to be required for autophagosome biogenesis [24]. Cargoes destined for autophagic degradation are poly-ubiquitinated so that adaptor proteins like p62 (sequestosome-1 SQSTM1) and NBR-1 (neighbour of BRCA1 gene 1 protein) can recognize and bind them [25, 26]. p62 is thought to recruit these cargoes to autophagosomes by binding to LC3-II [27]. The mature autophagosome then fuses with the lysosome, resulting in the degradation of its cargo.

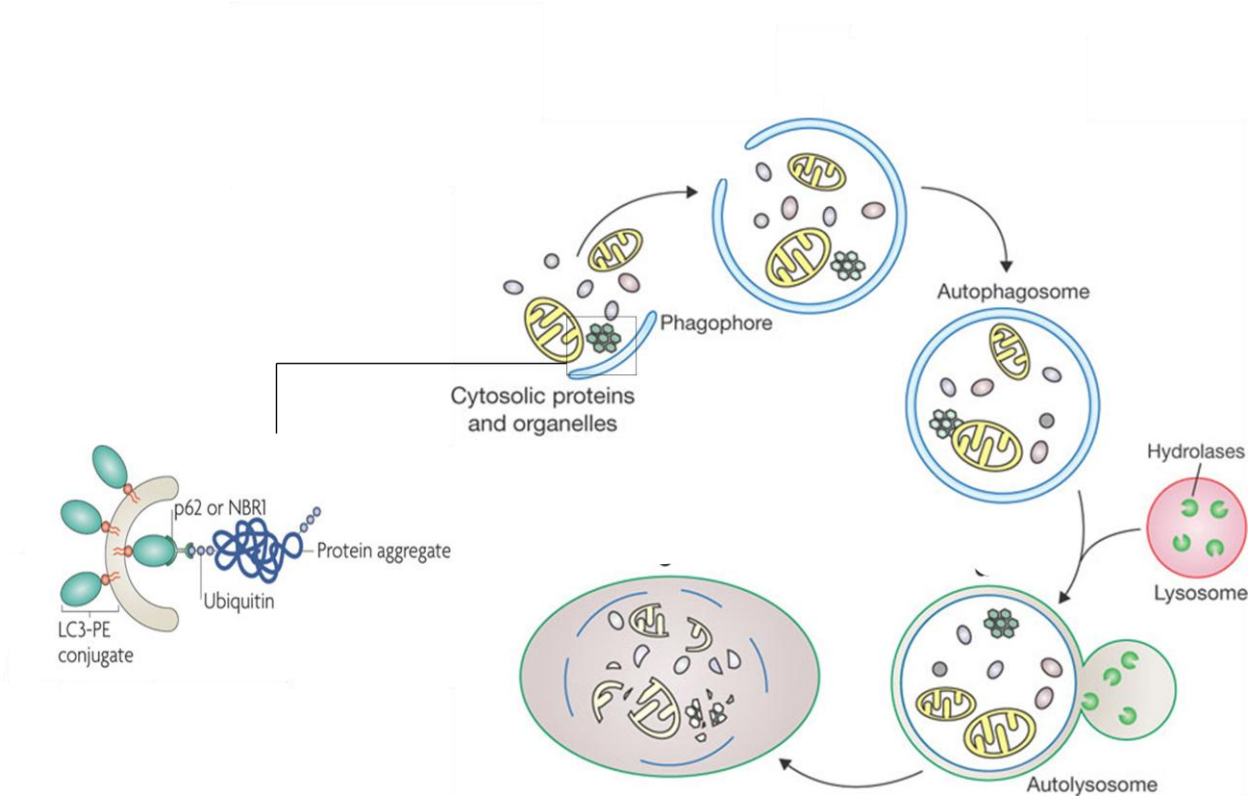
Studies have shown that cells can employ multiple mechanisms to sense intracellular amino acids. One well-known mechanism involves the activation of the kinase GCN2 (general control nonderepressible 2) by uncharged tRNAs, which accumulate during amino acid scarcity, and thus bind GCN2 [28]. Activated GCN2 then phosphorylates and inhibits a key translation initiation factor called eIF2α (eukaryotic initiation factor 2 alpha) [29]. A current hypothesis in the field is that intracellular amino acid sensing can initiate at lysosomes in an "inside-out" mechanism that requires the vacuolar H^+ ATPase at the lysosomal membrane and the presence of

amino acids in the lysosomal lumen [30] . The actual sensor for amino acids in this context has not been identified, but a recent study suggests that the lysosomal amino acid transporter SLC38A9 might act as a possible sensor for arginine [31].

B. Extracellular amino acid sensing by the heterodimeric G protein coupled receptor T1R1-T1R3

Amino acid sensing events upstream of the lysosome are less clear. The bidirectional amino acid transporter SLC7A5/SLC3A2 is thought to activate mTOR by facilitating the influx of leucine in exchange for the efflux of glutamine [32]. Glutamatergic activation of mTORC1 and ERK1/2 has been reported in neurons [33]. Findings such as these suggest roles for cell surface receptors and transporters in signaling to mTORC1. In comparison to intracellular amino acid sensing, extracellular amino acid sensing in contexts other than glutamatergic and dopaminergic neurotransmission in the brain has not received much attention. We found that the heterodimeric, seven transmembrane G protein coupled receptor (GPCR) Tas1R1-Tas1R3 (T1R1-T1R3) can act as a detector for amino acids in the extracellular milieu and convey information on amino acid availability to the mTOR and MAPK signaling pathways [34]. Along with Tas1R2 (T1R2), T1R1 and T1R3 belong to the T1R family of receptors within the class C family of GPCRs, which are characterized by the presence of a large extracellular domain called the Venus flytrap module that is involved in ligand binding. Members of this GPCR family typically form obligate homo-and heterodimers. Metabotropic glutamate receptors (mGluRs), the GABA-B receptor, the Ca2+-sensing receptor (CaSR), GPRC6A, and a few orphan receptors are also included in the class C family [35]. The Tas1Rs were first identified as taste receptors in gustatory cells of the tongue, where the T1R1-T1R3 heterodimer functions as the umami taste receptor [36] and the T1R2-T1R3 heterodimer acts as the sweet taste receptor [37]. T1R1-T1R3 is responsive to the L-, but not D-enantiomers of most of the 20 amino acid [38-40]. We found that these receptors were expressed in a wide variety of tissues in addition to the tongue, such as

small intestine, colon, muscle, kidney, parts of the brain, adipose tissue, liver, pancreas, and heart. Depletion of either T1R1 or T1R3 enhanced mTORC1 inhibition upon amino acid starvation, and reduced, but did not abolish mTORC1 activation upon subsequent stimulation with amino acids. Consistent with reducedmTORC1 activity, translation initiation was impaired and autophagy was increased in T1R3 knockdown cells. Amino acid-induced lysosomal mTOR localization appeared to be impaired in these cells. As mentioned earlier, little is known about how other signaling pathways might modulate lysosomal mTOR localization in response to amino acids. Multiple pathways are activated by T1R1-T1R3 in response to amino acids, and the effect of these signaling pathways on mTOR localization will be examined in Chapter 2. Finally, loss of T1R1/T1R3 induced multiple compensatory changes including decreased expression of mTOR inhibitors TSC2 and regulated-in-development-and-DNA-damage-responses-1 (REDD1), and increased mRNA expression of amino acid transporters. The effect of T1R3 silencing on gene expression in H9C2 rat cardiomyoblast cells will be discussed in detail in Chapter 2. Amino acids induce rapid, calcium dependent, transient activation of ERK1/2 in pancreatic beta cells through T1R1-T1R3. This ERK1/2 activation appears to enhance amino acid-induced mTOR activation [34]. This is consistent with previous work demonstrating MAPK-mediated enhancement of mTOR activity via phosphorylation of Raptor by ERK1/2 [41].



Adapted from Xie and Klionsky (2007) Nat Cell Biol and Tyedmers (2010) Nat Rev Mol Cell Biol

Fig 2: Overview of the process of autophagy. Protein aggregates, organelles and other cellular contents to be degraded are sequestered to the inner leaflet of an elongating membrane sac called the phagophore. This is achieved by the actions of the adaptor protein p62/NBR1 and a ubiquitin-like lipidated protein called LC3II, which is incorporated into the phagophore membrane as it elongates. Cargoes destined for degradation undergo poly-ubiquitination, and these poly-ubiquitin chains are recognized and bound by p62 /NBR1. p62 binds LC3II, thereby recruiting cargoes to the phagophore. The phagophore develops into a sealed, double membrane structure called the autophagosome. The mature autophagosome then undergoes fusion with lysosomes to form autolysosomes. The hydrolases in the acidic lumen of the lysosomes then degrade the contents of the autophagosome.

The broad goal of my work was to investigate downstream mechanisms that facilitate communication between T1R1-T1R3 at the cell surface and intracellular mTOR localization and signaling (Fig 3). Heterotrimeric G proteins mediate signal transduction by GPCRs. Ligand binding induces conformational changes in the receptor that promote its interaction with G proteins. GPCRs act as guanine nucleotide exchange factors (GEFs) for the Gα subunit, resulting in activation and dissociation of the heterotrimeric G proteins into Gα and Gβγ subunits. These dissociated subunits subsequently interact with and activate a variety of downstream effectors that generate second messengers such as calcium, inositol triphosphate (IP_3), and cyclic adenosine monophosphate (cAMP), which modulate the activity of signaling pathways to elicit the appropriate cellular response [42]. Studies have shown that T1R3 signals through the G protein gustducin (which comprises $G\alpha_{i,}$ $G\beta_{3,}$ and $G\gamma_{13}$ subunits) to function in sweet taste [43] and umami perception [44] in the tongue as well as chemosensing in the brush cells of the small intestine and pancreas [45, 46] and in enteroendocrine cells [47]. It has also been reported that T1R1-T1R3 can signal through $G\alpha_s$ in neuroblastoma cells [48], and potentially through $G\alpha_{14}$ in the posterior tongue [49]. It is unknown if gustducin or other G proteins can relay information on amino acids from T1R1-T1R3 to mTORC1. However, studies from our laboratory using pertussis toxin, a potent inhibitor of $G\alpha_i$, suggest that amino acid activation of mTORC1 does not require $G\alpha_i$. While this does not preclude the possibility of other G proteins playing a role, it is reasonable to speculate that G protein independent mechanisms might link T1R1-T1R3 to mTORC1. Indeed, GPCRs can generate G protein independent signals by coupling to adapter or scaffold proteins that link the receptor to non-G protein regulated effectors. The best characterized of these G protein-independent signaling pathways involves the arrestins, which are a family of GPCR-binding proteins. The arrestins were first discovered for their role in facilitating desensitization of GPCRs to their agonists by promoting receptor endocytosis in a clathrin dependent manner [50-52]. Subsequent studies showed that arrestins can act as scaffolds for E3 ubiquitin ligases to facilitate receptor ubiquitylation and

degradation [53, 54]. Arrestins have also been implicated in regulation of receptor trafficking via modulation of the activities of small GTPases involved in vesicle trafficking, such as the Rabs (Ras-related proteins) and Arfs (ADP- ribosylation factors) [55, 56]. Additionally, the arrestins can act as scaffolds for components of multiple signaling pathways, thereby facilitating signaling downstream of GPCRs through these pathways [57, 58]. It has recently been shown that arrestins are required for mTOR dependent protein synthesis stimulated by angiotensin AT1 receptor (AT1R) activation in an AKT and ERK1/2 dependent manner [59]. Another recent study has demonstrated that β-arrestin 1 promotes autophagy in the brain as a neuroprotective mechanism under conditions of cerebral ischemic stress [60]. Such findings highlight the arrestins as attractive candidates for G protein independent relaying of information on amino acid availability from T1R1-T1R3 to mTORC1. This idea will be further explored in Chapter 3.

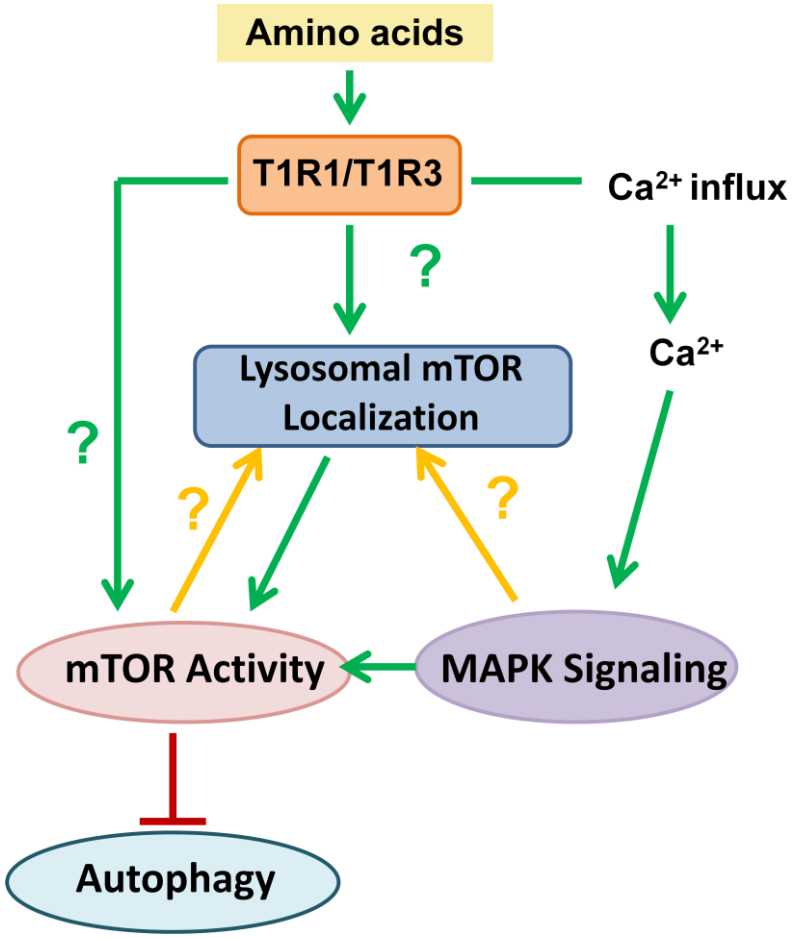


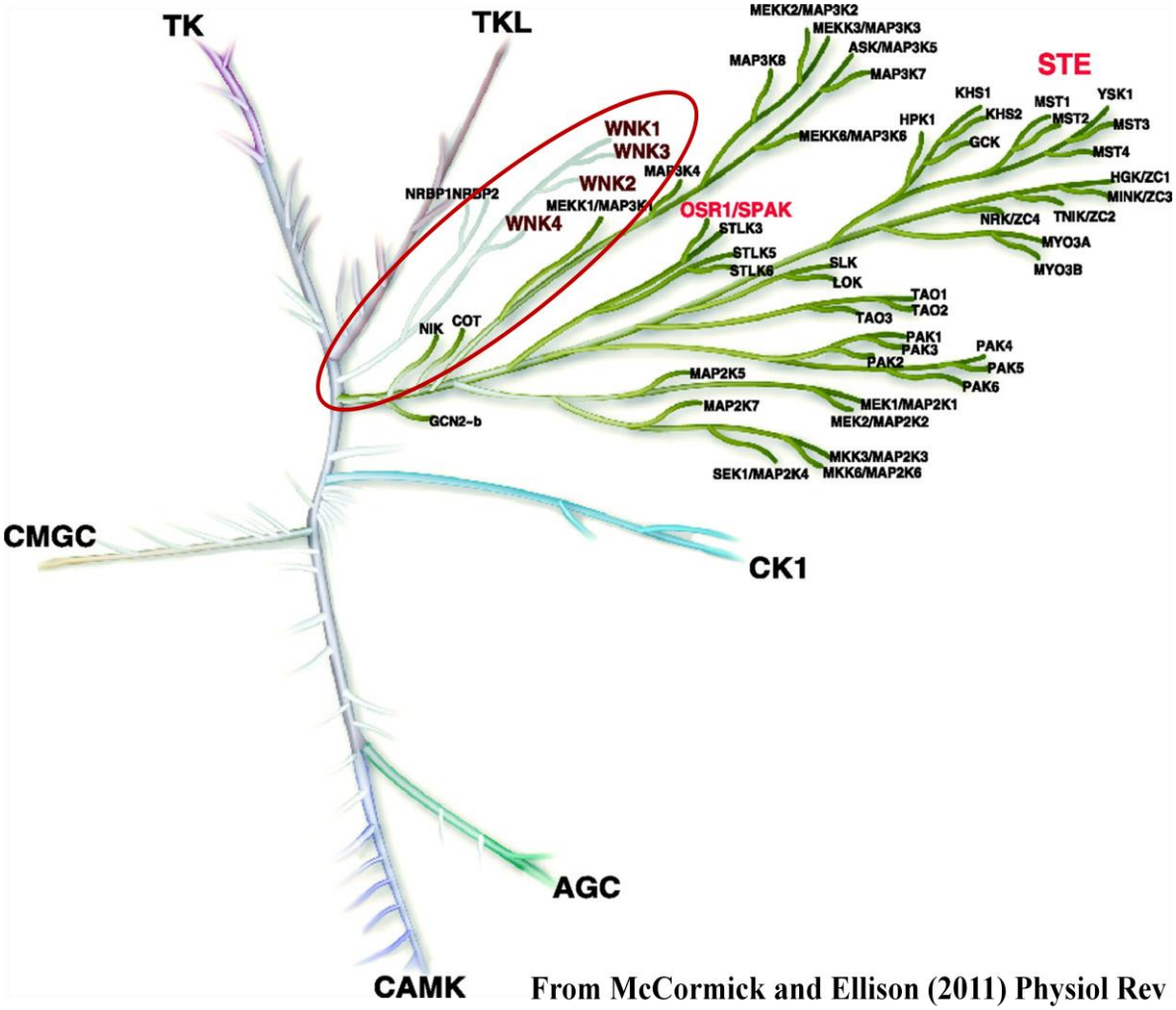
Fig 3: Role of the heterodimeric GPCR T1R1-T1R3 in amino acid- induced mTOR and MAPK signaling. Amino acids activate both mTOR and MAPK pathways through T1R1-T1R3. While stimulation of MAPK signaling downstream of this receptor is calcium dependent, mechanisms facilitating mTOR localization and activation are not clear. It is also unknown if mTOR and MAPK signaling participate in regulating mTOR localization.

PROJECT 2: STUDIES ON WNK1 PROTEIN DEGRADATION

A. With no lysine (WNK) kinases: A unique family of protein kinases with important physiological roles

Protein kinases are key mediators of cellular signaling. Collectively referred to as the kinome, this vast superfamily of proteins has been organized into families and subfamilies based on genomic DNA, complementary DNA, and expressed sequence tag (EST) sequences [61]. The WNK (With no lysine) family of protein kinases constitutes a unique branch of the kinome (Fig 4A) [62]. The WNK kinases were first isolated as part of a screen to identify novel members of the MAP kinase kinase family, and were so-named because of the unusual position of the catalytic lysine residue within its kinase domain that facilitates phosphoryl group transfer from ATP to the substrate[63]. This residue lies in beta-strand 2 (subdomain I) of the kinase domain in WNK1, and not beta-strand 3 (subdomain II), which is its location in all other protein kinases (Fig 4B) [64]. The WNK kinases appear to be highly conserved among multicellular organisms, with homologs identified in *Arabidopsis*, worms, flies, *Giardia*, frogs, zebrafish, mice and humans. Among unicellular organisms, WNK homologs were first found in some unicellular fungi like *Phycomyces*, but not in *Saccharomyces cerevisiae*. Mammals have four WNK kinases, WNK1, WNK2, WNK3 and WNK4 which range in size from approximately 2800 amino acids to 1200 amino acids (Fig 5) [65]. WNK1 is expressed in a wide variety of tissues, with higher expression reported in the testis, kidney, heart, brain, and skeletal muscle [66]. WNK2 is expressed mainly in the colon, heart, and brain [66, 67], while WNK3 shows low expression in kidney, brain, lung, liver and pancreas [66, 68]. WNK4 is expressed in the kidney, pancreas, colon, and skin [66, 69]. The four WNK family members share a conserved kinase domain with 85-90% sequence identity. Other conserved features include an autoinhibitory domain adjacent to the kinase domain that can suppress protein kinase activity [70], an autoinhibitory-related domain that may bind other proteins [71] and certain protein-interaction motifs, including coiled-coil domains and proline-rich motifs.

A



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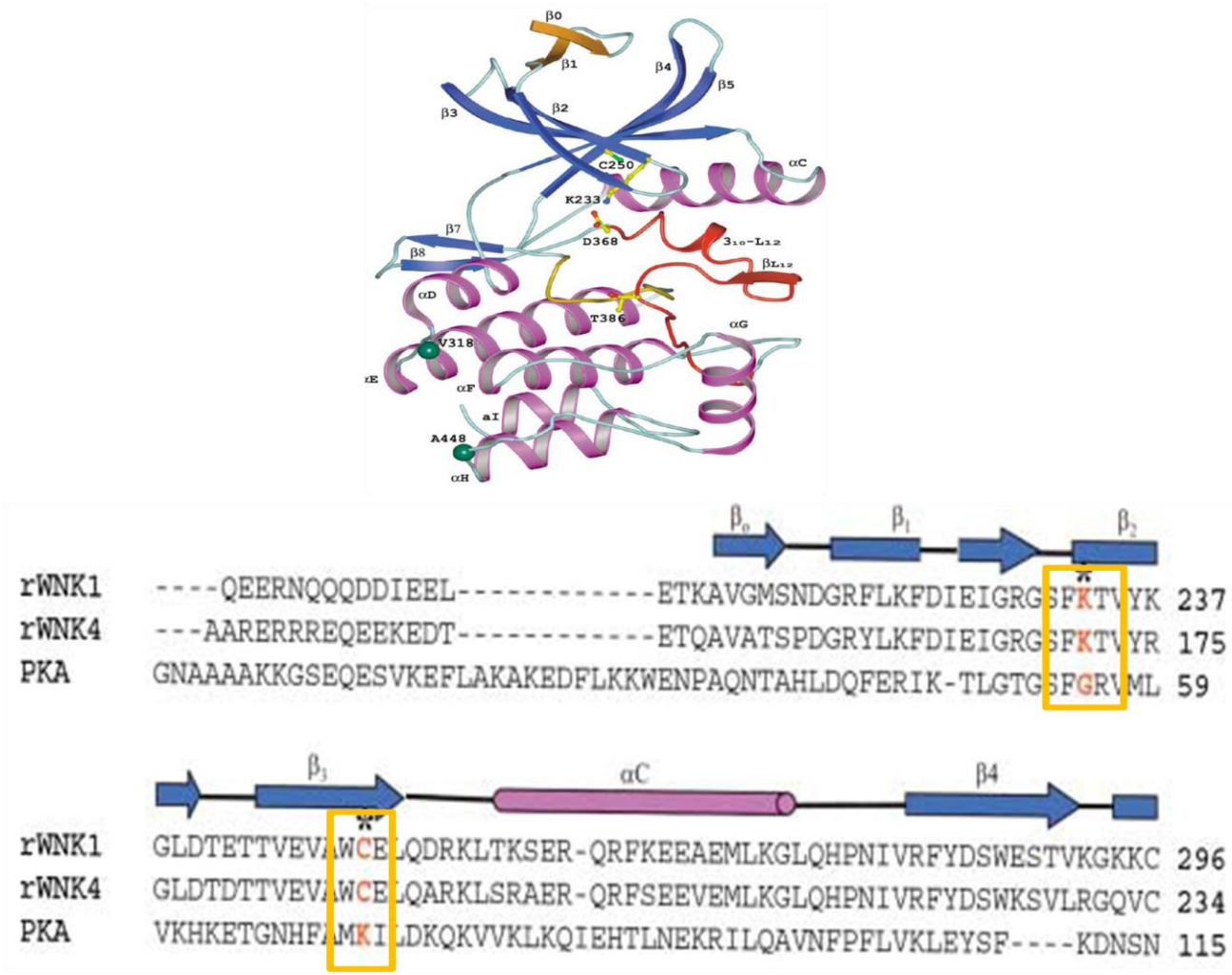

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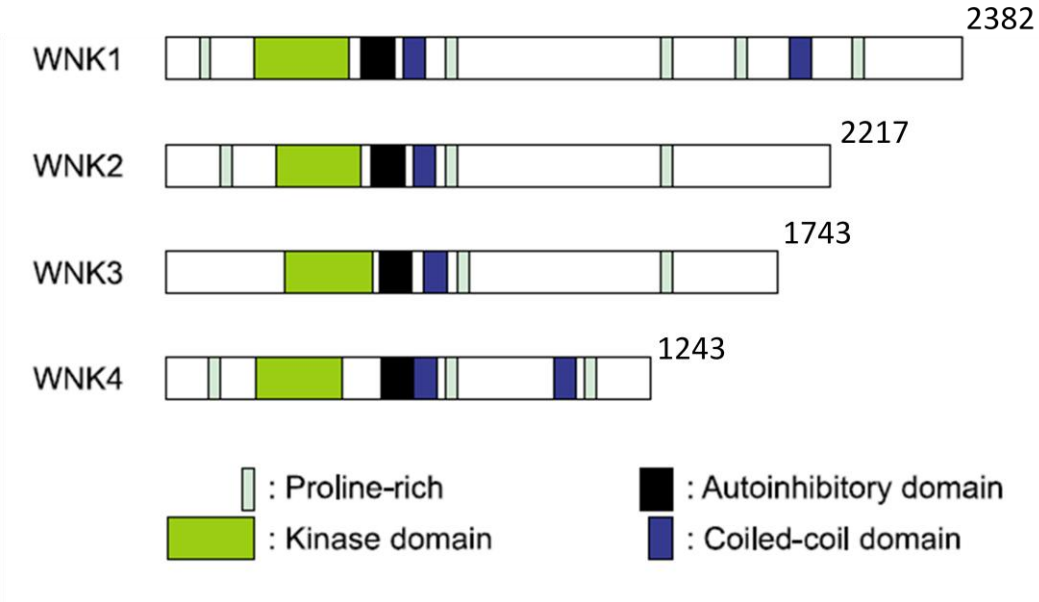
Fig 4: The WNKs are a unique family of protein kinases. (A) Representation of the human kinome showing the major families of kinases. WNKs (circled in red) comprise a distinct branch that is most closely related to the STE kinases. (B) Upper panel: Topology of the kinase domain of WNK1. Conserved beta strands are shown in blue and alpha helices are shown in magenta. The activation loop is shown in red and the catalytic loop in yellow. Cys250 in the β3 strand and K233 in the β2 strand are shown. Lower panel: Multiple sequence alignment of the kinase domain of WNK1 and WNK4 with that of a representative kinase protein kinase A (PKA). The catalytic lysine residue at position 250 in PKA is replaced by a cysteine in WNK kinases, with the catalytic lysine in WNKs residing at position 233. These sequence differences are highlighted by orange boxes.



Adapted from Huang *et al.* (2008) J. Cell Sci.

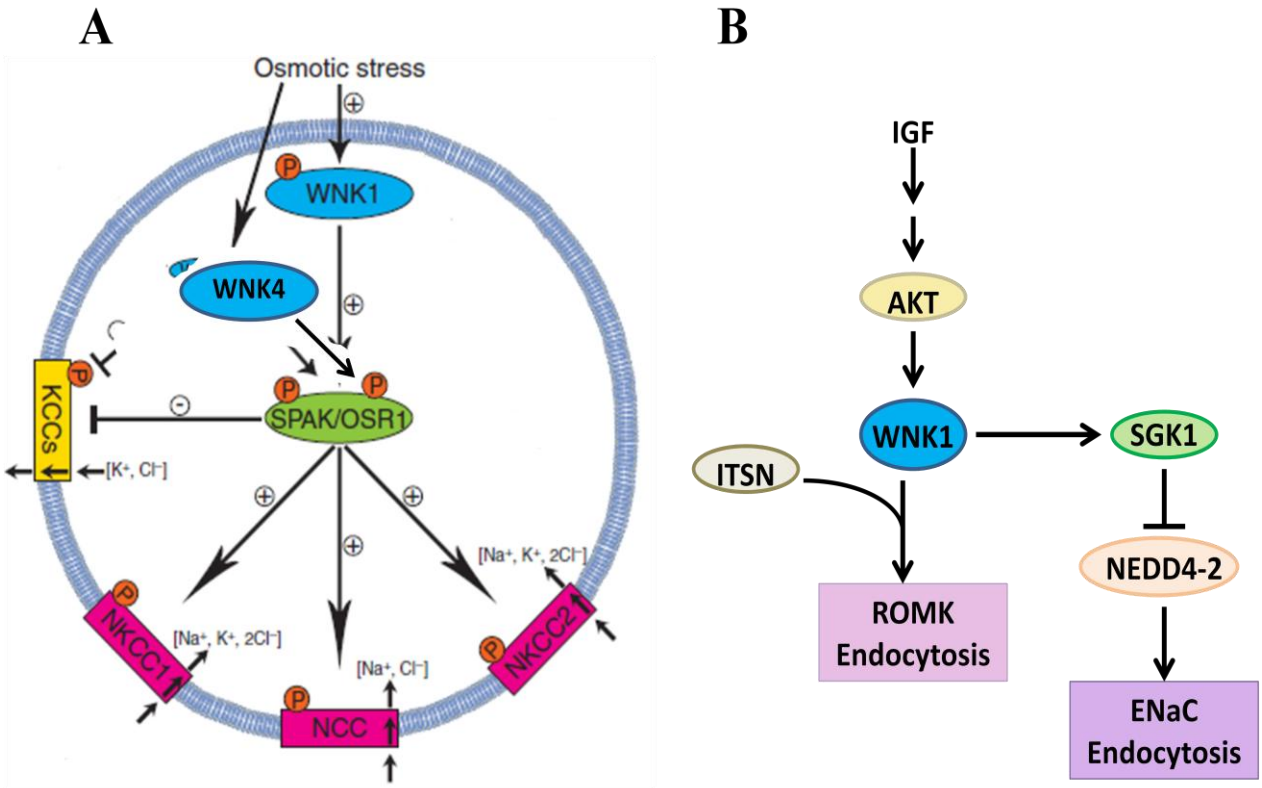
Fig 5: Domain structure of the four mammalian WNK kinases. Conserved domains are indicated by colored boxes. The highly conserved kinase domain spans approximately 270 amino acids near the N terminal end and is flanked by an autoinhibitory domain on its C terminal side. Multiple proline rich regions bearing PXXP motifs can serve as binding sites for proteins mediating signaling such as SH3 domain- containing proteins. Coiled coil domains serve as protein-protein interaction sites , and these might be especially important for WNK1 and WNK4, which each have a coiled coil domain in their C terminal regions in addition to the one adjacent to the autoinhibitory domain that is common to all four WNK kinases.

Apart from these domains, the remaining regions of WNK kinases do not share much similarity. These regions may contain protein interaction motifs that confer specificity and diversity of function for each of the WNK family members.

Soon after the discovery of WNK kinases, mutations in the genes encoding WNK1 and WNK4 were shown to cause a hereditary human disease called familial hyperkalemic hypertension (FHHt), also known as Gordon's syndrome, or, more commonly, pseudohypoaldosteronism type II (PHAII) [72]. Additionally, a number of polymorphisms in the genes WNK1 and WNK4 have been reported to be associated with blood pressure variability [73-77]. Consequently, research in the WNK field has focused mostly on understanding the role of WNK1 and WNK4 in regulating ion transport in the kidney. However, mutations in WNK family members have also been implicated in human diseases affecting tissues other than the kidney [78]. Mutations in WNK1 cause hereditary sensory neuropathy type II, an autosomal recessive disorder characterized by a loss of peripheral sensory nerves [79, 80]. Multiple studies have implicated WNK3 in development of schizophrenia and autism [81, 82]. Unbiased cancer genome sequencing studies have identified mutations in all four WNK family members in various types of cancers such as glioblastoma, multiple forms of lung cancer, melanoma, as well as breast, ovarian, and kidney cancer [83-87]. More recently, WNK1 expression in endothelial cells has been found to be essential for early angiogenesis in the developing mouse embryo. Mice with global or endothelial-specific WNK1 ablation die between embryonic day 10.5 to 12.5 due to multiple cardiovascular abnormalities such as smaller heart chambers with reduced myocardial trabeculation and failed yolk-sac vasculature remodeling [88, 89]. This variety of dysfunction in different organ systems indicates that WNK kinases have important and diverse physiological roles.

B. Cellular functions of WNK1

WNK1 plays a central role in cellular ion homeostasis. The only stimulus demonstrated to significantly increase WNK1 activity is osmotic stress [63, 90]. WNK1 can act as a sensor for chloride ions, which directly bind to WNK1 and stabilize its catalytically inactive form [91]. Under conditions of hypertonicity (0.5M NaCl or 0.5M sorbitol) or hypotonicity, WNK1 is activated by autophosphorylation at S382 and S378 within the activation loop of the kinase domain, as well as by suppression of autoinhibition [70, 92]. WNK1 then phosphorylates and activates two members of the sterile-20 family of kinases, oxidative stress responsive-1 (OSR1), and STE-20 related proline-, alanine-rich kinase (SPAK) at T185 and T233 respectively. These kinases then phosphorylate multiple members of a family of cation chloride cotransporters including the Na+K+2Cl- co-transporters 1 and 2 (NKCC1 and NKCC2), Na+Cl- co-transporter (NCC), and K+Cl- co-transporter (KCC) to modulate their activity and restore ion balance across the cell membrane [92, 93]. The other WNKs can also phosphorylate OSR1 and SPAK [94]. WNKs can also influence ion transport by modulating the membrane expression of the epithelial sodium channel (ENaC) [95] and renal outer medullary potassium channel (ROMK) [96]. Insulin-like growth factor 1 (IGF) stimulates AKT, which phosphorylates WNK1 at T58. WNK1 then activates the serum and glucocorticoid induced protein kinase SGK1 through a non-catalytic mechanism [97]. SGK1 phosphorylates and inhibits an E3 ubiquitin ligase that usually promotes ENaC endocytosis called neural precursor cell expressed developmentally downregulated 4-2 (NEDD4-2). This increases the membrane expression of ENaC, thereby promoting sodium reabsorption [98]. Upon treatment with insulin-like growth factor-1 (IGF-1) and activation of PI3 kinases, WNK1 promotes endocytosis of ROMK through a dynamin-dependent, clathrin-mediated pathway that requires interaction of WNK1 with the endocytic scaffold protein intersectin (ITSN) [99, 100]. Thus, WNK1 can control ion balance across the cell membrane through multiple mechanisms (Fig 6) [101].



Adapted from Richardson *et al.* (2008) *J. Cell Sci.*

Fig 6: Different mechanisms of WNK-mediated ion homeostasis. (A) Osmotic stress activates WNK1 and WNK4, which then phosphorylate and activate OSR1 and SPAK. These kinases then phosphorylate a variety of ion co transporters to modulate the flux of ions across the cell membrane. Phosphorylation promotes the activity of sodium/potassium/chloride cotransporters NKCC1, NKCC2 and NCC and inhibits the activity of potassium/chloride cotransporters (KCCs)**.** (B) Growth factor stimulation activates AKT, which phosphorylates WNK1 at T58. This promotes two actions of WNK1 on ion channel endocytosis. One pathway involves WNK1 promoting ROMK endocytosis in an intersectin and dynamin dependent manner. The other involves inhibition of ENaC endocytosis via kinase independent activation of SGK1 by WNK1, which promotes the phosphorylation and otherwise promotes ENaC endocytosis.

Cellular functions of WNK1 extend beyond ion homeostasis. Studies using pancreatic beta cells have uncovered roles for WNK1 in regulating exocytosis. A calcium-sensing membrane protein involved in membrane bending and fusion called synaptotagmin 2 interacts with and is phosphorylated by WNK1 in its calcium binding domains [102]. This may modulate the calcium-dependent interactions of synaptotagmin with membranes. Another mechanism by which WNK1 can regulate exocytosis is through interaction with the cytoplasmic scaffold protein Munc18. Unlike synaptotagmin, Munc18 does not appear to be phosphorylated by WNK1 [103]. MAPK signaling controls many vital cellular processes. Extracellular signal regulated kinase-5 (ERK5) is a MAPK that is activated by growth factors or stress, via phosphorylation by the MAP2K MEK5, the upstream regulator of which is the MAP3K MEKK2/3 [104]. WNK1 can bind to and activate MEKK2/3 in a kinase-independent manner, and this has been shown to promote ERK5 activation in response to epidermal growth factor (EGF) [105]. Another way in which WNK1 can modulate the cellular response to growth factors is by potentiating G protein-mediated PLCβ signaling [106]. The intracellular concentration of PIP_2 is the limiting factor for PLCβ activity and WNK1 increases PIP_2 synthesis by stimulating phosphatidylinositol 4-kinase IIIa (PI4KIIIa), a process promoted by the phosphorylation of WNK1 by AKT upon IGF stimulation. Transforming growth factor β (TGFβ) regulates cell proliferation, migration, differentiation and apoptosis through transcriptional regulator proteins called SMADs [107]. WNK1 can phosphorylate SMAD2 and depletion of WNK1 promotes SMAD-mediated changes in gene expression [108]. This suggests a role for WNK1 in regulating TGFβ signaling. As mentioned earlier, WNK1 knockout mice develop cardiovascular defects early in embryogenesis. It has recently been shown that WNK1 is required for proliferation, chemotaxis and migration of human umbilical vein endothelial cells (HUVECs) to form cords in vitro, with SPAK playing a role in proliferation and OSR1 playing a role in chemotaxis and

migration. The zinc finger transcription factor Slug appears to be involved in WNK1 mediated cord formation [109].

C. WNK1 expression and degradation

The human WNK1 gene is 160 kb and contains 32 exons. Transcriptional regulation of WNK1 is complex, with isoforms being generated by alternative promoter usage as well as by variation in splicing. Additionally, the presence of two 3' polyadenylation sites can generate variability in the stability and translation efficiency among these different isoforms. At least 10 different splice variants with tissue specific expression have been reported for human WNK1 [66, 79, 110, 111]. The full length form of WNK1 (L-WNK1) is derived from the proximal promoter and is ubiquitously expressed. The best characterized alternative isoform of WNK1 is a truncated variant expressed exclusively in the kidney known as kidney specific-WNK1 (KS-WNK1) [66, 110]. The transcript encoding KS-WNK1 is derived from an alternative promoter upstream of exon 4, and is identical to the full length WNK1 transcript beyond exon 5. KS-WNK1 can act a negative regulator of L-WNK1 [112], and regulates sodium reabsorption and potassium secretion in nephrons [113]. Putative transcription factors and transcriptional activators of WNK1 and KS-WNK1 have been predicted in silico, but await confirmation as bona fide regulators in vivo. Given the role of WNK1 in renal electrolyte balance and blood pressure control, studies on WNK1 and KS-WNK1 expression have focused on how alterations in dietary sodium/potassium and the mineralocorticoid aldosterone affect WNK1 expression. Manipulating dietary sodium or potassium affects the expression of KS-WNK1, but not L-WNK1 [114]. Aldosterone excess increases KS-WNK1 and L-WNK1 [114, 115]. Aldosterone might regulate KS-WNK1 through a putative glucocorticoid response element (GRE) that has been identified in the promoter region of KS-WNK1 but not L-WNK1 [110]. L-WNK1 expression can be regulated by the microRNA mir-192, the expression of which is controlled by aldosterone [115] and TGFβ.

PHAII is caused by mutations in WNK1 and WNK4 that lead to their increased expression [72]. The mutations in WNK1 are large deletions in intron 1, which causes increased expression of WNK1 in lymphocytes, increased KS-WNK1 and L-WNK1 expression in the distal convoluted tubule (DCT) of nephrons, and ectopic expression of KS-WNK1 [116]. Transcriptional insulators and repressors for mouse WNK1 have been identified in vitro in intron 1, but whether these mechanisms extend to gene expression of human WNK1 remains unknown [116]. In the case of WNK4, four missense mutations that cause PHAII have been identified, three of which are clustered within a region called the acidic degron motif encompassing amino acids 557-566 [72]. Recent studies have revealed that mutations in two genes Cullin 3 (CUL3) and Kelch like protein 3 (KLHL3) can also cause PHAII [117]. CUL3 is a scaffold protein that is a core component of cullin-RING-based BCR (BTB-CUL3-RBX1) E3 ubiquitin-protein ligase complexes which mediate the ubiquitination of various substrates [118]. KLHL3 is a member of the KLHL family of proteins that serve as substrate specific adapters for BCR complexes [119]. Recent studies have demonstrated that KLHL3 can bind WNK1 and WNK4 at their acidic degron motifs to facilitate ubiquitination by the KLHL3-CUL3-RBX1 complex, which promotes degradation of WNK1 and WNK4 (Fig 7) [120]. Disease-causing mutations in KLHL3 abolish its ability to bind to WNK4. Thus, excess WNK4 expression and activity is thought to be the basis of the molecular pathophysiology of PHAII caused by KLHL3 and CUL3 mutations. [120-123]. In response to volume depletion, angiotensin signaling via AT1R activates protein kinase C α and β (PKCα and PKCβ), which phosphorylate KLHL3 to abrogate its binding to WNK4 [124]. It has been shown that another member of the KLHL family, KLHL2, can regulate WNK3 expression to control vascular tonus in smooth muscle cells [125]. Findings such as these highlight the importance of controlling the expression of WNK family members for cellular and physiological homeostasis.

The studies mentioned above identify specific proteins and signaling events involved in WNK4 degradation, but it is not clear if all of these mechanisms extend to WNK1. Both short-term and chronic deletions of CUL3 loss in mice increase WNK4 expression to similar extents. However, acute loss of CUL3 increases WNK1 to a much greater extent than chronic loss does. Perhaps chronic CUL3 loss can induce compensatory mechanisms that promote WNK1 degradation, but not WNK4 degradation [126]. These observations demonstrate that WNK1 expression is tightly regulated and may involve other molecular players in addition to CUL3 and KLHL3.

Preliminary data from our laboratory suggest that other E3 ubiquitin ligases may also interact with WNK1. The role of one such E3 ligase, UBR5 (ubiquitin protein ligase E3 component N-recognin 5) in WNK1 expression and function will be investigated in Chapter 3. UBR5, also called EDD (E3 ligase identified by differential display) was first identified in human T47D breast cancer cells as a transcript encoding a 300 kilodaton protein, and is the mammalian homolog of the Drosophila tumor suppressor protein Hyd (hyperplastic discs protein) [127]. A member of the HECT (Homologous to E6-AP carboxyl terminus) domain containing family of E3 ligases, UBR5 belongs to a class of E3 ligases called the N-recognins, which typically recognize N terminal degrons. In addition to its HECT domain, which possesses E3 ligase activity, UBR5 contains a PABC domain (Polyadenylate binding protein C terminal domain) and a Zinc-finger domain. UBR5 has been shown to play a role in regulating micro-RNA mediated gene silencing which is independent of its E3 ligase activity and is mediated by its PABC domain [128]. UBR5 has been demonstrated to promote the degradation of various proteins, including nuclear receptors like estrogen receptor α and pregnane-X receptor, metabolic enzymes like PEPCK (phosphoenol pyruvate carboxy kinase), and phosphatases like PP2Ac [129-132]. Like WNK1 knockout mice, UBR5 knockout mice display failed yolk-sac vasculature remodeling and chorioallantoic fusion, and die between E10-E12 [133].

Although specific molecular players like CUL3 and KLHL3 have been identified as regulators of WNK1 protein amount, the roles of different cellular protein degradation pathways in controlling WNK1 stability have not been investigated. Cells employ four major pathways to degrade proteins: (1) the ubiquitin-proteasome system (2) lysosomal degradation by autophagy or by fusion of endosomes bearing internalized cell surface proteins with lysosomes (3) non-lysosomal proteolysis by cytoplasmic proteases, and (4) endoplasmic-reticulum associated degradation. The 26S proteasome is a 2.5 megadalton multi-subunit complex composed of a 20S proteolytic core and one or two 19S regulatory particles. The 20S core consists of four hetero-heptameric rings that are stacked to form a cylindrical structure with a central lumen. These rings are composed of alpha and beta subunits, and the beta subunits possess different peptide bond hydrolyzing activities, with their catalytic residues facing the central channel of the 20S core [134]. The 19S particle serves as the recognition site for ubiquitinated substrates, and promotes de-ubiquitination, unfolding, and translocation of those substrates into the core in an ATP-dependent fashion [135, 136]. Proteins that are destined to undergo proteasomal degradation are typically marked by poly-ubiquitination, but studies have shown that mono-ubiquitination and multi-monoubiquitination can also target proteins to the proteasome [137, 138]. The 26S proteasome is thought to degrade mostly short-lived regulatory proteins. Long-lived proteins, organelles and protein aggregates are degraded by lysosomes through the process of autophagy. Based on how cargoes are recruited to lysosomes, autophagy can be classified into three types: macro-autphagy, micro-autophagy, and chaperone-mediated autophagy [139]. Macro-autophagy has been discussed in Project 1. Micro-autophagy differs from macro-autophagy in that cargoes are directly engulfed by lysosomes through invaginations of lysosomal membranes and do not require autophagosome formation for their sequestration. The process can be both selective and non-selective in cargo recruitment, and has been reported to contribute to degradation of peroxisomes and parts of the nucleus [140-142].

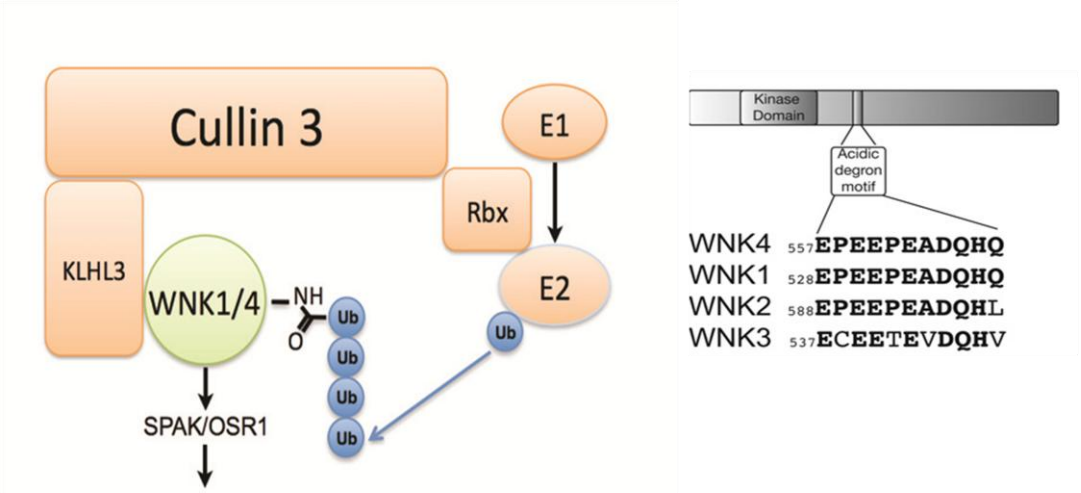


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Fig 7: Cullin3 and KLHL3 regulate WNK1/WNK4. KLHL3 can recruit WNK1 and WNK4 to the BCR (BTB-CUL3-RBX1) E3 ligase complex, which then catalyzes the transfer of ubiquitin from E2 proteins to specific lysine residues on WNK4/WNK1. KLHL3 binds WNK4 and WNK1 at their acidic degron motif, which is near the autoinhibitory domain.

Chaperone-mediated autophagy is a highly selective process in which the chaperone Hsc70 (Heat shock cognate 70) recognizes and binds cytosolic proteins bearing KFERQ motifs and then delivers these proteins to lysosomal surfaces, where they bind to the lysosomal membrane protein LAMP2A, which oligomerizes to form a channel-like structure. The protein then undergoes unfolding and translocation through LAMP2A from the cytosol into the lysosomal lumen, where it is degraded by lysosomal hydrolases [143]. Proteins that have been identified as CMA substrates include GAPDH (glyceraldehyde-3-phosphate dehydrogenase), ribonuclease A, HIF1α (hypoxia inducible factor 1), and LRRK2 (Leucine rich repeat kinase 2) [144-146]. Another system of proteolysis involves a family of non-lysosomal calcium-dependent cysteine proteases called the calpains, which comprise fifteen members, nine of which are ubiquitous in expression [147]. These proteases exert their effects through limited proteolysis of substrates, unlike the extensive proteolysis that occurs in proteasomes and lysosomes. Calpains can act on a wide variety of substrates, and sites of cleavage have been identified *in vitro* for some of these substrates [148]. While a specific recognition motif for calpains has not been identified, *in vitro* peptide cleavage and sequencing studies as well as bioinformatic analyses have revealed amino acid sequence preferences that influence recognition of substrates by calpain [149, 150]. The most well-known preference displayed by calpains is for hydrophobic amino acids. Finally, ERAD is a quality control process that monitors the synthesis and folding of proteins made in the ER and mediates the degradation of mis-folded proteins. Once they are tagged for degradation, mis-folded proteins are unfolded and translocated out of the ER into the cytosol, where they are degraded by the proteasome .

Chapter 4 will describe my efforts to examine the roles of the proteasome, lysosomes, calpains, and protein chaperones in WNK1 degradation, and to determine whether UBR5 plays a role in regulating WNK1 stability.

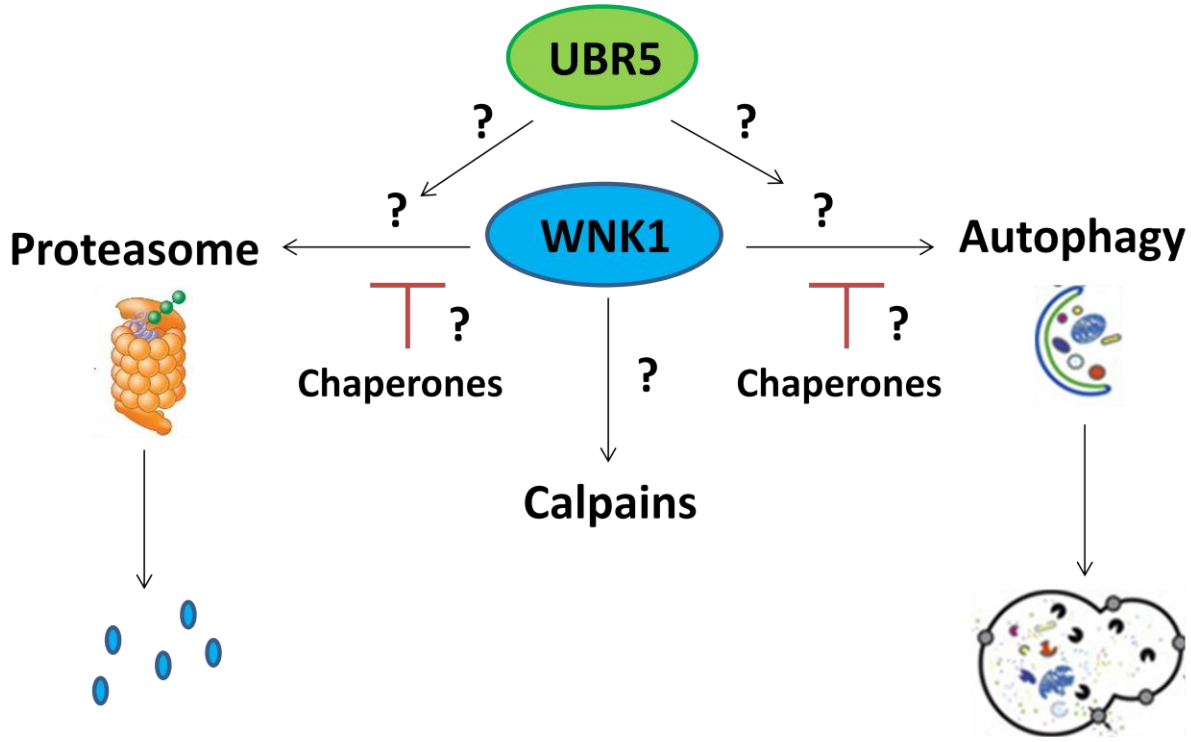


Fig 8: Unaddressed questions about WNK1 degradation. Proteasomal degradation, lysosomal degradation by autophagy, and calpain-mediated proteolysis are three major mechanisms of protein disposal in cells. Which of these pathways contribute to WNK1 degradation? Do chaperones play a role in promoting WNK1 stability? Does UBR5 affect WNK1 degradation?

CHAPTER 2:

Investigating gene expression and mTOR localization in the cellular response to amino acids

MATERIALS AND METHODS:

Cell Culture, drug treatments and transfections:

H9C2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1mM glutamine at 37˚C and 5% CO_2. For knockdown experiments, 130,000 cells per well were transfected with 10nM negative control siRNA (Sigma) or T1R3 siRNA (Sigma) using Lipofectamine RNAiMAX reagent (Life Technologies), and Opti-MEM reduced serum medium (Life Technologies) as per the manufacturer's instructions. HeLa cells or Hep3B cells were maintained in DMEM at 37˚C and 5% CO_2. For amino acid starvation, DMEM was removed, cells were washed once with Krebs-Ringer HEPES Buffer (KRBH) (115mM NaCl, 5mM KCl, 24mM $NaHCO_3$, 1mM $MgCl_2$-$6H_2O$, 2.5mM $CaCl_2$-$2H_2O$, 25mM HEPES pH 7.4) containing 4.5 mM glucose and 0.1% BSA, and then incubated in KRBH for the indicated times before a mixture of amino acids (AA) was applied for 30 minutes. Cells were treated with PD0325901, rapamycin, KU0063794 (Selleck Chemicals), or DMSO as control for the indicated time periods and doses.

RNA extraction, Reverse Transcription, and Quantitative PCR:

RNA was extracted 72 hours after siRNA treatment using Tri-reagent (Ambion) as per the manufacturer's instructions. 2 μg RNA was reverse transcribed using the iTAQ™ DNA polymerase cDNA synthesis kit (BioRad). qPCR was performed using the Bio-Rad CFX-96 Real Time PCR instrument with SYBR green. Primers used were as follows: 18S (forward 5'-CGCGGTTCTATTTTGTTGGT-3'); (reverse 5'-AGTCGGCATCGTTTATGGTC-3'), Slc3a2 (forward 5'-GCCCAATTCACAAGAACCAG-3'); (reverse 5'-CAGAGCCTCCTTCATTTTGG-3'), Slc7a11 (forward 5'GATGGTTCTAAATAGCACGAGT-3'); reverse (5'-GGGCAACCCCATTAGATTTGT-3'), Slc7a3 (forward 5'-TCCA GATTTCTTTGCCTTGG-3');

(reverse 5'-CCTCTTTCGTGAGCTTCCAG-3'), Slc16a1 (forward 5'-TCTGTAACACGGTGCAGGAA-3'); (reverse 5'-GGAG CCAGGGTAGAGAGGAA-3').

Immunofluorescence Microscopy:

Cells were grown on glass coverslips and treated as described above. They were then washed once with phosphate buffered saline (PBS) and fixed with 4% para-formaldehyde (PFA) in phosphate PBS for 10 minutes at 37˚C. Coverslips were washed three times with PBS to remove excess PFA. Fixed cells were then permeabilized using 0.5% Triton in PBS for 5 minutes at 4˚C and coverslips were washed three times with PBS to remove excess Triton. Blocking was performed with normal goat serum (NGS) (Life Technologies) for 1 hour at room temperature. Incubation with primary antibodies to mTOR (Cell Signaling Technology, 1:200) and LAMP2 (Abcam, 1:200) in NGS was performed at 4˚C overnight. Following 3 washes with PBS to remove excess primary antibodies, coverslips were incubated with anti-mouse and anti-rabbit antibodies (Life Technologies 1:2000) diluted in NGS for 2 hours at room temperature. After washing three times with PBS to remove excess secondary antibodies and once with distilled water to remove salts, coverslips were mounted on glass slides using Dapi-Fluoromount G (Southern Biotech), which contained DAPI for visualization of nuclei. Confocal images were obtained using Leica TCS SP5 confocal microscope with a slice thickness of 0.2μm. Images were analyzed using ImageJ software.

SECTION I: T1R3 DEPLETION IMPACTS GENE EXPRESSION OF AMINO ACID TRANSPORTERS

BACKGROUND:

Altering gene transcription is an important mechanism for cellular adaptation to changing nutritional status. Amino acid deprivation is known to activate the expression of several genes involved in amino acid biosynthesis and other cellular processes [151]. Promoter analysis of two such genes, CCAAT/enhancer-binding protein homologous protein (CHOP) and asparagine synthetase (AS), has revealed that a cis-element, designated the amino acid response element (AARE) in the CHOP gene or the nutrient sensing response element-1 (NSRE-1) in the AS gene, regulates the induction of these genes in response to deprivation of several individual amino acids [152, 153]. Several transcription factors such as activating transcription factor 2 (ATF2), ATF3, ATF4, ATF7, C/EBPα, C/EBPβ, C/EBPγ, and C/EBPδ have been demonstrated to interact with these elements in vitro [154]. In addition to transcription factors like CHOP and enzymes like AS, amino acid transporters are also regulated by amino acid abundance. Studies have shown that AARE's are involved in transcriptional regulation of two amino acid transporters, a cationic amino acid transporter, CAT1 (SLC7A1), and a sodium-coupled neutral amino acid transporter, SNAT2 (SLC38A2) in response to amino acid deprivation [155, 156].

Because amino acids activate mTOR, regulators of their transport could also serve as regulators of mTOR signaling. Consistent with this idea, bidirectional transport of amino acids, particularly the shuffling of glutamine in and out of cells, has been implicated in the regulation of mTOR and autophagy. Members of the solute-linked carrier (SLC) family of transporter proteins that control amino acid uptake and efflux such as SLC1A5, SLC7A5 and SLC3A2 appear to be key players in this process. SLC1A5 regulates glutamine uptake and loss of SLC1A5 function inhibits cell growth and activates autophagy. SLC7A5/SLC3A2 is a bidirectional transporter, facilitating the

simultaneous efflux of glutamine out of cells and uptake of leucine and other essential amino acids into cells.

Cell surface receptor expression and function can influence gene expression in the nucleus, which can then modulate signaling in the cytosol. An unaddressed question was whether T1R3 employed transcriptional mechanisms to impinge on mTOR signaling. As mentioned previously, mTOR activity is reduced upon T1R3 depletion. Given the role of SLC1A5 and SLC7A5/SLC3A2 in mTOR signaling, could T1R3 regulate mTOR activity by regulating the expression of these transporters? Also, could T1R3 regulate the expression of other amino acid transporters? To address these questions, I assessed the effect of T1R3 loss on the expression of SLC3A2, SLC1A5, and other known transporters including SLC7A11 (cystine-glutamate antiporter), SLC7A3 (cationic amino acid transporter), SLC16A1 (proton-coupled monocarboxylate transporter), and SLC6A6 (sodium and chloride-dependent taurine transporter).

RESULTS AND DISCUSSION:

qPCR analysis of RNA from H9C2 cells treated with control or T1R3 siRNA revealed that mRNA expression of SLC1A5, SLC7A11, and SLC16A1 were up-regulated approximately 4 fold, while that SLC3A2 was up-regulated by approximately 2 fold. SLC7A3 expression was the most affected, being increased 14 fold. SLC6A6 expression was slightly decreased. These observations indicate that the reduction of mTOR activity upon T1R3 loss cannot be attributed to decreased expression of SLC1A5 or SLC7A5/SLC3A2. The overall trend of increased transporter expression suggests that T1R3 loss may be interpreted by cells as an amino acid starvation stress signal and this induces different compensatory mechanisms to combat this perceived stress (Fig 9).

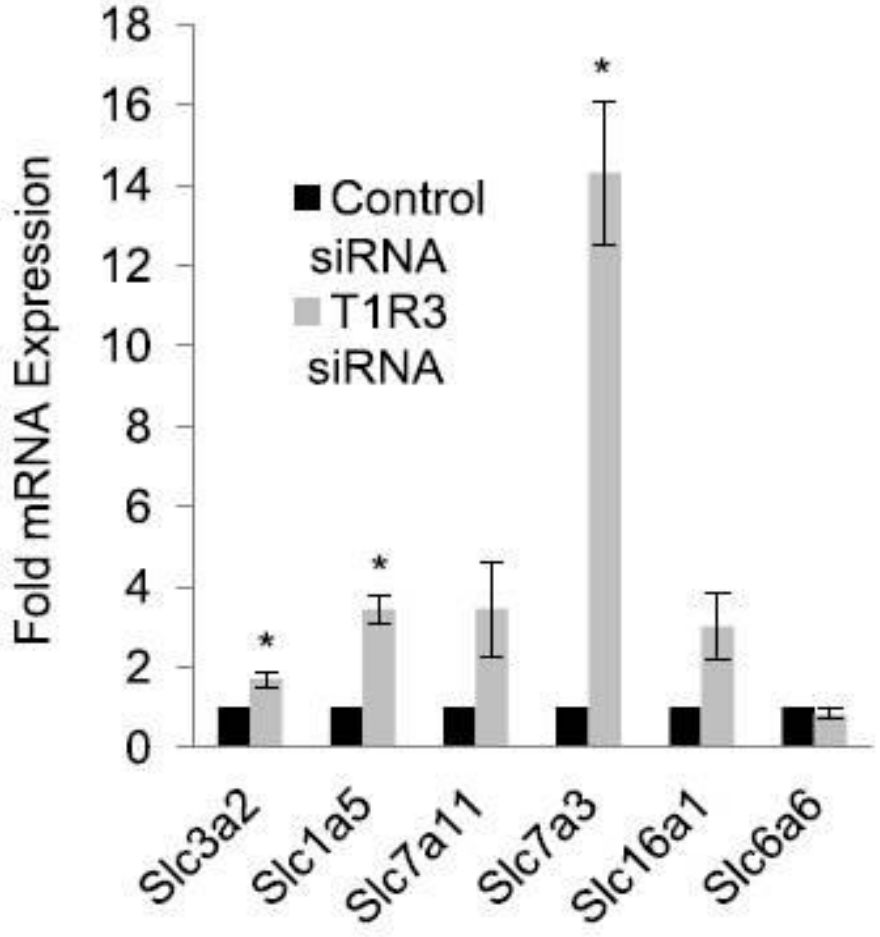


Fig 9: T1R3 knockdown increases mRNA expression of amino acid transporters RNA extracted from H9C2 cardiomyoblast cells transfected with control siRNA or T1R3 siRNA was analyzed by qPCR. Data are the mean +/- SEM of three independent experiments. *$p < 0.05$

The increased expression of SLC1A5 and SLC3A2 may represent one compensatory mechanism induced to maintain mTOR signaling upon perceived starvation. Another such mechanism is the reduction of expression of mTOR inhibiting proteins TSC2 and REDD1 upon T1R3 loss [34].

SLC7A3 is a member of the cationic amino acid transporter (CAT) family of proteins and is therefore also called CAT3. Association of CAT family function to mTOR signaling has been demonstrated in *Drosophila.* CAT1 and CAT3 are homologs of the Drosophila growth modifier gene *Slimfast*, and depletion of *Slimfast* results in growth defects resembling those associated with nutrient deprivation or genetic deficiency in the mTOR pathway [157]. In the context of neuronal function, CAT1 and CAT3 have been shown to mediate the effects of the neurotransmitter NMDA (N-methyl-D-aspartate) on mTOR dependent protein synthesis and nerve growth [158]. As mentioned in Chapter 1 (Project 1), the lysosomal arginine sensor SLC38A9 has recently been implicated in signaling arginine sufficiency to mTOR [31]. Multiple studies have linked the arginine degrading enzymes arginase-1 and arginase-2 to mTOR and autophagy regulation in different contexts [159-161]. These findings indicate that arginine can employ multiple mechanisms to regulate mTOR activity in addition to general pathways used by all amino acids. A recent study suggests that arginine can regulate autophagy independent of mTOR [162]. Thus, cationic amino acids can engage diverse signaling pathways to regulate cellular nutrient responses. Studies suggest that SLC7A3 can play a role in signaling modules other than the mTOR pathway. The uptake of arginine by CAT family members is an important source of substrate for the synthesis of the second messenger nitric oxide in cells [163]. A recent study has demonstrated that ablation of SLC7A3 in zebrafish leads to abnormal lipid retention in the liver during fasting, with impaired nitric oxide synthesis and impaired APMK-PPARα (peroxisome proliferator activated receptor α) signaling underlying the phenotype [164]. Thus, it is possible that T1R3 depletion might alter signaling of other pathways in addition to the mTOR pathway, and cells may employ increased SLC7A3 expression to combat these changes as well.

SLC7A11 (also called xCT) transports the anionic form of cystine into the cell in exchange for glutamine efflux. Its expression has been demonstrated to be induced under amino acid starvation [165]. Therefore, the increase in its expression is indicative of T1R3 loss mimicking starvation. SLC7A11 has been implicated in normal and pathological extra-synaptic glutamatergic signaling in the brain [166], and is emerging as a potential regulator of glutamate signaling in bone [167]. In light of these findings, it is reasonable to infer that increased SLC7A11 in the context of T1R3 depletion may be a mechanism to specifically adjust glutamate signaling. The uptake of cystine by SLC7A11 has been shown to be important for glutathione synthesis [168], which is required for protection against cellular oxidative stress. Hence, oxidative stress also increases expression of this transporter.

SLC16A1 (also called monocarboxylate transporter 1 or MCT1) catalyzes the import of key metabolites such as lactate and pyruvate, as well as acetate, beta-hydroxy butyrate, and branched chain oxo-acids derived from valine, leucine and isoleucine. SLC16A1 is a key regulator of metabolism. It appears to be involved in the intestinal absorption of nutrients and certain drugs [169]. It is highly expressed in glycolytic tumors, and has been demonstrated to be an effective therapeutic target for such tumors [170]. Its expression is specifically repressed in pancreatic beta cells to prevent inappropriate insulin release in response to exercised-induced lactate production by muscles, and mutations in the promoter region of the gene cause congenital hyperinsulinism [171, 172]. SLC16A1 expression has been demonstrated to increase during fasting [173] and ketosis [174]. Thus, the increase of SLC16A1 mRNA in the context of T1R3 depletion is consistent with the idea that cells perceive and respond to T1R3 loss as a starvation signal. SLC16A1 is a target of the transcription factors Myc [175] and PPARα [176]. In a micro-array experiment analyzing gene expression in MIN6 cells, T1R3 knockdown appeared to increase Myc expression. It is possible that increased Myc expression upon T1R3 depletion could drive increased SLC16A1 expression.

SLC6A6 (also called the taurine transporter TauT) is a sodium-dependent transporter that mediates the uptake of the cysteine-derived sulfonic acid taurine and beta-alanine. Although it is not incorporated into proteins, taurine is ubiquitous, and studies in SLC6A6 knockout mice have shown that taurine transport is important for normal functioning of different organs. [177]. Taurine has physiological roles in the heart and in skeletal muscle [178]. For instance, taurine can protect against chronic heart failure by counteracting the harmful effects of catecholamines and angiotensin II [179], and can reduce cardiac damage due to myocardial infarction through antioxidant and osmoregulatory actions [180]. Through modulation of ion channel gating and calcium homeostasis in skeletal muscle tissue, taurine can also have beneficial effects on muscular dysfunction [181]. Assessing the effect of T1R3 loss on SLC6A6 expression in H9C2 cardiomyoblast cells seemed pertinent in light of this information. In contrast to the other transporters, SLC6A6 expression was modestly reduced upon T1R3 loss. Thus, T1R3 might not play a major role in taurine sensing and transport.

In addition to transcriptional regulation, transporter expression is can be regulated post-transcriptionally, and functionality is often regulated by modulating membrane expression via endocytosis. The impact of these changes in mRNA expression on total protein expression and membrane expression of each of these transporters remains to be examined.

SECTION II: INTERROGATING ERK AND mTOR INPUT INTO mTOR LOCALIZATION CONTROL

BACKGROUND:

Translocation of mTOR from the cytosol to lysosomal surfaces is critical for its activation. Under conditions of amino acid starvation, mTORC1 appears to be dispersed in the cytosol, as assessed by immunofluorescence. Upon stimulation with amino acids, mTORC1 distribution appears punctate, and these puncta appear to colocalize with late endosomes and lysosomes. It was observed that T1R3 depletion in HeLa cells resulted in dispersed cytosolic distribution of lysosomes, and impaired amino acid-induced lysosomal mTOR localization [34]. How T1R3 could impinge on mTOR localization and lysosome positioning was not clear. Various molecular players like the Rag GTPases, components of the Ragulator complex, and the TTT-RUVBL1/2 complex have been shown to be required for this localization and for assembly of the mTORC1 complex. However, the contributions of nutrient-responsive signaling pathways, such as the MAP kinase pathway, the PI3K-AKT pathway, and the mTOR pathway itself to regulation of mTOR localization have not been examined. Maturation and positioning of late endosomes/lysosomes have been found to be important for regulating mTORC1 and autophagy [182, 183]. Conversely, mTORC1 has been found to be required for the maturation of lysosomes [184]. Chronic inhibition of ERK1/2 has been shown to alter the cytosolic distribution of lysosomes from a punctate pattern to a more dispersed pattern [185]. Amino acid-induced mTOR and ERK1/2 activation is reduced by T1R3 knockdown. Therefore, one hypothesis that emerges from these data is that T1R3 might influence mTOR localization via MAPK and mTOR signaling. While the idea of mTOR activity possibly controlling mTORC1 localization may seem counterintuitive, there is evidence in the literature for the activity of certain kinases controlling the localization of complexes containing those kinases [186].

RESULTS AND DISCUSSION:

I used pharmacological inhibition of MAPK and mTOR pathways to interrogate the role of these pathways in mTOR localization. Treatment of Hep3B cells with PD0325901, a specific inhibitor of MEK1/2, for one hour did not impair amino acid-induced mTOR localization and did not induce lysosomal dispersion (Fig 10). This is in contrast with findings in other studies [185], which treated cells with the inhibitor for 24 hours or longer and used different cell lines. Rapamycin treatment had an unexpected effect on mTOR localization. Amino-acid induced lysosomal localization of mTOR was not impaired by rapamycin treatment. However, under conditions of starvation, rapamycin promoted the persistence of mTOR at lysosomes unlike what occurred in untreated cells, in which mTOR was dissociated from lysosomes (Fig 11A). Rapamycin treatment did not alter total mTOR or LAMP2 protein in cells (Fig 11B). This observation was reproducible in several different cell lines, including HeLa as well as normal and transformed human bronchial epithelial cells (HBEC). Treatment with another mTOR inhibitor KU0063794 did not affect mTOR localization during either amino acid starvation or amino acid stimulation (Fig 12).

Rapamycin and KU0063794 have different mechanisms of action. KU0063794 is a small molecule ATP competitor that inhibits both mTORC1 and mTORC2 [187]. Therefore, the lack of effect of KU0063794 on mTOR localization suggests that mTOR kinase activity is not required for mTOR to dissociate from lysosomes or for mTOR to associate with lysosomes. Rapamycin is a macrolide antibiotic that binds FKBP12 (FK-binding protein 12). This complex then binds to a conserved region of mTOR called the FRB domain [188]. Studies have shown that rapamycin disrupts the association of the mTORC1 component Raptor with mTOR [189]. Raptor binds the mTOR substrates p70 S6 kinase and 4EBP1 and facilitates their phosphorylation [190]. Therefore, rapamycin is an allosteric inhibitor of mTOR activity that acts

by blocking access to substrates. Addiotionally, studies have suggested that functional mTORC1 is dimeric [13, 191], and a cryo- electron microscopy study has shown that rapamycin can disrupt these dimers in vitro [191]. Although mTORC2 was initially believed to be insensitive to inhibition by rapamycin, it has been shown that chronic rapamycin treatment can also cause disassembly and deactivation of mTORC2 [192].

One possible explanation for rapamycin-induced persistence of mTOR at lysosomes during starvation could be that monomeric mTORC1 generated by rapamycin treatment might be able to associate more strongly with lysosomes under amino acid starvation conditions than dimeric mTORC1. Another possibility is that rapamycin-FKBP12 binding could either induce a conformational change that exposes a region of the protein that is otherwise occluded, or block interaction of other proteins in addition to Raptor, and these events promote abnormal association of mTOR with proteins at the lysosomal surface. While molecular players regulating translocation of mTORC1 to the lysosome have been intensely studied, less is known about mechanisms regulating dissociation of mTORC1 from lysosomes upon starvation and other stresses. Recent studies have shown that stress-responsive proteins called Sestrins can negatively regulate the GATOR2 complex to suppress RagB-dependent mTORC1 lysosomal localization [193, 194] during amino acid starvation. Whether and how rapamycin could inhibit the actions of these proteins during starvation is not clear. Perhaps the extraction of mTOR from membranes is an active process that requires association with other proteins through the FRB domain. In summary, rapamycin-induced lysosomal mTOR accumulation suggests that there are aspects of mTORC1 localization control that await discovery.

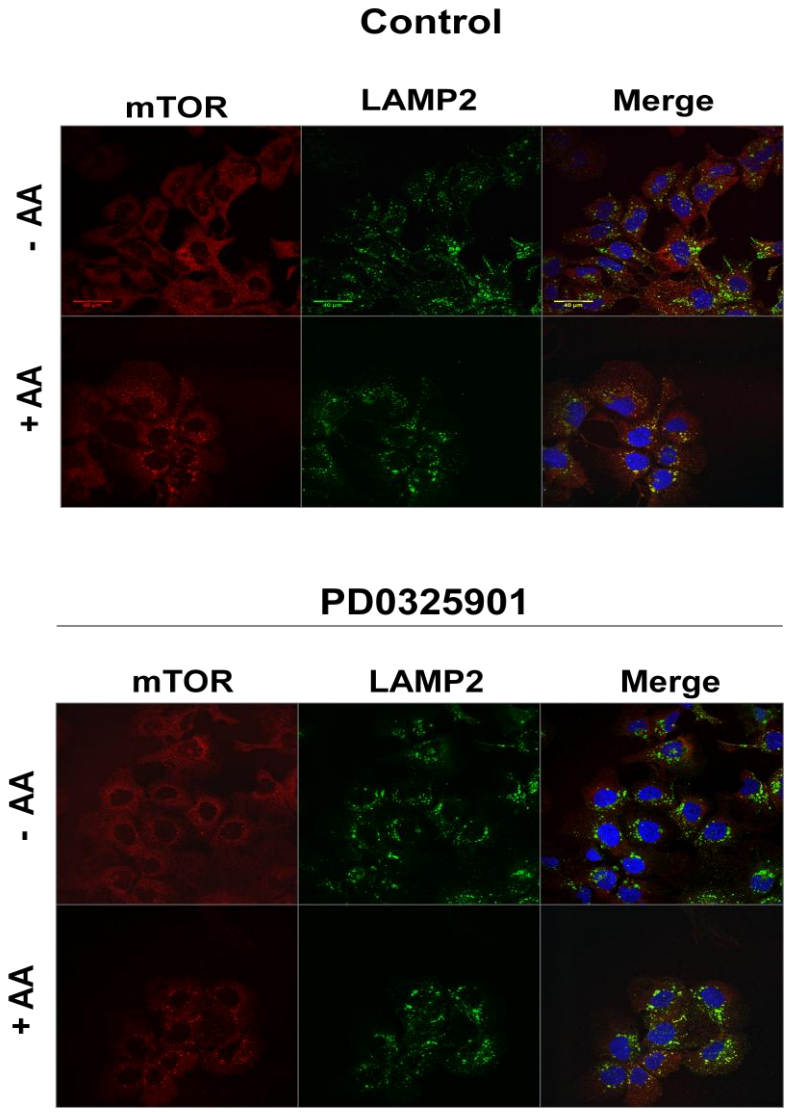
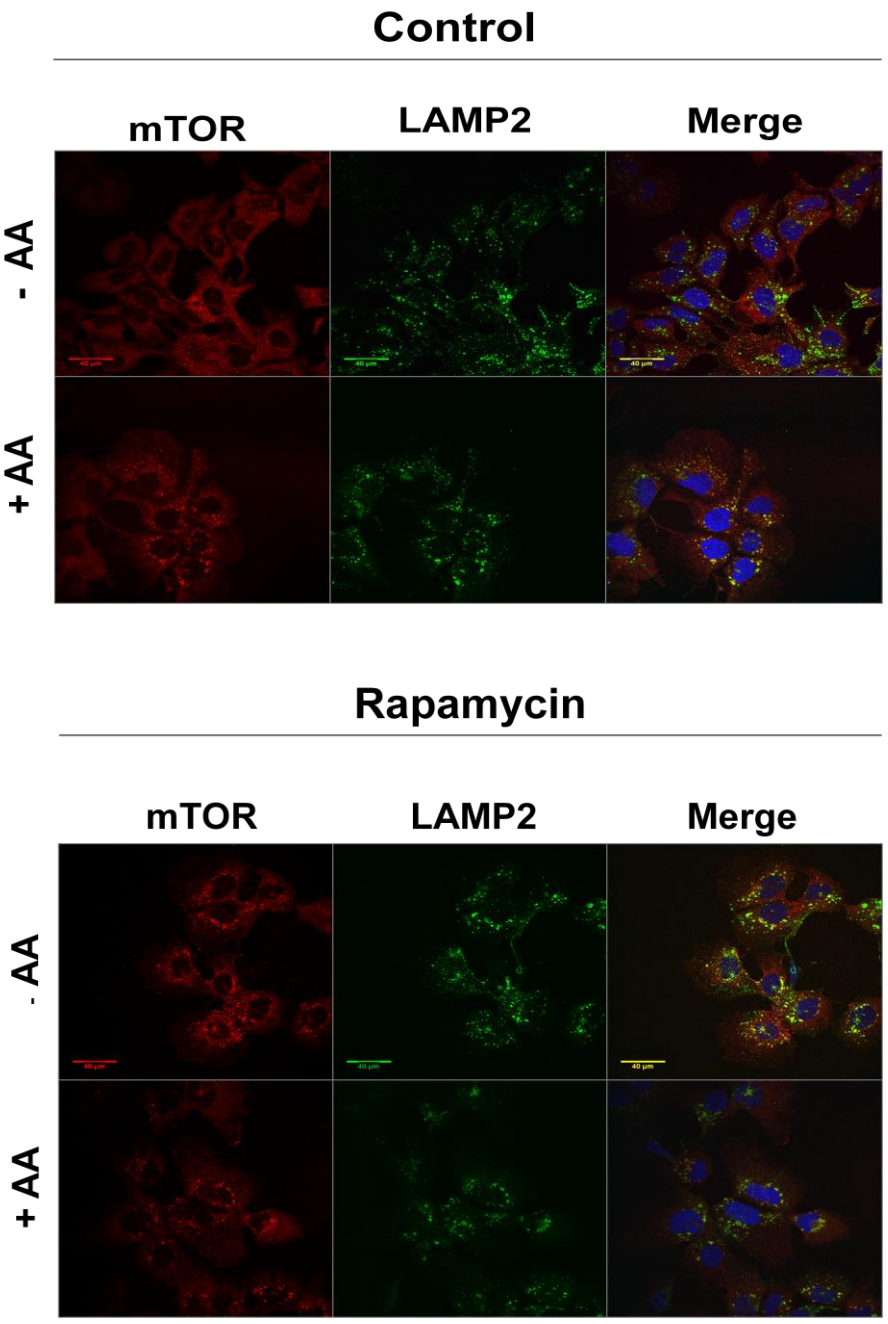


Fig 10: Pharmacological inhibition of MEK does not affect mTOR localization Hep3B cells were starved in KRBH for 6 hours before addition of amino acid (+AA) or water (-AA) for 30 minutes. Cells were treated with PD0325901 or DMSO at a final concentration of 100 nM for one hour prior to addition of amino acids or water.


Control
mTOR
LAMP2
Merge
- AA
+ AA
Rapamycin
mTOR
LAMP2
Merge
- AA
+ AA

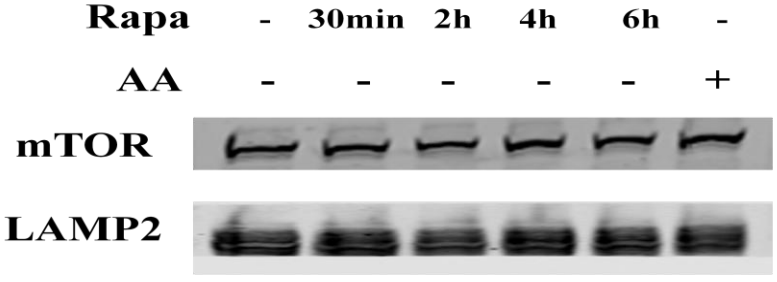

Rapa - 30min 2h 4h 6h -
AA - - - - - +
mTOR
LAMP2

Fig 11: Pharmacological inhibition of mTOR by rapamycin affects mTOR localization under starvation (A) Hep3B cells were starved in KRBH for six hours before addition of amino acid (+AA) or water (-AA) for 30 minutes. Cells were treated with rapamcyin or DMSO at a final concentration of 50 nM for 30 minutes prior to addition of amino acids or water. This is a representative image of 6 independent experiments. (B) Hep3B cells were starved in KRBH for 6 hours and treated with rapamycin or DMSO at a final concentration of 50 nM for the indicated time periods before harvest. Amino acids were added after six hour starvation for 30 minutes before harvest. Cell lysates were analysed by immunoblotting for the indicated proteins.

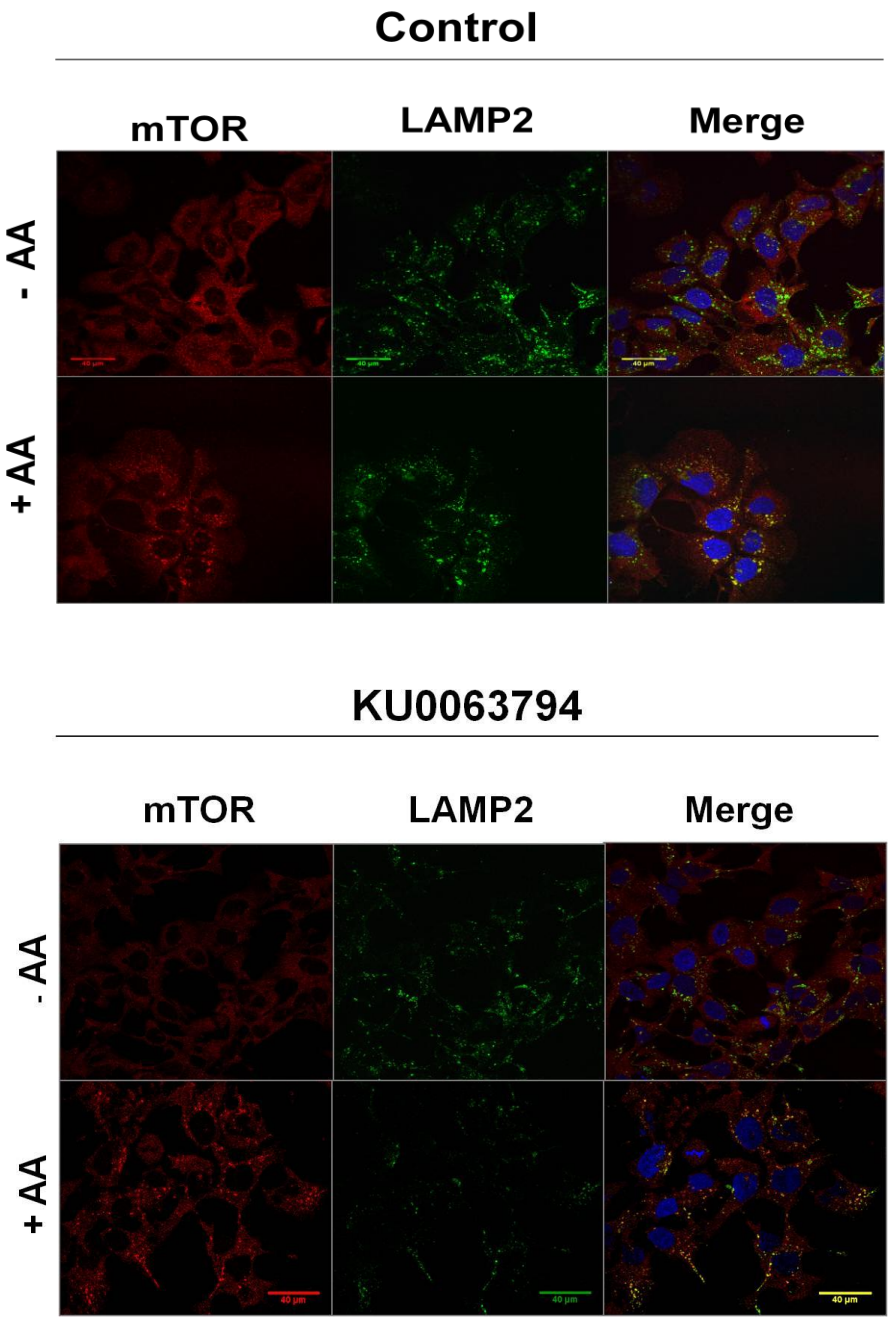


Fig 12: Pharmacological inhibition of mTOR by KU0063794 does not affect mTOR localization. Hep3B cells were starved in KRBH for six hours before addition of amino acid (+AA) or water (-AA) for 30 minutes. Cells were treated with KU0063794 or DMSO at a final concentration of 500 nM nM for 30 minutes prior to addition of amino acids or water.

CHAPTER 3:

Exploring the role of β-arrestin 2 in mTORC1 signaling, autophagy and T1R3 expression.

MATERIALS AND METHODS:

Cell culture, amino acid manipulation, and drug treatments:

HeLa cells and H9C2 rat cardiomyoblast cells were maintained in DMEM containing 10% FBS and 1mM glutamine at 37˚C and 5% CO_2. Human bronchial epithelial cells (HBEC3KT) were maintained in keratinocyte serum free medium (KSFM) supplemented with epidermal growth factor (EGF) and bovine pituitary extract (Life Technologies), while RasV12-p53 null HBEC (HBEC3KTRL) were maintained in Roswell Park Memorial Institute Medium (RPMI) (Life Technologies) containing 5% FBS at 37˚C and 5% CO_2. For amino acid starvation, medium was removed, cells were washed once with KRBH containing 4.5 mM glucose and 0.1% BSA, and then incubated in KRBH for the indicated times before a mixture of amino acids was applied for 30 minutes. Cells were treated with PD0325901, rapamycin , KU0063794 (Selleck Chemicals), or DMSO as control for the indicated time periods and doses.

Transfections:

For knockdown experiments, cells were transfected with 20nM negative control siRNA (Sigma) or β-arrestin 2 siRNA (Sigma) using Lipofectamine RNAiMAX reagent (Life Technologies), and Opti-MEM reduced serum medium (Life Technologies) as per the manufacturer's instructions. Knockdown experiments were performed in 6 well dishes using 130,000, 150,000 and 180,000 cells/well for H9C2 cells, HeLa cells and HBEC3KTRL cells, and HBEC3KT cells, respectively. Seventy-two hours after transfection, cells were subjected to amino acid starvation and re-feeding or drug treatments.

Preparation of cell lysates, SDS-PAGE, and immunoblotting:

Cells were washed once in PBS and then harvested in ice cold lysis buffer (137mM NaCl, 25mM Tris, 1% Triton, 10% glycerol, 200nM Na_3VO_4, 200nM β-glycerophosphate) containing protease inhibitors and phenylmethanesulfonyl fluoride (PMSF), on ice. After mixing lysis buffer and cells by pipetting 10 times on ice, lysates were centrifuged at 13200 rcf for 10 minutes. Supernatants were collected and mixed with 5X Laemmli sample buffer. Lysates to be analyzed for T1R3/T1R1 protein expression were not heated. All other lysates were subjected to heating at 70˚C for 10 minutes. For analysis of LC3, lysates were resolved on 15% - Tricine gels. For analysis of all other proteins, 10% acrylamide gels were used for SDS-Polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred from gels onto PVDF membranes for LC3 analysis and nitrocellulose membranes for other proteins at 250mA overnight or 750mA for 3 hours at 4˚C. Membranes were subjected to blocking at room temperature for 30 minutes using 5% milk in Tris buffer saline (TBS) or Odyssey blocking buffer (LI-COR) and then incubated overnight at 4˚C with primary antibodies against T1R1 (Abcam, 1:1000), T1R3 (Abcam 1:1000), β-arrestin1/2 (Cell Signaling 1:1000), p62 (Santa Cruz 1:1000), phospho S6 Ser240/244 (Cell Signaling 1:2000), phospho S6 Ser235/236 (Cell Signaling 1:1000), S6 (Cell Signaling 1:500), ERK1/2 (Y691 1:1000 or Cell Signaling 1:500), pERK1/2 (Cell Signaling 1:1000), GAPDH (Santa Cruz 1:1000), and LC3 (MBL 1:500). After three washes with TBS-Tween (TBST), membranes were incubated with anti-rabbit and anti-mouse secondary antibodies (LICOR 1:10,000) for one hour at room temperature, followed by three washes with TSBT. Images of blots were obtained using the LI-COR Odyssey IR Imaging System.

BACKGROUND:

The rapid and transient activation of ERK1/2 by amino acids involves signaling mediated by calcium and $G\alpha_q$. However, the molecular players relaying signals from T1R1-T1R3 to mTORC1 were not known. As mentioned in Chapter 1 (Project 1), $G\alpha_i$ has been implicated in signaling downstream of T1R1-T1R3 in some tissues. However, experiments performed in our laboratory using pertussis toxin, an inhibitor of $G\alpha_i$, suggest that $G\alpha_i$ does not mediate mTORC1 activation by amino acids in pancreatic beta cells. While this does not rule out the participation of other G proteins, it does present the possibility of G-protein independent signaling mechanisms mediating communication from T1R1-T1R3 to mTOR. As discussed previously, the β-arrestins are attractive candidates for such mechanisms, owing to their role as scaffolds for components of various signaling pathways. It has been demonstrated that the calcium sensing receptor (CaSR), which is a member of the Class C family of GPCRs to which T1R1 and T1R3 belong, can activate ERK1/2 in response to various agonists by recruiting β-arrestins [195]. Given their well-established role in GPCR endocytosis, β-arrestins could also regulate T1R1-T1R3 signaling by modulating its internalization.

The first question I attempted to answer was whether T1R1-T1R3 could bind β-arrestin1/2. To this end, I attempted to determine whether arrestins could co-immunoprecipitate with T1R1-T1R3, and immunofluorescence experiments to assess the effect of arrestin knockdown on T1R1-T1R3 subcellular localization. However, lack of antibodies that are reliable for immunoprecipitation and immunofluorescence for T1R3 and β-arrestins rendered these experiments inconclusive. Therefore, the approach I adopted was to examine the effects of β-arrestin knockdown on mTORC1 activity and autophagy. As mentioned previously, mTORC1 phosphorylates p70 S6 kinase, which in turn phosphorylates S6 at several serine residues, including 235/236 and 240/244. Therefore, I used S6 phosphorylation as a read-out of mTORC1

activity. Autophagy results in degradation of the adapter protein p62, and autophagosome formation requires conversion of LC3I to LC3II. I assessed p62 degradation and LC3II formation as markers of autophagy, as this is a widely employed strategy in the autophagy field [196, 197].

RESULTS AND DISCUSSION:

β-arrestin 2 can promote mTORC1 activity

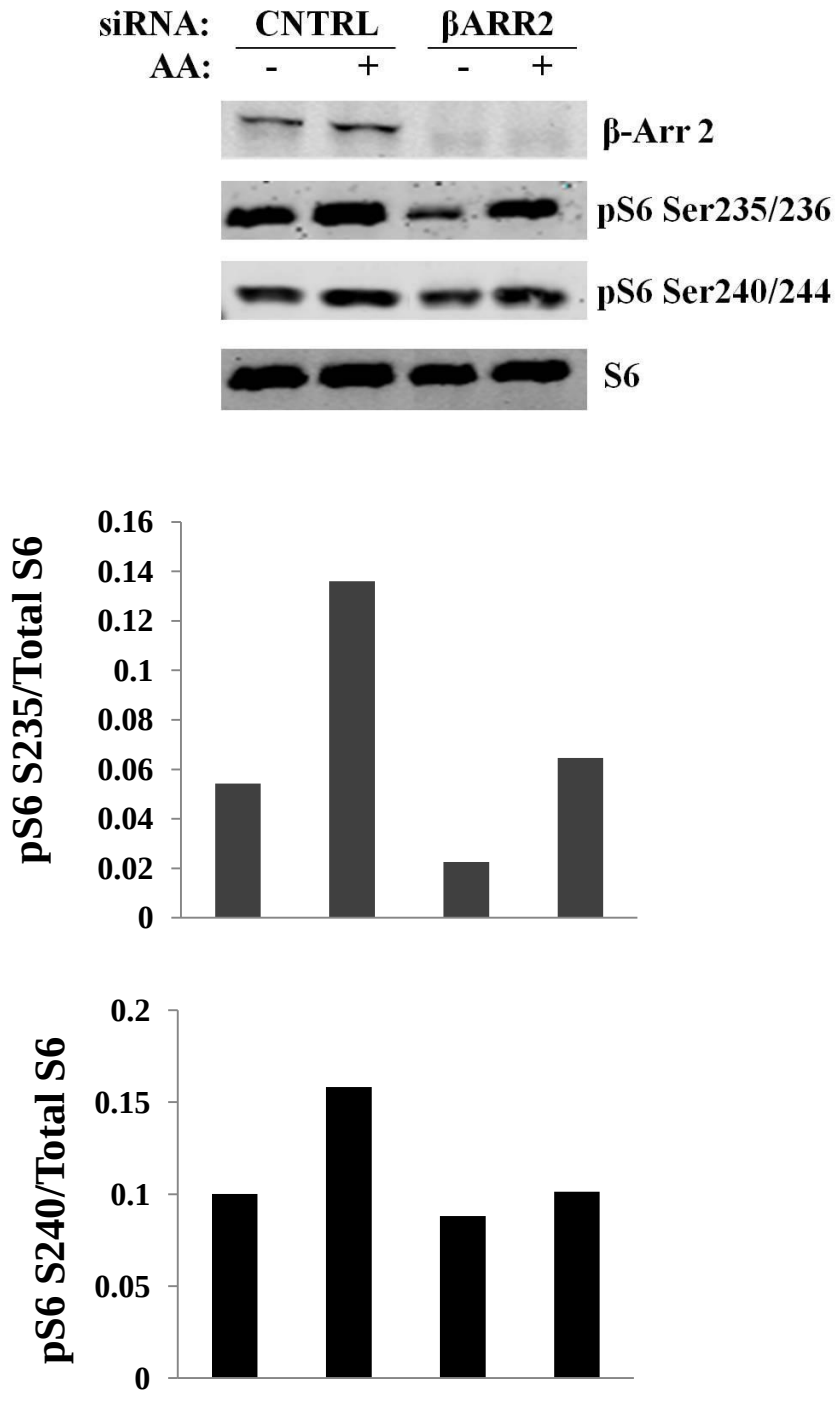
Amino acid deprivation represses mTORC1 activity, which parallels decreased S6 phosphorylation and re-feeding with amino acids re-activates mTORC1, which parallels with increased S6 phosphorylation. H9C2 cells from which β-arrestin 2 had been depleted displayed lower S6 phosphorylation as a result of amino acid deprivation compared to control cells. Knockdown of β-arrestin 2 reduced, but did not ablate, the amino-acid induced increase in S6 phosphorylation observed in control cells. This effect was more pronounced on phosphorylation of Ser235/236 than on Ser240/244 (Fig 13A). While mTORC1 can phosphorylate S6 at both of these sites, studies suggest that these sites are distinct in their regulation and possible functional outputs. There is evidence for mTORC1-independent phosphorylation for each of these sites, especially for Ser235/236. This might explain the difference in impact of β-arrestin 2 knockdown on the two sites. Reduced S6 phosphorylation at Ser240/244 was also observed in HeLa cells (Fig 13B). This reduction was comparable to the reduction in S6 phosphorylation caused by T1R1 knockdown, and was not as severe as reduction in S6 phosphorylation caused by Raptor knockdown, which served as a positive control for mTORC1 activity reduction. β-arrestin 2 knockdown also reduced S6 phosphorylation induced by stimulation with serum and amino acids together after starvation in HeLa cells (Fig 14). However, this reduction was less than that observed in the context of stimulation with amino acids alone. Taken together, these data suggest

that β-arrestin 2 may play a role in promoting the response of mTOR to amino acids and growth factors.

β-arrestin 2 can negatively regulate autophagy

Because mTORC1 inhibits autophagy, repression of mTORC1 by amino acid starvation increases autophagy. This increases degradation of p62, thereby reducing total p62 protein, and increases the conversion of LC3I to its cleaved, lipidated form LC3II. While the amount of LC3II parallels with the number of autophagosomes, LC3II is itself degraded by autophagy [198]. This complicates the interpretation of LC3 immunoblotting data. Also, the LC3II amount at a given time does not reflect autophagic flux. To use LC3II as a measure of autophagic flux, it is important to measure the amount of LC3-II delivered to lysosomes by comparing LC3-II levels in the presence and absence of lysosomal protease inhibitors such as chloroquine or bafilomycin [199]. β-arrestin 2 knockdown reduces p62 protein in HeLa cells under starvation, and this reduction is comparable to that caused by T1R1 knockdown and Raptor knockdown (Fig 14A). Next, I assessed the effect of silencing β-arrestin 2 on conversion of LC3I to LC3II in the presence and absence of bafilomycin in HeLa cells. β-arrestin 2 knockdown increased the amount of LC3II in both DMSO and bafilomyin treated cells (Fig 14B). This indicates that loss of β-arrestin 2 increases autophagic flux. Similar results were obtained in H9C2 cells, in which the amount of LC3II was increased by β-arrestin 2 knockdown under both unstarved (Fig 14C) and starved conditions (Fig 14D). Collectively, these data suggest that β-arrestin 2 may negatively regulate autophagy, similar to the role of T1R1-T1R3. It is possible that β-arrestin 2 can suppress autophagy in an mTOR-dependent fashion. However, autophagy can also be regulated by mTOR independent mechanisms [200-202].

A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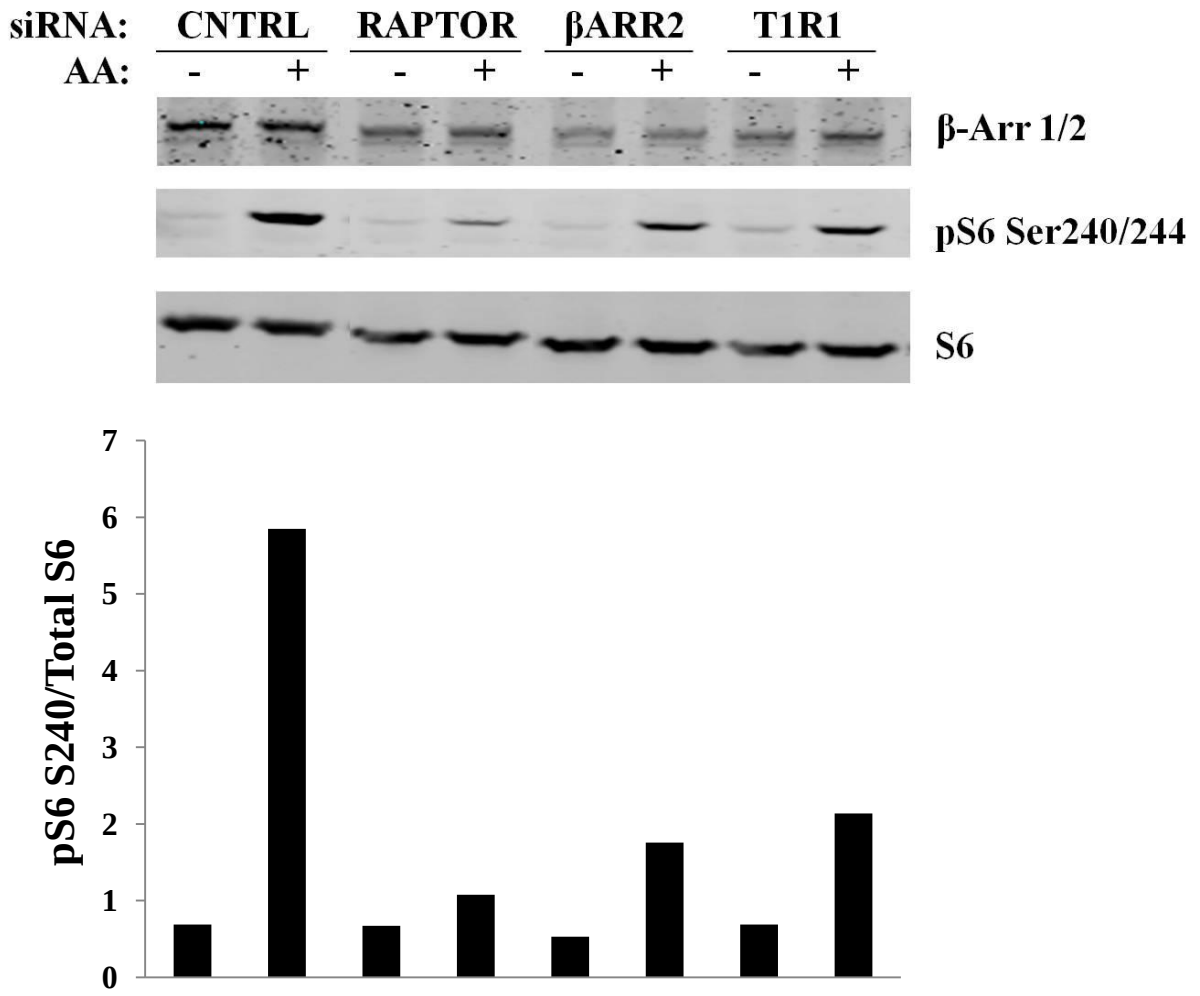
CNTRL
βARR2
AA:
-
+
-
+
β-Arr 2
pS6 Ser235/236
pS6 Ser240/244
S6
pS6 S235/Total S6
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
pS6 S240/Total S6
0.2
0.15
0.1
0.05
0

B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CNTRL
RAPTOR
βARR2
T1R1
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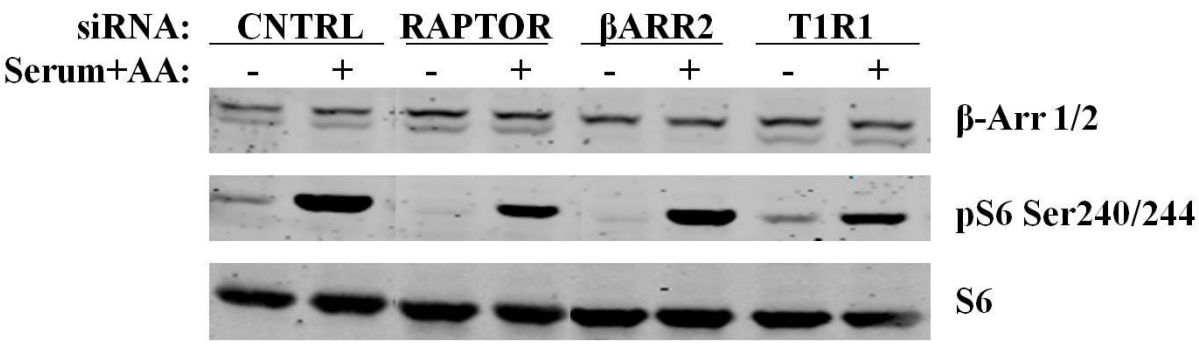
S6
pS6 S240/Total S6
0
1
2
3
4
5
6
7

C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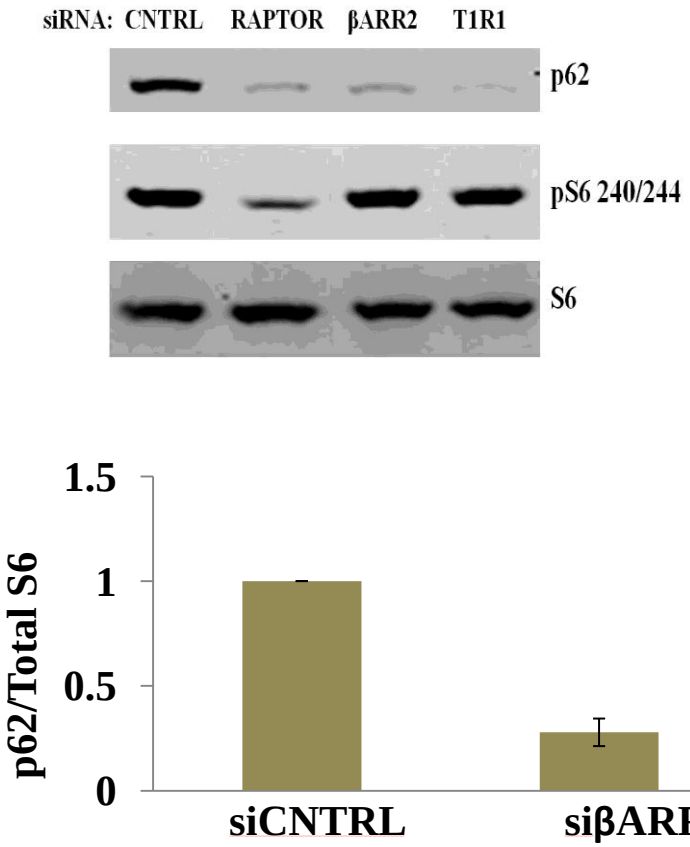
CNTRL
RAPTOR
βARR2
T1R1
Serum+AA:
- + - + - + - +
β-Arr 1/2
pS6 Ser240/244
S6

Fig 13: Beta-arrestin 2 knockdown reduces amino acid and growth factor-induced S6 phosphorylation in H9C2 and HeLa cells. (A) H9C2 cells treated with β-arrestin 2 siRNA or control siRNA were starved in KRBH for 2h prior to stimulation with amino acids (+AA) or water (-AA) for 30 minutes. Cells were then harvested and lysates were analyzed by immunoblotting for the indicated proteins. Quantifications are shown below the blots. Data are the mean of two experiments. (B) HeLa cells treated with the indicated siRNAs were starved in KRBH for 4h prior to stimulation with amino acids (+AA) or water (-AA). Cells were then harvested and lysates were analyzed by immunoblotting for the indicated proteins. Quantifications are shown below the blots. Data are the mean of two experiments. (C) HeLa cells treated with the indicated siRNAs were starved in KRBH for 4h prior to the addition of complete growth medium containing serum and amino acids for 30 minutes. Cells were then harvested and lysates were analyzed by immunoblotting for the indicated proteins.

A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pS6 240/244
S6
p62/Total S6
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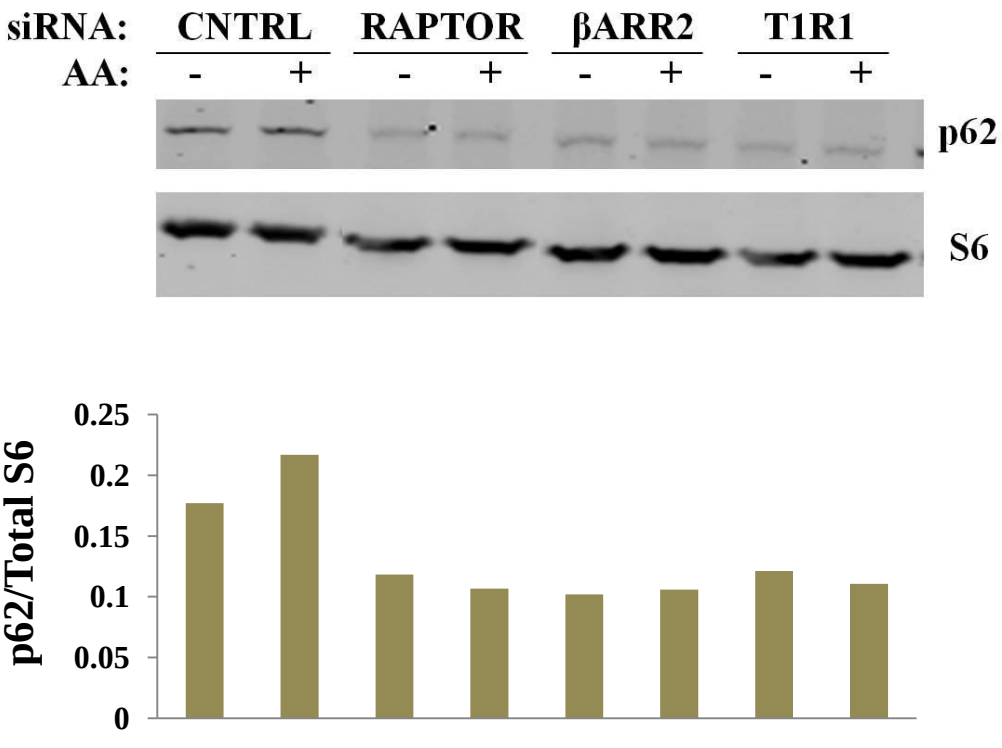
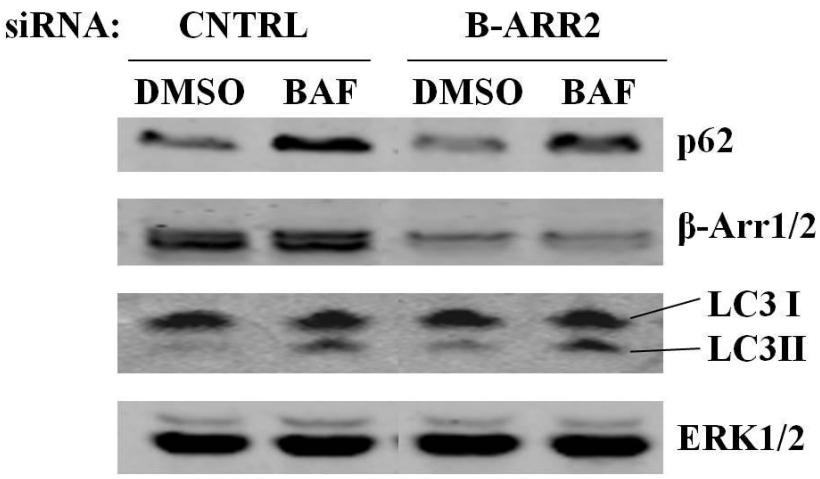
1
0.5
0
siCNTRL
siβARR2

B


siRNA: CNTRL RAPTOR βARR2 T1R1
AA: - + - + - + - +
p62
S6
p62/Total S6
0.25
0.2
0.15
0.1
0.05
0

C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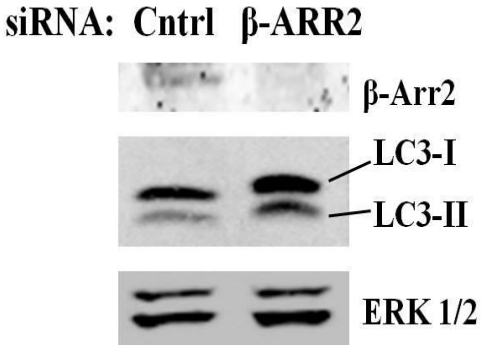
CNTRL
B-ARR2
DMSO
BAF
DMSO
BAF
p62
β-Arr1/2
LC3 I
LC3II
ERK1/2

D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Cntrl
β-ARR2
β-Arr2
LC3-I
LC3-II
ERK 1/2

E

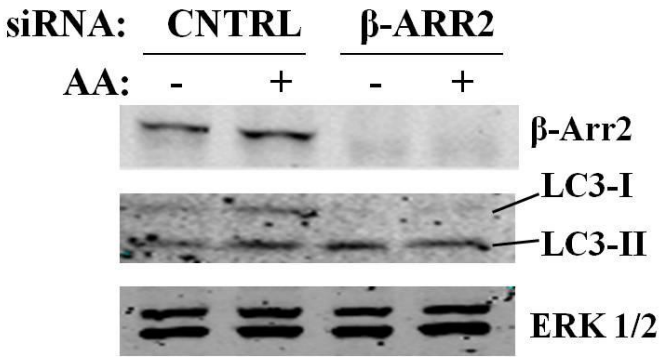

siRNA:
CNTRL
β-ARR2
AA:
-
+
-
+
β-Arr2
LC3-I
LC3-II
ERK 1/2

Fig 14: Beta-arrestin 2 knockdown increases autophagy in H9C2 and HeLa cells. (A) HeLa cells treated with the indicated siRNAs were maintained in complete growth medium. Cells were then harvested and lysates were analyzed by immunoblotting for the indicated proteins. Quantification is shown below the blots. Data are the mean +/- SEM of three independent experiments. (B) HeLa cells treated with the indicated siRNAs were starved in KRBH for 4h prior to stimulation with amino acids (+AA) or water (-AA) for 30 minutes. Cells were then harvested and lysates were analyzed by immunoblotting for the indicated proteins. Quantifications are shown below the blots. Data are the mean of two experiments. (C) HeLa cells treated with β-arrestin 2 siRNA or control siRNA were treated with 100 nM bafilomycin or DMSO for 4 hours. Cells were then harvested and lysates were analyzed by immunoblotting for the indicated proteins. (D) H9C2 cells treated with beta-arrestin 2 siRNA or control siRNA grown in complete growth medium and (E) H9C2 cells treated with beta-arrestin siRNA or control siRNA were starved in KRBH for 2 hours before stimulation with amino acids (+AA) or water (-AA) for 30 minutes. Cells were then harvested and lysates were analyzed by immunoblotting for the indicated proteins.

ERK1/2 may be involved in negative regulation of autophagy by β-arrestin 2

MAP kinases such as p38, JNKs (cJun N terminal kinases), and ERK1/2 play roles in autophagy regulation in different contexts [203-205]. β-arrestins are well-known signaling scaffolds for MAPK pathways and have been shown to bind c-Raf, MEK1, ERK2, JNK3, ASK1 (apoptosis signal-regulated kinase 1), and p38 [206-209]. In light of this information, I assessed the effect of β-arrestin 2 knockdown on phosphorylation of ERK1/2. Depletion of β-arrestin 2 increased phosphorylation of ERK1/2 in HeLa cells and H9C2 cells (Fig 15A). Inhibition of MEK by PD0325901 reduced the amount of LC3II and increased p62 protein in β-arrestin 2 knockdown cells, but not in control cells (Fig 15B). This suggests that increased ERK1/2 signaling might contribute to the increased autophagy caused by arrestin depletion. If this is correct, then cells displaying constitutively high ERK1/2 phosphorylation should be immune to the effects of arrestin knockdown on autophagy. To test this idea, I used human bronchial epithelial cells (HBEC) that were normal (HBEC3KT) or that were p53 null and bearing activating mutations in Ras (HBEC3KTRL). Ras is upstream of Raf, which, in turn, is upstream of MEK1/2. Depletion of β-arrestin 2 decreased p62 protein in HBEC3KT, but did not affect p62 protein in HBEC3KTRL, suggesting that arrestins can influence autophagy in HBEC3KT, but not HBEC3KTRL (Fig 16). These observations provide further evidence for the role of ERK1/2 in autophagy regulation by arrestins. Because β-arrestin 1 and 2 typically promote MAPK signaling downstream of GPCRs in response to agonists, the increased ERK1/2 phosphorylation upon arrestin knockdown observed in this work may appear puzzling. It is possible that β-arrestin 2 knockdown promotes stress-induced ERK1/2 activity, which participates in increasing autophagy. This will be further discussed in Chapter 5 (Future Directions). Simultaneous treatment with PD0325901 and bafilomycin will be required in order to determine whether autophagosome formation (as assessed by LC3-II formation) is altered by MEK inhibition.

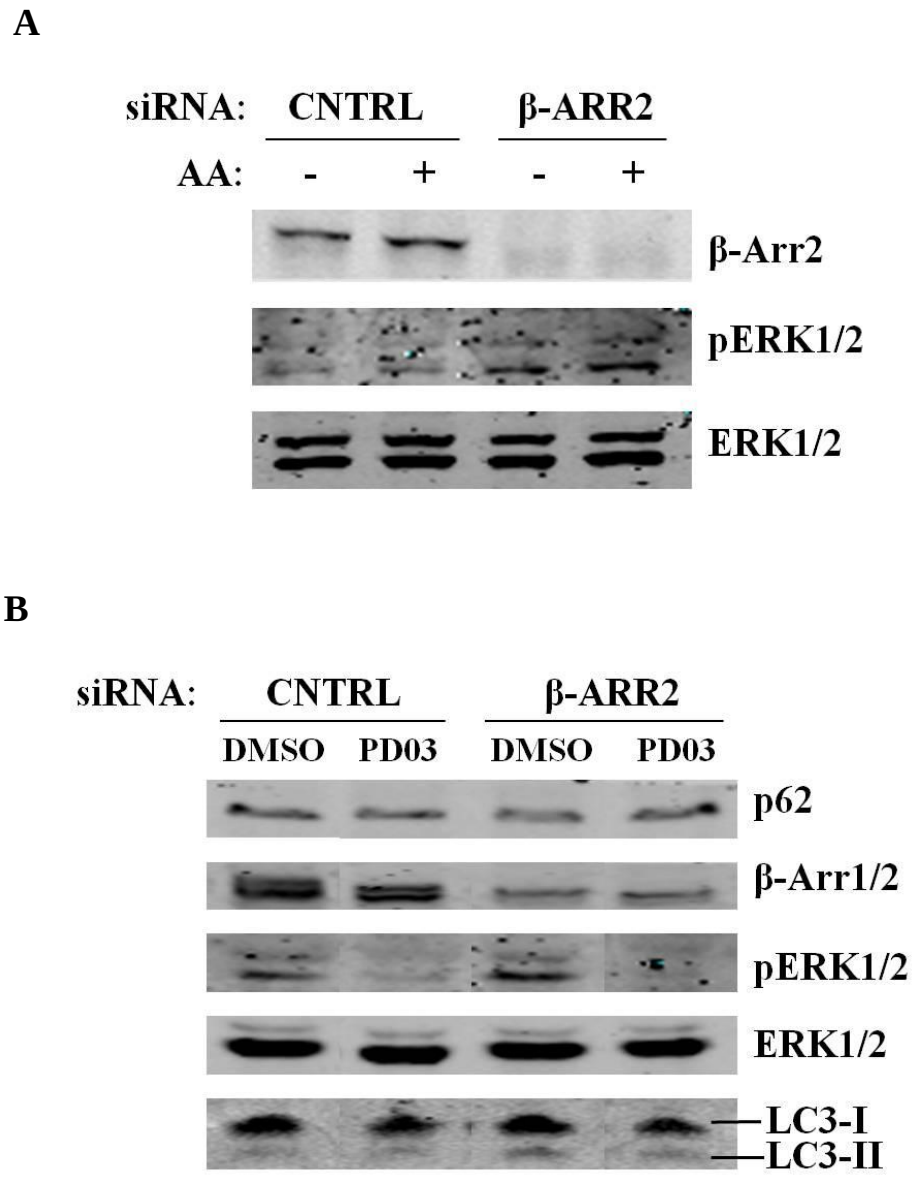


Fig 15: Beta-arrestin 2 knockdown increases ERK1/2 phosphorylation in H9C2 and HeLa cells. (A) H9C2 cells treated with β-arrestin 2 or control siRNA were starved in KRBH for 2 hours prior to stimulation with amino acids (+AA) or water (-AA) for 30 minutes. Cells were then harvested and lysates were analyzed by immunoblotting for the indicated proteins. (B) HeLa cells treated with β-arrestin 2 siRNA or control siRNA were treated with 100 nM PD0325901 or DMSO for 4 hours before harvest. Lysates were analyzed by immunoblotting for the indicated proteins.

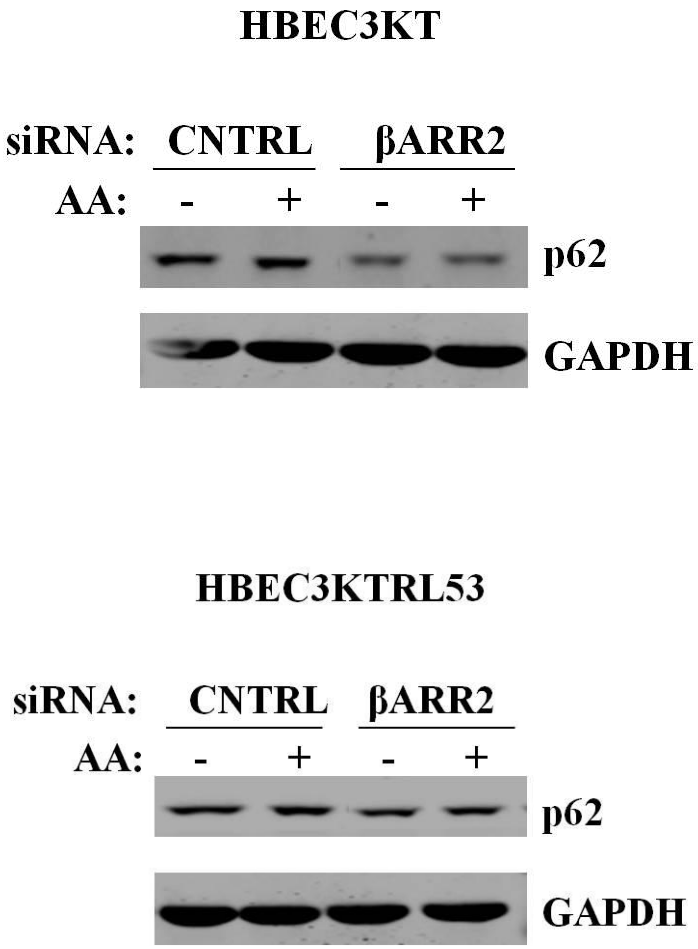


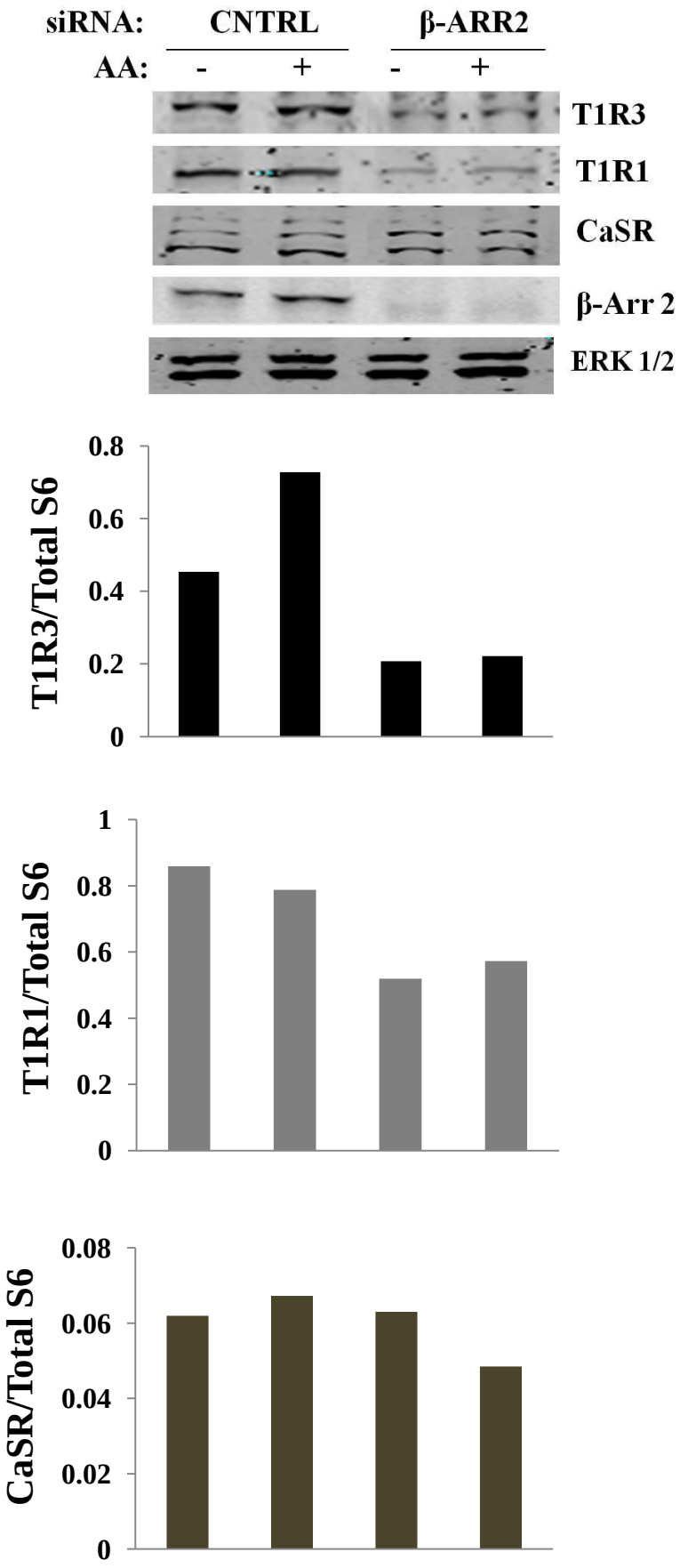
Fig 16: Beta-arrestin 2 knockdown reduces p62 in normal but not Ras-transformed HBEC. Human bronchial epithelial cells (HBEC3KT) or Rasv12 p53 null HBEC3KT (HBEC3KTRL) cells treated with β-arrestin 2 or control siRNA were starved in KRBH for 4h prior to stimulation with amino acids (+AA) or water (-AA) for 30 minutes. Cells were then harvested and lysates were analyzed by immunoblotting for the indicated proteins.

β-arrestin 2 positively regulates total cellular protein expression of T1R1-T1R3

As mentioned in Chapter 1 (Project 1), the arrestins can control membrane expression of GPCRs via endocytosis. Most studies demonstrating this have assessed membrane fractions for receptor expression. Not much is known about the impact of arrestins on total receptor protein expression. Because some of the cellular consequences of β-arrestin 2 knockdown appeared to phenocopy those observed upon T1R3/T1R1 knockdown, I examined the total cellular expression of T1R1 and T1R3. Surprisingly, β-arrestin 2 knockdown markedly decreased the expression of T1R1 amd T1R3 in H9C2 cells (Fig 17A). This decrease in total receptor expression did not appear to be a non-specific effect on all GPCRs, as the expression of CaSR, another member of the class C family of GPCRs to which T1R1 amd T1R3 belong, was not affected by β-arrestin 2 knockdown. This change in expression could not be attributed to decreased mRNA expression, as qPCR analysis revealed no significant difference in the amounts of T1R1 transcripts or T1R3 transcripts (Fig 17B). Therefore, it would appear that β-arrestin 2 depletion increases degradation of T1R1 and T1R3 proteins. Internalized cell surface receptors can either undergo degradation by fusion of the endosome with a lysosome, or be recycled back to the plasma membrane. They may also persist in endosomes without being subjected to immediate degradation, where they may participate in intracellular signaling. Therefore, the amount of receptor present in a cell at any given time could be thought of as being determined by the relative rates of degradation and recycling, apart from mRNA transcription and translation. Different cellular stimuli and stresses can induce different sets of interacting partners to bind a receptor and orchestrate its fate. Arrestins have been implicated not only in receptor internalization and degradation, but also in receptor trafficking and recycling to the plasma membrane. It is possible that under normal conditions, β-arrestin 2 promotes T1R1-T1R3 recycling, thereby maintaining total cellular amounts of the receptors. Loss of β-arrestin 2 might

alter the trafficking of T1R1 and T1R3 such that a degradative pathway is favored over recycling pathways.

A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CNTRL
β-ARR2
AA:
-
+
-
+
T1R3
T1R1
CaSR
β-Arr 2
ERK 1/2
T1R3/Total S6
0.8
0.6
0.4
0.2
0
T1R1/Total S6
1
0.8
0.6
0.4
0.2
0
CaSR/Total S6
0.08
0.06
0.04
0.02
0

B

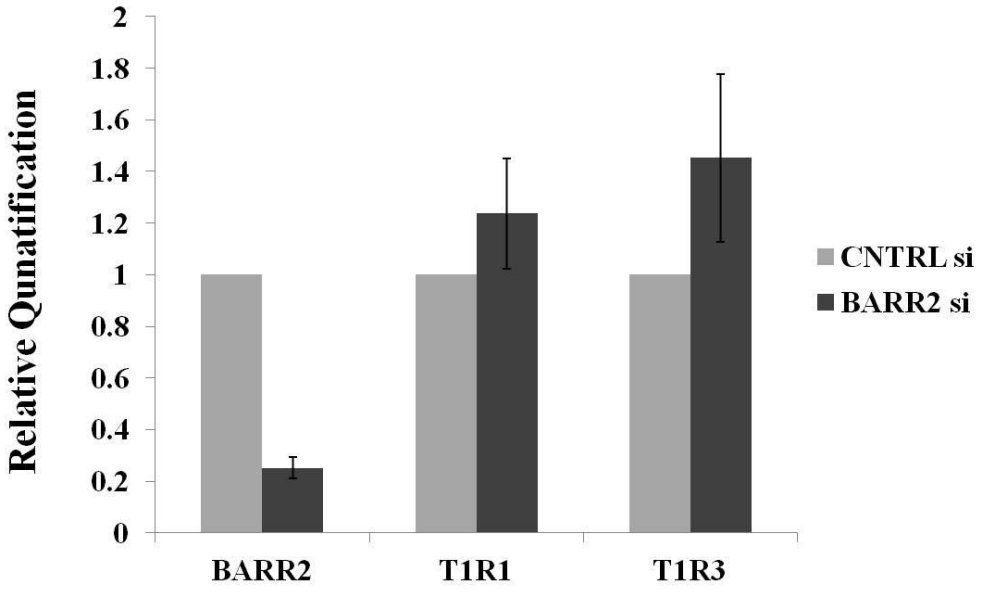

Fig 17: Beta-arrestin 2 knockdown reduces total protein but not mRNA expression of T1R1 and T1R3 in H9C2 cells. (A) H9C2 cells treated with beta-arrestin 2 siRNA or control siRNA were starved in KRBH for 2h prior to stimulation with amino acids (+AA) or water (-AA) for 30 min. Cells were then harvested and lysates were analyzed by immunoblotting for the indicated proteins. The blot shown here is representative of two experiments. Quantifications are shown below the blots. Data are the mean of two experiments. (B) cDNA obtained by reverse transcription of RNA isolated from H9C2 cells treated with beta-arrestin 2 siRNA or control siRNA was analyzed by qPCR for expression of T1R1 and T1R3 mRNA. Data are represented as the mean +/- SEM of three independent experiments.

CHAPTER 4:
Studies on regulation of WNK1 degradation

MATERIALS AND METHODS:

Cell culture, drug treatments, and transfections:

HeLa cells or HEK293T cells were maintained in DMEM containing 10% FBS and 1mM glutamine. MG132 (LC Laboratories), bafilomycin (COMPANY), cycloheximide (COMPANY), geldanamycin (LC Laboratories), and MDL28170 (LC Laboratories) were used at the indicated doses and times. For knockdown experiments, HeLa or HEK293T cells were transfected with 2nM control siRNA or UBR5 siRNA using Lipofectamine RNAiMax (Life Technologies) and OptiMEM (Life Technologies) as per the manufacturer's instructions. 72 hours later, cells were subjected to drug treatments. One million cells/10 cm dish or 180,000 cells/ well of a 6-well dish were used for knockdown experiments. For overexpression experiments, HEK293T or HeLa cells plated 24 hours prior to transfection were transfected with plasmid DNA (5-10 ug DNA per 10 cm dish) using Lipofectamine 2000 (Life Technologies) and OptiMEM (Life Technologies) according to the manufacturer's instructions.

Preparation of cell lysates, SDS-PAGE, and Immunoblotting:

After washing once with PBS, cells were harvested in lysis buffer (150mM NaCl, 1-2% SDS and 25mM Tris pH 7.4) Lysates were passaged through a 25 gauge needle five times, mixed with 5X Laemmli sample buffer, and boiled for three minutes. Lysates were analyzed using 8% or 10% acrylamide gels for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred from gels onto nitrocellulose membranes at 300mA overnight or 750mA for four hours at 4˚C. Membranes were subjected to blocking at room temperature for 30 minutes using Odyssey blocking buffer (LI-COR) and then incubated overnight at 4˚C with primary antibodies against WNK1 (Cell signaling 1:500 or Q256 or Q255), UBR5 (Santa Cruz 1:500), p62 (Santa Cruz 1:1000), p53 (Santa Cruz 1:500), OSR (Cell Signaling 1:1000 or U5439), SPAK (Cell Signaling 1:500), β-Actin (Sigma 1:2000), Myc-tag (Sigma 1:1000), HA-tag (Roche 1:500 or

Cell signaling 1:500), FLAG-tag (Sigma 1:1000), KLHL3 (ProteinTech 1:500). After three washes with TBS-Tween (TBST), membranes were incubated with anti-rabbit, anti-goat or anti-mouse secondary antibodies (LICOR, Jackson Antibodies 1:10,000) for one hour at room temperature, followed by three washes with TSBT. Images of blots were obtained using the LI-COR Odyssey IR Imaging System.

Immunoprecipitation:

For immunoprecipitation experiments, cells were washed once in PBS and then harvested in ice cold lysis buffer (137mM NaCl, 25mM Tris, 1% Triton, 10% glycerol, 200nM Na_3VO_4, 200nM β-glycerophosphate) containing protease inhibitors and phenylmethanesulfonyl fluoride (PMSF), on ice. After mixing lysis buffer and cells by pipetting 10 times on ice, lysates were centrifuged at 13200 rcf for 10 minutes. Supernatants were collected and protein concentrations were determined by the BCA assay. One-three mg of protein in 500 μL- 1 mL of lysate was used per tube. Lysates were incubated overnight at 4˚C with antibodies to WNK1 (Q256 bleed 6 1:100), OSR (U5439 1:100), FLAG (Sigma 1:100), Myc-tag (Sigma 1:100). Protein-antibody complexes were pulled down with 40μL of Protein A Sepharose beads (GE Healthcare) per tube for one hour at 4˚C. For immunoprecipitation of endogenous WNK1, OSR1, and FLAG-tagged constructs, immunoprecipitates were washed three times with lysis buffer, resuspended in lysis buffer mixed with 5X Laemmli buffer, and boiled for three minutes. For 1-491 WNK1 Myc immunoprecipitates, beads were washed three times with wash buffer (500 mM NaCl, 1% Triton, and 10mM Tris pH 7.4), once with lysis buffer, resuspended in lysis buffer with 5X Laemmli, and boiled for three minutes. Eluted proteins were analyzed by SDS-PAGE and immunoblotting as described above.

SECTION I: Examining the role of different protein degradation pathways in regulation of WNK1 stability.

BACKGROUND:

The amount of a protein at a given time in a cell is governed by the rates of synthesis and degradation of that protein at that time. Little is known about the turnover of the WNK1 protein. The half-life of a protein can be determined by monitoring its degradation after blocking protein synthesis using inhibitors like cycloheximide. Another approach is to perform pulse chase experiments using S^{35}-methionine to radiolabel proteins and then monitor their degradation. Such studies have not been reported for WNK1. As mentioned in Chapter 1 (Project 2), it has been shown that WNK1 can be ubiquitinated in vitro by the KLHL3-CUL3-RBX1 E3 ligase complex, and mutations in CUL3 and KLHL3 that cause PHA II cause increased WNK4 protein expression. However, these studies have not addressed whether ubiquitination of WNK1 targets it for degradation by the proteasome or by autophagy in lysosomes. Additionally, the contribution of other degradation pathways, such as lysosome-independent proteolysis, has not been explored. Structural information on WNK1 apart from the kinase domain and autoinhibitory domain is lacking. While motif prediction softwares suggest the presence of coiled coil domains, much of the remaining regions appear to be unstructured. Unstructured regions of proteins are usually more susceptible to proteolysis than structured regions. For this reason, proteins with unstructured regions are often stabilized by association with chaperone proteins. It is unknown if chaperones are involved in regulating WNK1 stability. The aim of this section of my work is to examine the contribution of different protein degradation pathways to regulation of WNK1 stability. To this end, I assessed the effects of pharmacological inhibition of these pathways on cellular WNK1 protein amount.

RESULTS AND DISCUSSION:

WNK1 protein degradation exhibits fractional exponential decay kinetics

I examined the stability of WNK1 in HeLa cells by measuring a time course of WNK1 protein amounts remaining after treatment with cycloheximide. One approach to study protein degradation is to monitor protein degradation after blocking protein synthesis using inhibitors like cycloheximide. In these experiments, WNK1 displayed a half-life of approximately three hours (Fig 18A). Studies characterizing protein degradation often make the assumption that degradation follows a pattern of exponential decay ($\propto \exp(-t)$), which results in a linear degradation curve if the fraction of remaining protein is plotted on a log scale versus time. However, such a linear plot was not obtained for the degradation of WNK1 (Fig 18B). Linearization was obtained, however, when the log of fraction of WNK1 remaining is plotted versus the square root of time (Fig 18C). This reveals that WNK1 protein degradation displays characteristics of fractional exponential decay ($\propto \exp(-\sqrt{t})$) rather than the exponential decay pattern of degradation exhibited by many other proteins reported in the literature. Fractional exponential decay is slower than exponential decay. This supports previous experiments performed in our laboratory that suggest that WNK1 is a relatively stable protein. Patterns of fractional exponential decay have been observed and modeled for some biophysical and biochemical phenomena, but have not been reported in the context of protein degradation [210-212]. At a dynamical level, it is thought that exponential decay is indicative of trapping-limited diffusion, while fractional exponential decay is indicative of diffusion-limited trapping [213]. Applying this concept to the process of protein degradation, one might envision subcellular organelles/macromolecular complexes like the proteasome, lysosomes and other membranous compartments, and large protein complexes to act as traps for proteins diffusing in the

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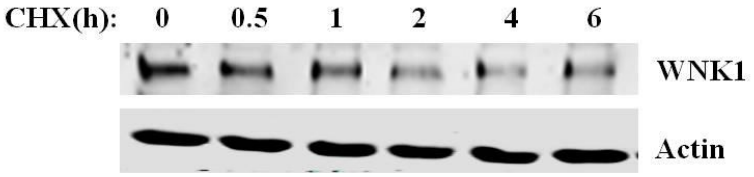
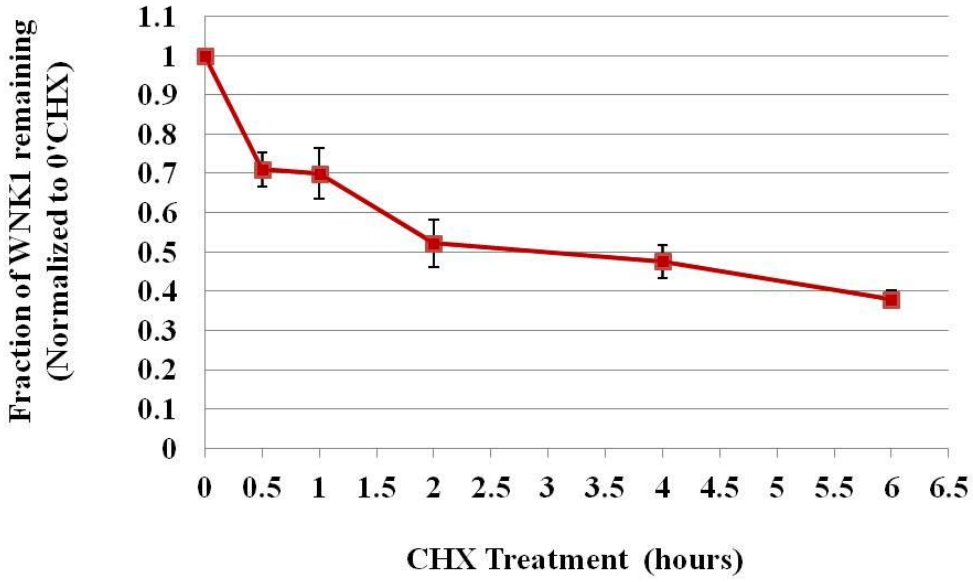
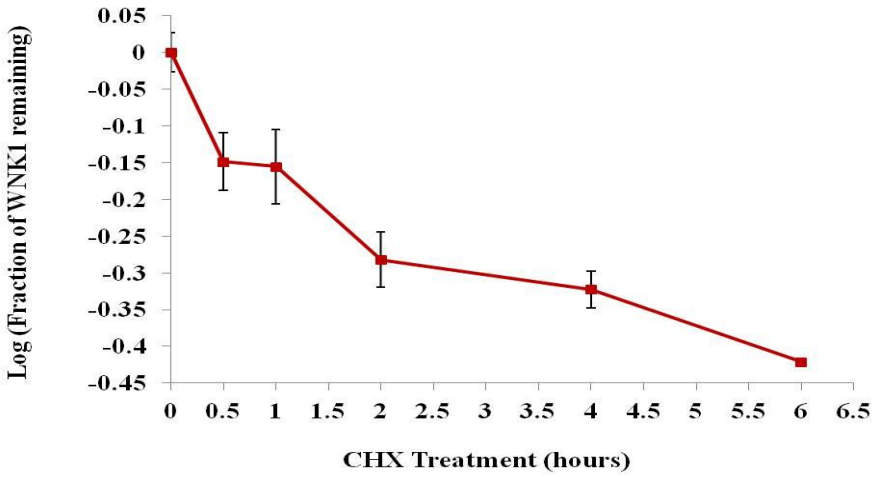

CHX(h): 0 0.5 1 2 4 6
WNK1
Actin

B


Fraction of WNK1 remaining (Normalized to 0'CHX)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5
CHX Treatment (hours)

C



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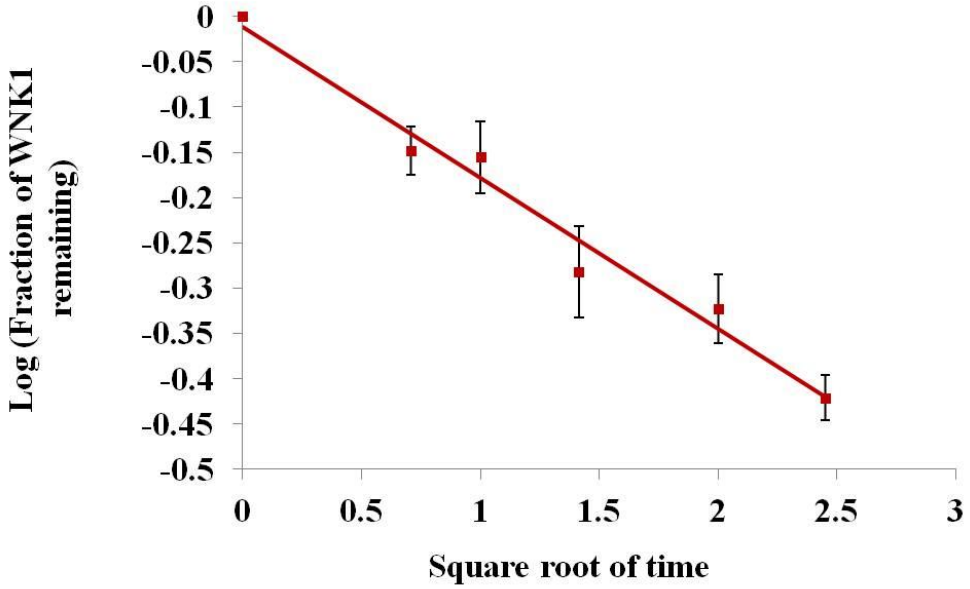

Fig 18: WNK1 has a half life of three hours and exhibits fractional decay kinetics. (A) HeLa cells treated were treated with 100 μM cycloheximide (CHX) for the indicated times before harvest. Cells were treated with DMSO for the zero hour time point. Lysates were analyzed by immunoblotting for the indicated proteins. Shown here is a representative blot from experiments in Fig. 26(A) in which cells were treated with control siRNA. (B) Graphical representation of data obtained by quantification of blots in (A). Data are represented as the mean +/- SEM of three independent experiments. (C) Semi-log plot of data in (D) Log-power plot of data in (B).

cytosol, with degradation occurring at these traps. It could then be hypothesized that a protein which is captured irreversibly by many such traps and degraded therein could exhibit exponential decay, while a protein that is captured for a limited time and/or by fewer traps would exhibit fractional exponential decay. Microscopy studies using GFP-WNK1 have shown that WNK1 can shuttle rapidly between the cytosol and vesicular compartments of cells in response to osmotic stress [214]. Perhaps WNK1 can reside in complexes at different sub-cellular locations, and this could influence the kinetics of its degradation. Cycloheximide treatment for longer periods of time than were used for these experiments would be useful to determine whether fractional exponential decay kinetics are maintained during degradation over multiple half lives.

WNK1 protein amount is sensitive to lysosomal, but not proteasomal inhibition in HeLa cells

Inhibition of proteasomal activity leads to the accumulation of ubiquitin- conjugated proteins in cells. The pharmacological agent most widely used for this purpose is MG132. I assessed the effect of MG132 treatment on WNK1 protein amount in HeLa cells. MG132 treatment for four hours did not significantly affect WNK1 protein expression in cells (Fig 19 A and B). The tumor suppressor p53 displays rapid turnover and is known to undergo proteasomal degradation [215, 216]. Therefore, I used p53 as a positive control to ascertain the functionality of MG132 in these experiments. MG132 treatment resulted in p53 accumulation (Fig 20A), thus confirming that four hour treatment with MG132 did inhibit proteasomal degradation of proteins. Preliminary experiments with another proteasome inhibitor, bortezomib, showed similar results. This suggests that proteasomal degradation might not be the primary mechanism regulating WNK1 stability. It is also possible that a small fraction of WNK1 might undergo proteasomal degradation through a process that is slower than that observed for typical proteasomal

substrates. If this is the case, then proteasomal contribution to WNK1 degradation may not be easily distinguished by treatment with inhibitors in cells.

It is thought that short-lived regulatory proteins are targeted for proteasomal degradation while long-lived proteins are targeted for lysosomal degradation by autophagy. To examine the contribution of autophagy to WNK1 degradation, I assessed the effect of autophagy inhibition on WNK1 protein amount in HeLa cells. Autophagy can be suppressed by multiple inhibitors that impair lysosome function through different mechanisms. One such inhibitor is bafilomycin, which specifically blocks the activity of the vacuolar type H^+-ATPase responsible for maintaining the acidic pH of the lysosomal lumen [217]. Because lysosomal hydrolases require an acidic pH for activity, this inhibits degradation of cargoes delivered to lysosomes. Other well-known inhibitors include lysosomotropic agents like chloroquine and ammonium chloride, which alkalinize the lysosomal lumen, and protease/peptidase inhibitors like leupeptin. Treatment with bafilomycin increased WNK1 amount in HeLa cells (Fig 20 A and B). Similar results were obtained upon chloroquine treatment. This suggests that WNK1 can undergo autophagic degradation. As mentioned in Chapter 1 (Project 2), three different types of autophagy have been indentified in cells: macro-autophagy, micro-autophagy, and chaperone mediated autophagy. Which of these forms of autophagy is involved in WNK1 degradation remains to be explored, and will be discussed in Chapter 5 (Future Directions).

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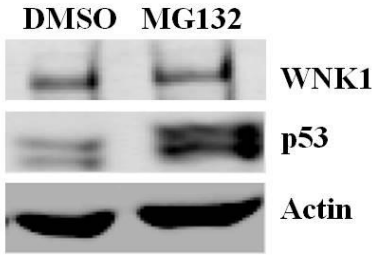

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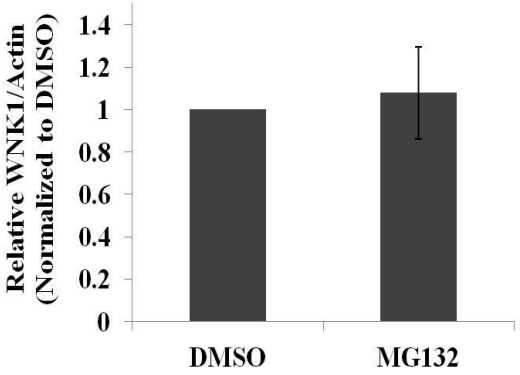
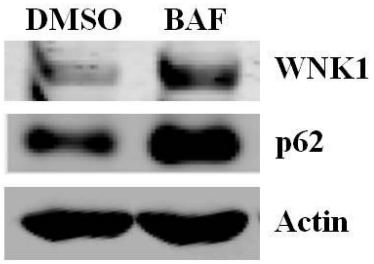


Fig 19: Proteasomal inhibition does not significantly affect WNK1 protein amount. (A) HeLa cells were treated with 10μM MG132 or DMSO for four hours prior to harvest. Cell lysates were analyzed by immunoblotting for the indicated proteins.(B) Quantification of blots in (A). Data are represented as the mean +/- SEM of three independent experiments.

A



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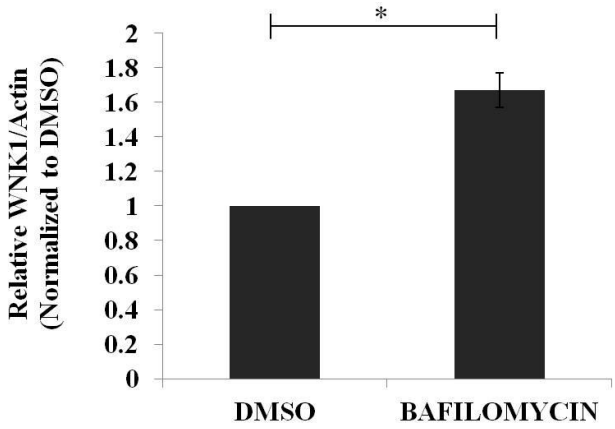


Fig 20: Inhibition of autophagy increases WNK1 protein amount. (A) HeLa cells were treated with 500nM bafilomycin (BAF) or DMSO for four hours prior to harvest. Cell lysates were analyzed by immunoblotting for the indicated proteins.(B) Quantification of blots in (A). Data are represented as the mean +/- SEM of three independent experiments. *$p<0.05$

Hsp90 inhibition increases WNK1 protein

The heat shock protein family of molecular chaperones is involved in regulating protein quality through various mechanisms. In many cases, chaperones bind to and protect their client proteins from degradation. For instance, Hsp90 (Heat shock protein 90) has been shown to associate with and protect IKK (Iκ B kinase) from autophagic degradation [218]. Additionally, Hsp90 is critical for maintaining the stability of the large kinase, LRRK2 (Leucine rich repeat kinase 2) which has been reported to undergo proteasomal degradation and chaperone mediated autophagy. [146, 219]. In view of this information, I tested the idea that Hsp90 may play a role in WNK1 stability by assessing the effect of pharmacological inhibition of Hsp90 on WNK1 protein amounts in cells. AKT has been shown to undergo degradation upon Hsp90- inhibition, and was therefore used as a positive control to ascertain the functionality of geldanamycin. Geldanamycin and its analogs are the most widely used inhibitors of Hsp90, preventing its association with client proteins by inhibiting its ATP-ase activity [220]. If Hsp90 promotes WNK1 stability, its inhibition should reduce WNK1 protein amount in cells. However, HeLa cells treated with increasing concentrations of geldanamycin displayed a dose dependent increase in WNK1 protein amount (Fig 21). One explanation for this observation could be that Hsp90 inhibition decreases the stability of proteins that mediate WNK1 degradation, such as CUL3 and KLHL3. However, preliminary experiments suggest that this does not underlie the increase in WNK1 protein. Several studies have shown that Hsp90 inhibition induces expression of another protein chaperone called Hsp70 (Heat shock protein 70) [221-224]. It is possible that Hsp70 could protect WNK1 from degradation upon its induction by Hsp90 inhibition.

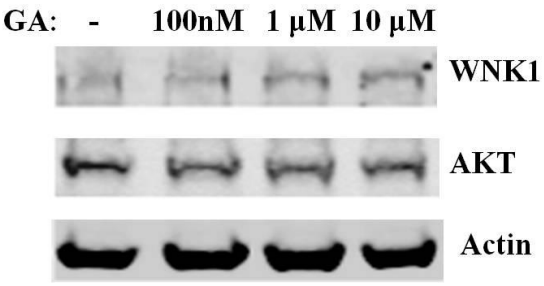


Fig 21: Inhibition of Hsp90 increases WNK1 protein amount HeLa cells were treated with the indicated doses of geldanamycin (GA) or DMSO for four hours. Cell lysates were analyzed by immunoblotting for the indicated proteins. This blot is representative of two independent experiments.

Experimental approaches to test this idea will be described in Chapter 5 (Future Directions).

Calpain inhibition increases WNK1 protein

Cycloheximide treatment for six hours in HeLa cells resulted in the appearance of discrete bands at molecular weights of approximately 130-150 kilodaltons, which are much smaller in size than full length WNK1 at 230 kilodaltons (Fig 22). This suggested that degradation of WNK1 may involve limited proteolysis by proteases that do not act on unfolded proteins. Because the antibody used for immunoblotting recognizes an epitope in the amino terminal region of WNK1, it can be inferred that the observed bands arise from cleavage at sites which are at least 130-150 residues from the amino terminal of WNK1. As mentioned in Chapter 1 (Project 2), calpains are non-lysosomal cysteine proteases that are activated by intracellular calcium. Calpains have been shown to mediate degradation of kinases such as PKCα, PKCδ, and class IA PI3 kinases [225-227]. Therefore, calpains appeared to be attractive candidates for mediators of WNK1 proteolysis. I tested this idea by assessing the effect of the calpain inhibitor MDL28170 (calpain inhibitor III) on WNK1 protein amount in cells. Treatment with MDL28170 increased WNK1 protein amount in a dose- dependent manner in HeLa cells (Fig 23), suggesting that calpains can promote WNK1 degradation. Experimental strategies to explore this novel connection between calpains and WNK1 will be described in Chapter 5 (Future Directions).

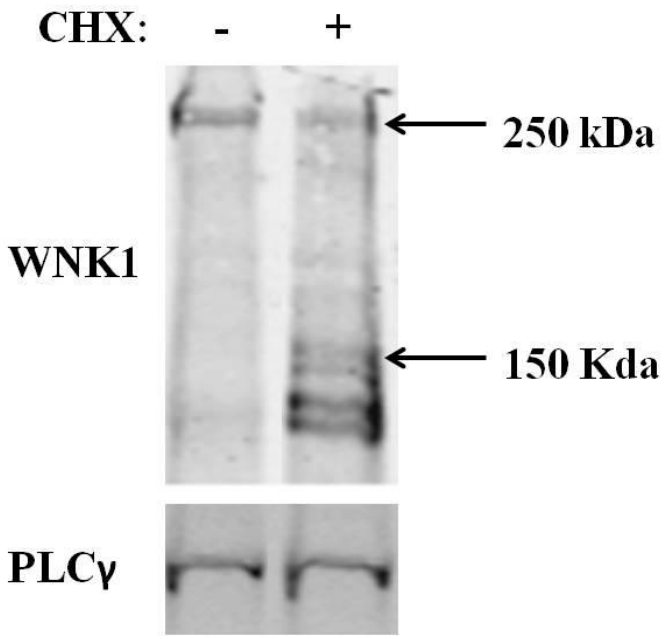


Fig 22: WNK1 may undergo limited proteolytic cleavage. HeLa cells were treated with 100 μM cycloheximide (CHX) for 6 hours. Cell lysates were analyzed by immunoblotting for the indicated proteins. PLCγ was used as a loading control. This blot is representative of three independent experiments.

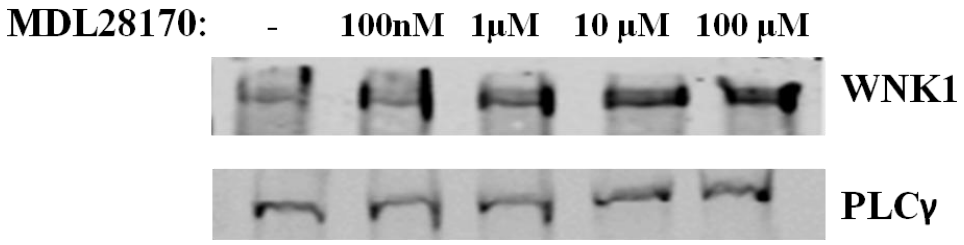


Fig 23: Inhibition of calpains by MDL28170 increases WNK1 protein . HeLa cells were treated with the indicated doses of geldanamycin (GA) or DMSO for four hours. Cell lysates were analyzed by immunoblotting for the indicated proteins. PLCγ was used as a loading control. This blot is representative of two independent experiments.

Summary:

This section has examined the half-life of WNK1 and the contribution of proteasomal, lysosomal and calpain-mediated degradation pathways to the regulation of WNK1 stability. The contribution of protein chaperones has also been explored. Analysis of data from cycloheximide chase experiments revealed fractional degradation kinetics for the WNK1 protein. Pharmacological inhibition of different protein degradation pathways demonstrated the involvement of lysosomes and calpains in WNK1 degradation. Proteasomal inhibition did not significantly affect WNK1 protein amounts. Inhibition of Hsp90 increased WNK1 protein expression, suggesting possible roles for chaperones and/or kinases regulated by Hsp90 in WNK1 stabilization. In conclusion, these findings provide new insights into the complex regulation of WNK1 degradation.

SECTION II: The role of the E3 ligase UBR5 in WNK1 degradation

BACKGROUND:

Experiments performed in our laboratory showed that the large E3 ubiquitin ligase UBR5 can co-immunoprecipitate with OSR1 and that this interaction required WNK1. This suggested that WNK1 may also co-immunoprecipitate with UBR5 and that this association may have consequences for WNK1 stability. As mentioned previously, UBR5 has been implicated in regulation of protein stability. Most of these studies have not determined whether UBR5 promotes proteasomal or lysosomal degradation of its substrates. In this context, it is worth noting that UBR4 (ubiquitin ligase E3 component n-recognin 4), which is related to UBR5, has been reported to participate in autophagy regulation.

RESULTS AND DISCUSSION:

UBR5 co-immunoprecipitates with WNK1 and negatively regulates its stability

I performed immunoprecipitation experiments using an anti-WNK1 antibody to determine whether UBR5 could co-immunoprecipitate with WNK1 and whether this interaction required OSR1 and SPAK. UBR5 co-immunoprecipitated with WNK1 and this interaction did not require OSR1 and SPAK (Fig 24). UBR5 depletion in HeLa cells increased WNK1 protein by 23% (Fig 25 A and B). This difference appears modest, but when considered in view of the complex regulation of cellular WNK1 protein amount and of its potential role as a scaffold, it is possible that small increases in WNK1 protein might have biologically significant consequences. I assessed the effect of UBR5 depletion on WNK1 half-life using cycloheximide chase

experiments described in Section I. Knockdown of UBR5 increased the half-life of WNK1 to approximately eight hours (Fig 26). These data suggest that UBR5 negatively regulates WNK1 stability.

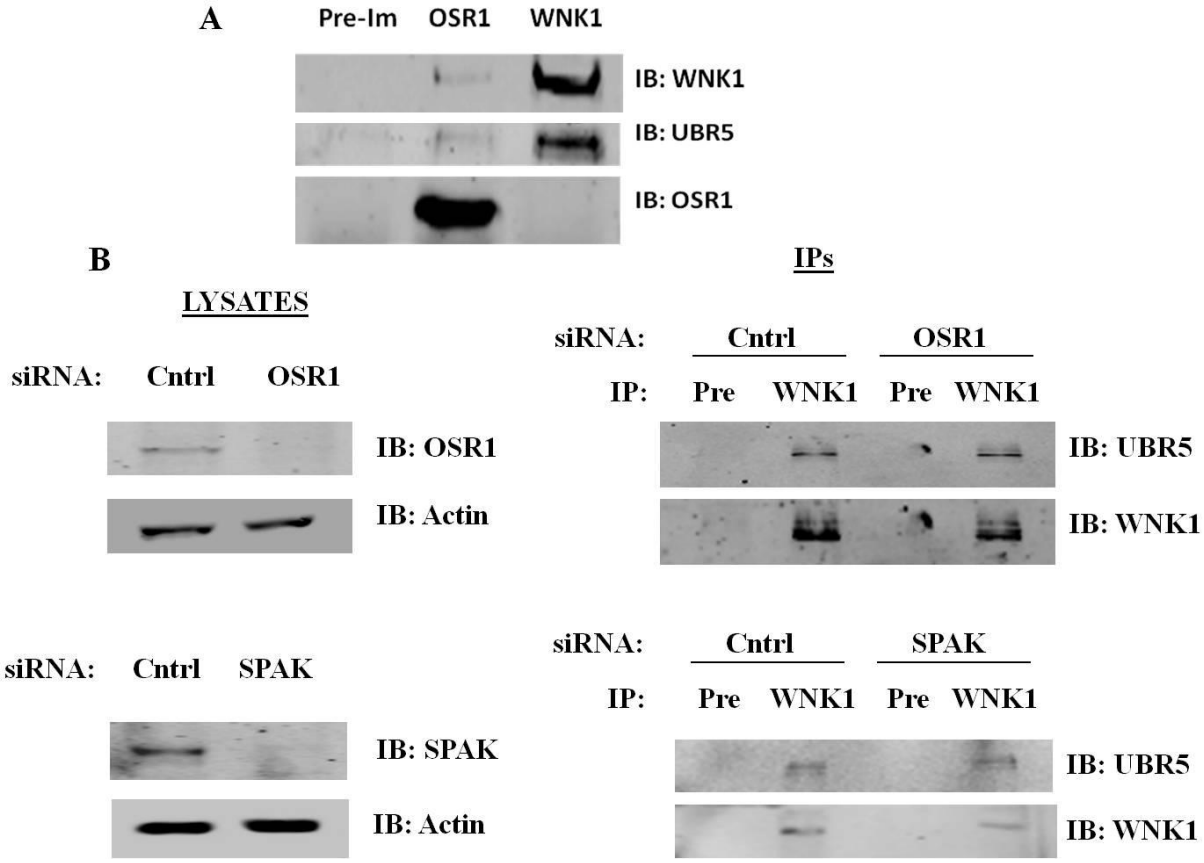
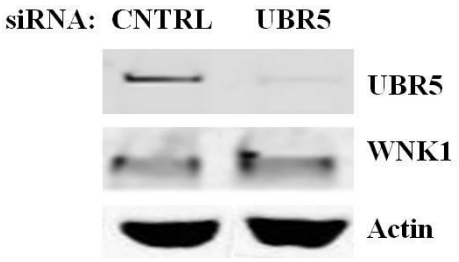


Fig 24: UBR5 co-immunoprecipitates with WNK1 independent of OSR1 and SPAK. (A) UBR5 co-immunoprecipitates with WNK1 and OSR1. HeLa cell lysates were subjected to immunoprecipitation using a WNK1 antibody. Immunoprecipitates were analyzed by immunoblotting for the indicated proteins (B) Lysates of HeLa cells treated with control siRNA , OSR1 siRNA or SPAK siRNA were subjected to immunoprecipitation using WNK1 antibodies. Immunoprecipitates were analyzed by immunoblotting for the indicated proteins. Blots shown are representative of three independent experiments.

A



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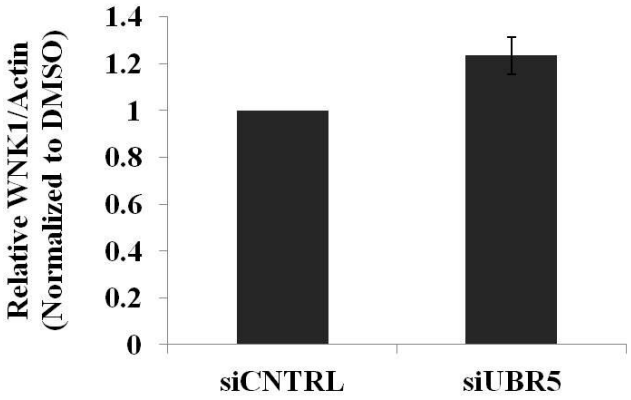
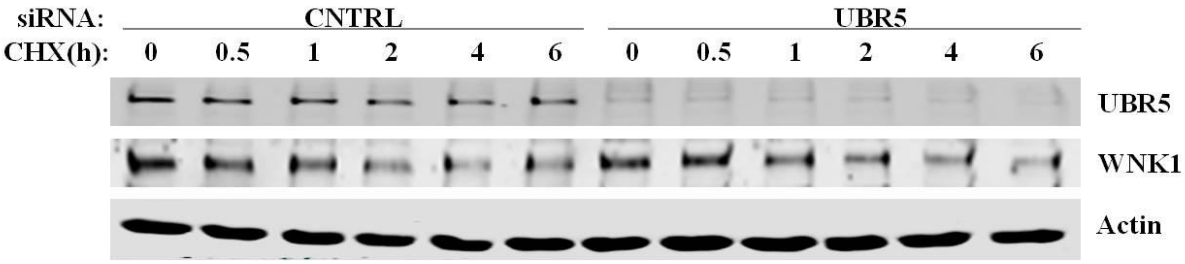
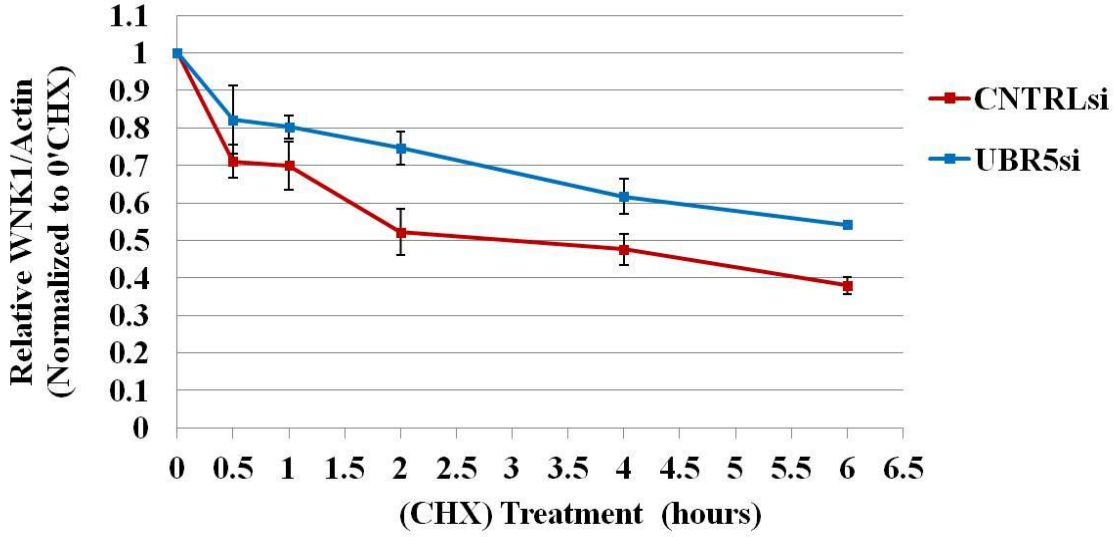


Fig 25: UBR5 depletion increases WNK1 protein. (A) HeLa cells treated with control siRNA or UBR5 siRNA were harvested 72 hours after transfection and lysates were analyzed by immunoblotting for the indicated proteins. (B) Quantification of blots in (A). Data are represented as the mean +/- SEM of three independent experiments. p= 0.05

A


siRNA:
CNTRL
UBR5
CHX(h):
0 0.5 1 2 4 6 0 0.5 1 2 4 6
UBR5
WNK1
Actin

B


Relative WNK1/Actin (Normalized to 0'CHX)
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6 6.5
(CHX) Treatment (hours)
CNTRLsi
UBR5si

C

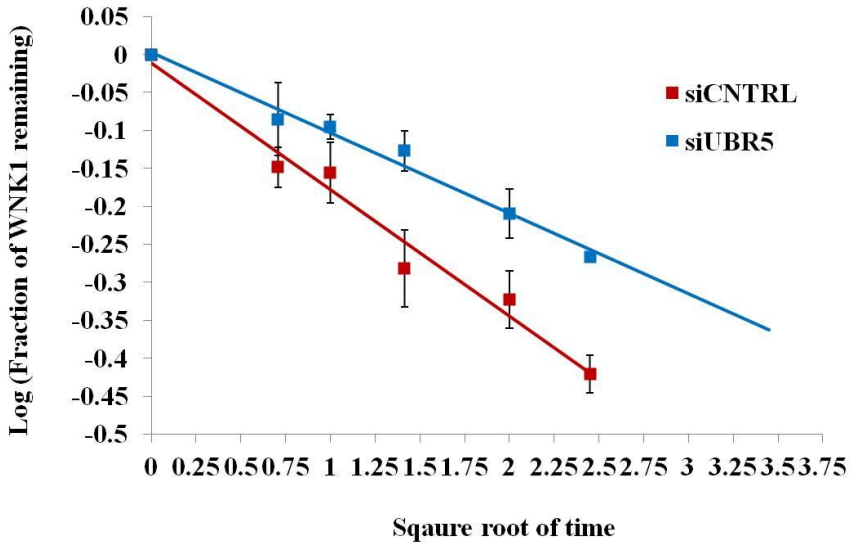


Fig 26: UBR5 depletion increases WNK1 protein stability. (A) HeLa cells transfected with control siRNA or UBR5 siRNA were treated with 100μM cycloheximide (CHX) for the indicated times prior to harvest. Cell lysates were analyzed by immunoblotting for the indicated proteins. (B) Quantification of blots in (A). Data are represented as the mean +/- SEM of three independent experiments. (C) Log-power plot of data in (B).

UBR5 may affect ubiquitination of WNK1 through regulation of KLHL3

Next, I attempted to determine whether UBR5 regulates the ubiquitination of endogenous WNK1, but was unable to address this question due to multiple technical difficulties that precluded reliable immunoprecipitation of ubiquitin and of WNK1 under the denaturing conditions recommended for such experiments and detection of endogenous ubiquitin by immunoblotting. Attempts to overexpress tagged full-length WNK1 to overcome these issues were not successful. Therefore, I assessed the effect of UBR5 depletion on ubiquitination of an overexpressed fragment of WNK1 containing the first 491 residues, which encompasses the kinase domain. HA-tagged ubiquitin was co-expressed with Myc-tagged 1-491 WNK1 to enable visualization of ubiquitin. UBR5 knockdown increased the expression of 1-491 WNK1 in cells and appeared to decrease ubiquitination of immunoprecipitated 1-491 WNK1 (Fig 27). While it is possible that the regulation of expression and ubiquitination of 1-491 WNK1 may not parallel that of full length endogenous WNK1, this does not exclude the possibility of UBR5 regulating ubiquitination of WNK1 at lysine residues within the 1-491 region of full length endogenous WNK1.

Identifying protein regions required for mediating association between two proteins can provide valuable clues about the regulation and function of the association. To identify the region of WNK1 required for its interaction with UBR5, I overexpressed FLAG- or Myc-tagged fragments of WNK1 that encompassed different regions of the protein (Fig 28A) in cells and tested their ability to co-immunoprecipitate with endogenous UBR5. These experiments revealed that UBR5 can co-immunoprecipitate with a C terminal fragment of WNK1 encompassing residues 1850-2126 and not with N terminal fragments (Fig 28B). It is possible that ubiquitination of lysine residues within or near this region of WNK1 may be regulated by UBR5. However, this finding also suggests that the effect of UBR5 depletion observed previously on

ubiquitination of the N terminal 1-491 WNK1 fragment may be indirect, as UBR5 cannot bind to that fragment.

As mentioned in Chapter 1 (Project 2), the KLHL3-CUL3-RBX1 complex can bind to an acidic degron motif near the N terminal region of WNK1 and mediate ubiquitination of WNK1 at multiple lysine residues, including some residues that lie within 1-491. One possibility is that UBR5 can regulate expression and/or function of KLHL3 or CUL3 and thereby influence ubiquitination of lysine residues near the N terminus of WNK1. I tested the effect of UBR5 depletion on KLHL3 protein expression in HeLa cells and found that silencing UBR5 reduced KLHL3 expression by 50 percent (Fig 29). Thus, UBR5 positively regulates KLHL3 expression. Experiments to uncover the mechanism of KLHL3 regulation by UBR5 will be discussed in Chapter 5 (Future Directions). This reduction in KLHL3 might explain the observed reduction in ubiquitination of 1-491 WNK1 upon UBR5 knockdown.

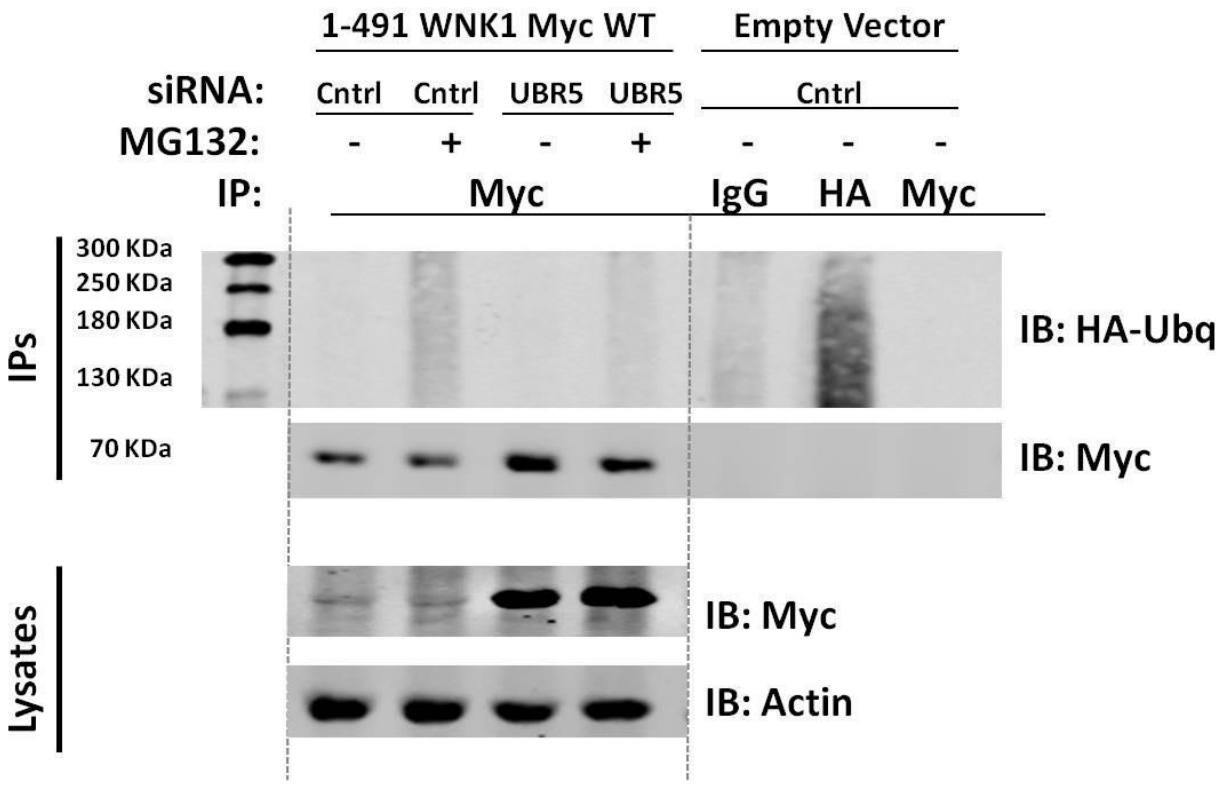


Fig 27: UBR5 depletion increases 1-491 WNK1 expression and reduces ubiquitination of 1-491 WNK1. HEK293T cells were treated with control siRNA or UBR5 siRNA. After 24 hours, cells were transfected with plasmids encoding HA-ubiquitin and 1-491 WNK1 Myc. After 48 hours, cells were treated with 10 μM MG132 for 4 hours prior to harvest. Lysates were subjected to immunoprecipitation using a Myc-tag antibody and immunoprecipitates were analyzed by immunoblotting for the indicated proteins.

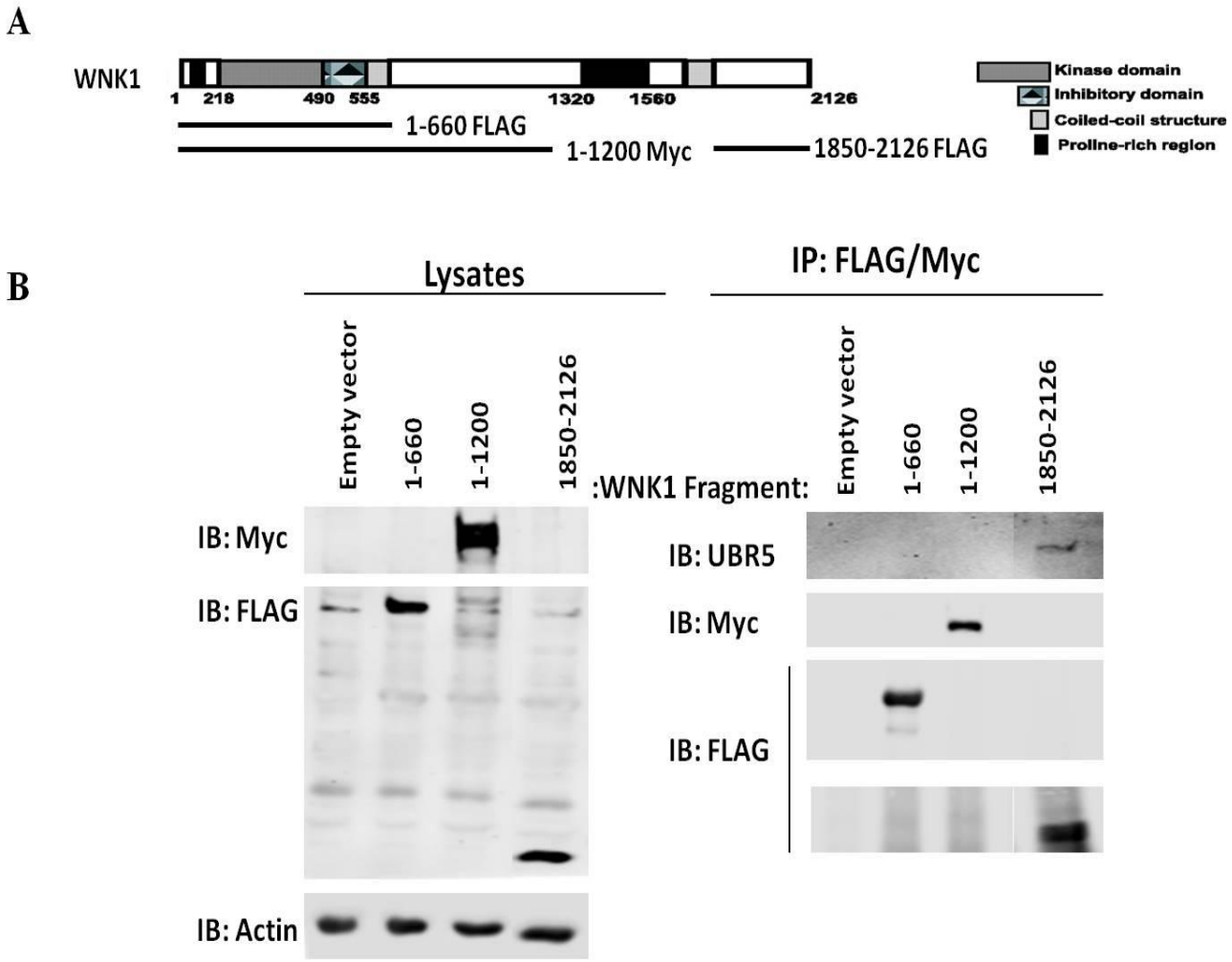
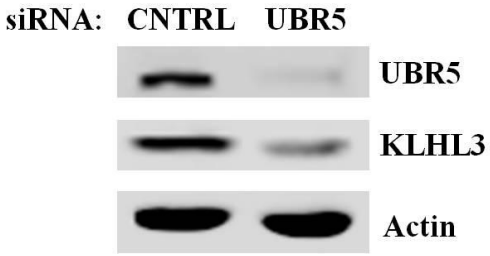


Fig 28: UBR5 co-immunoprecipitates with a C terminal fragment of WNK1. (A) Schematic representation of the various fragments of WNK1 tested for co-immunoprecipitation with endogenous UBR5 (B) Fragments shown in (A) were overexpressed for 48 hours in HEK293T cells and lysates were then subjected to immunoprecipitation with anti-Myc-tag or anti-FLAG-tag antibodies. Immunoprecipitates were then analyzed by immunoblotting for the indicated proteins.

A



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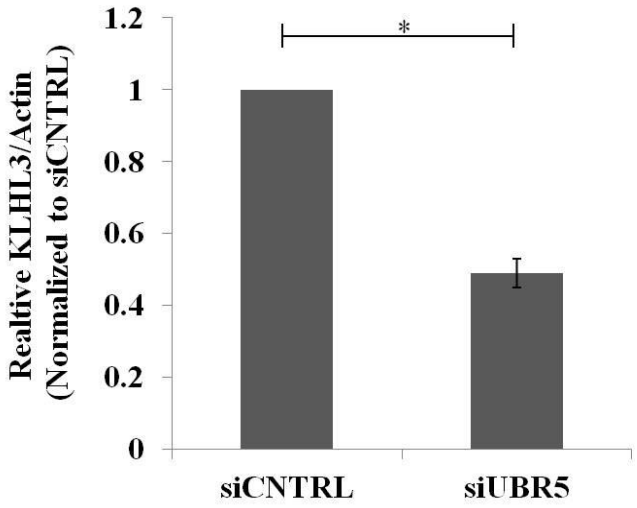


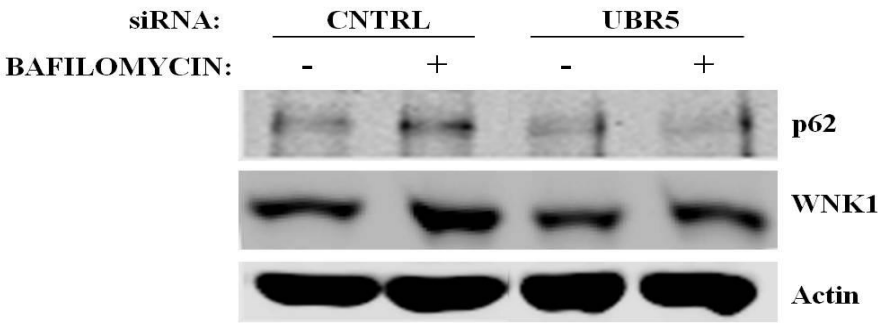
Fig 29: UBR5 depletion reduces KLHL3 protein. (A) HeLa cells treated with control siRNA or UBR5 siRNA were harvested 72 hours after transfection and lysates were analyzed by immunoblotting for the indicated proteins. (B) Quantification of blots in (A). Data are represented as the mean +/- SEM of three independent experiments. *$p<0.05$

UBR5 may promote autophagic degradation of WNK1

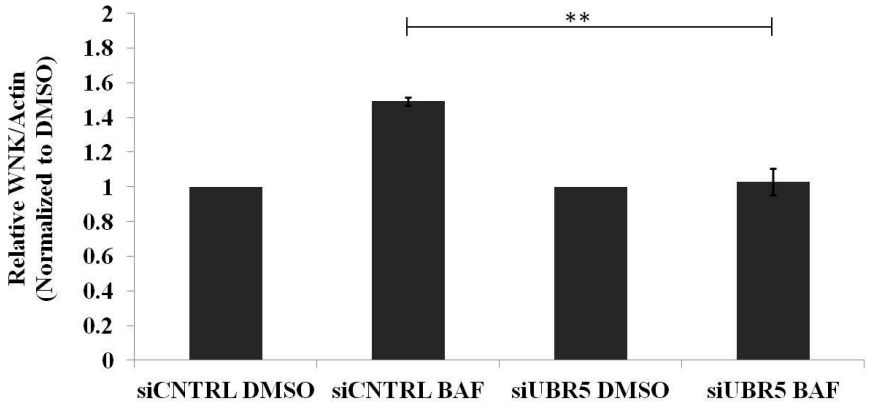
As mentioned in Section I, WNK1 protein expression can be regulated by autophagy. Because cargoes are marked for lysosomal degradation by poly-ubiquitination and UBR5 may regulate WNK1 ubiquitination, I hypothesized that UBR5 might promote autophagic degradation. To test this hypothesis, I assessed the effect of UBR5 depletion on autophagic degradation of WNK1. Knockdown of UBR5 reduced the bafilomycin-induced increase in WNK1 protein amounts compared to cells treated with control siRNA (Fig 30 A and B). Because bafilomycin inhibits lysosomal hydrolases, cargoes destined for autophagic degradation that reach lysosomes will accumulate therein. As a result, the extent of accumulation of a protein induced by bafilomycin treatment can be thought of as indicative of the amount of the protein that reaches lysosomes. Therefore, these results suggest that UBR5 depletion reduces the ability of WNK1 to be recruited to lysosomes, thereby reducing the flux of WNK1 through autophagy. Thus, UBR5 may promote autophagic degradation of WNK1.

UBR5 may exert this effect by specifically affecting recruitment of WNK1 to autophagosomes or it may affect steps in the general process of autophagy, leading to effects on WNK1 degradation. To examine the latter possibility, I examined the effect of UBR5 depletion on bafilomycin-induced p62 protein accumulation. Knockdown of UBR5 did reduce p62 accumulation, but this difference was not significant, suggesting that general defects in autophagy may not underlie the effect observed on WNK1 accumulation (Fig 30 C).

A


siRNA:
CNTRL
UBR5
BAFILOMYCIN:
-
+
-
+
p62
WNK1
Actin

B


Relative WNK/Actin (Normalized to DMSO)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
siCNTRL DMSO
siCNTRL BAF
siUBR5 DMSO
siUBR5 BAF

C

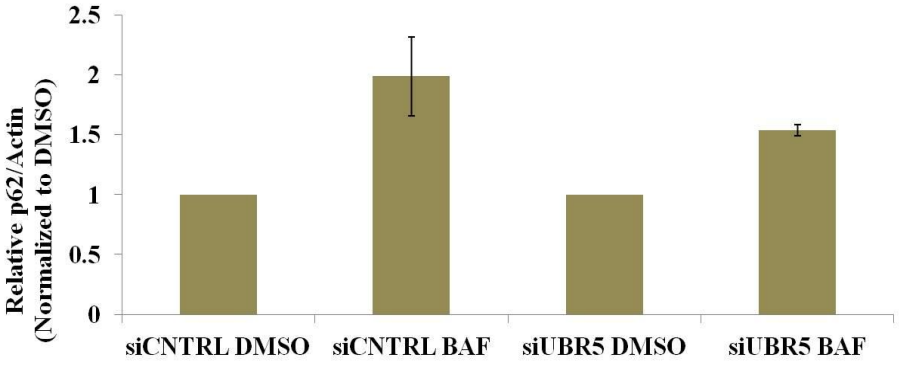

Relative p62/Actin (Normalized to DMSO)
2.5
2
1.5
1
0.5
0
siCNTRL DMSO
siCNTRL BAF
siUBR5 DMSO
siUBR5 BAF

Fig 30: UBR5 promotes autophagic degradation of WNK1 (A) HeLa cells treated with control siRNA or UBR5 siRNA were treated with 500 nM bafilomycin or DMSO for four hours prior to harvest. Lysates were analyzed by immunoblotting for the indicated proteins. (B and C) Quantification of blots in (A). Data are represented as the mean +/- SEM of three independent experiments. **$p<0.01$

Summary:

This section has examined the interaction of the E3 ligase UBR5 with WNK1 and examined the role of this E3 ligase in regulating WNK1 stability. UBR5 co-immunoprecipitates with WNK1 and this interaction could be mediated by the C terminal region of WNK1. UBR5 can regulate the ubiquitination of an overexpressed N terminal fragment of WNK1, most likely through positive regulation of KLHL3 expression. Finally, UBR5 promotes autophagic degradation of WNK1. In summary, these findings identify UBR5 as a new modulator of WNK1 stability.

CHAPTER 5:
Future Directions

Project 1: Future perspectives on the mechanisms involved in regulation of mTORC1 activity, autophagy and T1R1-T1R3 expression by β-arrestin 2

How does β-arrestin2 impinge on mTOR signaling? As shown in Chapter 4, knockdown of β-arrestin 2 decreases amino acid and growth factor-induced S6 phosphorylation, decreases p62 protein, and increases LC3-II formation. These findings point to a role for β-arrestin 2 in promoting mTORC1 activity and suppressing autophagy. Because kinases other than p70 S6 kinase can also phosphorylate S6, it is important to assess the phosphorylation of mTOR substrates p70 S6 kinase and 4EBP1. While preliminary data suggest that 4EBP1 phosphorylation is affected by β-arrestin 2 knockdown, the effect on phosphorylation of p70 S6 kinase remains to be assessed.

This study has used siRNA mediated silencing for depletion of β-arrestin 2 in cells. However, it will be important to determine whether the effects observed in this study also occur upon long term depletion of β-arrestin 2. This would require examination of mTORC1 activity and autophagy in β-arrestin 2 knockout MEFs (mouse embryonic fibroblasts) and in cells depleted of β-arrestin 2 by CRISPR-mediated gene editing. Additionally, it remains to be determined if the effects of β-arrestin 2 knockdown can be rescued by expression of an siRNA-resistant version of the protein.

What are the mechanisms through which β-arrestin 2 can affect mTORC1 activity? One possibility is that amino-acid- induced mTORC1 localization might be impaired in arrestin knockdown cells. However, preliminary experiments using immunofluorescence microscopy suggest that this is not the case. mTORC1 signaling requires normal lysosome maturation and function. Therefore, it could be hypothesized that β-arrestin 2 promotes normal lysosome function to facilitate mTORC1 signaling. Preliminary experiments indicate that β-arrestin 2 knockdown decreases the expression of lysosomal protein LAMP1 (Lysosome associated

membrane protein 1) and, to a lesser extent, LAMP2 (Lysosome associated membrane protein 2). It is possible that expression of other lysosomal proteins may be also affected, which could lead to impaired lysosomal function. Lysosome function depends on the maintenance of the acidic pH of the lysosomal lumen. For this reason, it will be important to examine the effect of β-arrestin 2 knockdown on lysosomal acidification. Acidotropic dyes for qualitative measurement of lysosomal pH by fluorescence intensity, as well as ratiometric dyes for quantification of pH are widely used to assess lysosomal acidification [228]. Some lysosomal proteins are specifically involved in communicating amino acid availability to mTORC1. These include the arginine transporter SLC38A9 and the V-type H^+ ATPase. Because the effects of β-arrestin 2 depletion were the most pronounced in the context of amino acid-induced mTORC1 activity, it is possible that β-arrestin 2 might influence the function of these molecular players to modulate the responsiveness of mTORC1 to amino acids.

Alternatively, it is possible that β-arrestin 2 could modulate the activity of upstream regulators of mTORC1. As mentioned previously, it has been reported that arrestins are required for mTOR dependent protein synthesis stimulated by angiotensin AT1 receptor activation and that this involves the activity of an arrestin-bound pool of AKT. AKT is involved in activation of mTORC1 in response to growth factors, but not amino acids. Therefore, while AKT scaffolding by arrestins might be involved in growth factor-induced mTORC1 activation, it may not contribute to amino acid-induced mTORC1 activation. DEPTOR (DEP domain containing mTOR-interacting protein) and PRAS40 (40 kilodalton proline rich AKT substrate) have been shown to bind mTORC1 and inhibit its activity [229, 230]. Perhaps β-arrestin 2 might interact with these proteins to sequester them away from mTORC1. This idea can be tested by immunoprecipitation experiments.

How does β-arrestin 2 participate in autophagy regulation? Autophagy is a complex process involving many steps, each of which can be regulated by different mechanisms. β-arrestin 2 appears to be involved in preventing excessive autophagy in both nutrient-rich conditions and starvation conditions. Preliminary data in HeLa cells suggest that administration of amino acids to cells growing in complete growth medium suppresses autophagy, and that this is impaired upon β-arrestin 2 depletion. While β-arrestin 2 knockdown affects mTORC1 activity during amino acid starvation and re-feeding, it does not strongly impact mTORC1 activity in cells growing in complete medium. Taken together, these findings suggest that β-arrestin 2 regulates autophagy through both mTORC1- dependent and mTORC1-independent pathways. As mentioned previously in Chapter 3, numerous studies have reported that autophagy can be regulated by mTOR independent mechanisms which are governed by second messengers such cAMP, inositol, IP_3, and calcium [200-202]. As a first step to investigating the impact of β-arrestin 2 on these pathways, the concentrations of these messengers could be measured in cells subjected to arrestin depletion under different nutritional conditions.

Studies have implicated ERK1/2 in promoting autophagy in an mTOR-independent fashion [205, 231]. Findings in this study point to the possibility of ERK1/2 involvement in the increased autophagy induced by β-arrestin 2 depletion. However, the cause of increased ERK1/2 upon arrestin depletion remains unclear. It is unlikely that increased MEK1/2 activity can account for increased ERK1/2 phosphorylation in this context, because the arrestins have been reported to promote ERK1/2 activity by scaffolding MEK1/2 [206]. However, the scaffolding activities of arrestins are highly context-dependent, and they have also been reported to act as scaffolds for phosphatases such as PP2A (protein phosphatase 2) and PTEN (phosphatase and tensin homolog) [232, 233]. PP2A has been shown to regulate autophagy in yeast [234]. It would be interesting to assess the effect of arrestin loss on the activities of these phosphatases as well as members of the MAP kinase phosphatase family. If the activity any of these phosphatases is

found to be decreased by arrestin depletion, it will be important to determine whether this reduced activity contributes to the increased autophagy induced by arrestin depletion. This can be achieved by testing the ability of a constitutively active form of the phosphatase to reduce autophagy when overexpressed in cells depleted of β-arrestin 2. Other MAP kinases like p38 and JNK have also been reported to regulate autophagy and can be scaffolded by arrestins [209]. Therefore, it will be important to assess the activity of these kinases upon arrestin depletion under various nutritional conditions to examine their contribution, if any, to the increased autophagy phenotype.

Which step (or steps) in autophagy is (are) influenced by β-arrestin 2? Since ULK1 and AMPK play central roles in autophagy induction, their activation and function upon arrestin depletion should be assessed. The binding of Vps34 to Beclin-1 is an important step for the initiation of autophagy. It has been reported that β-arrestin 1 can bind to each of these proteins and promote their interaction to mediate neuroprotective autophagy [60]. It is possible that β-arrestin 2 may also have similar scaffolding capacities for autophagy regulators that modulate their function. Perhaps β-arrestin 2 could suppress the Vps34-Beclin-1 interaction. This question could be tested by immunoprecipitation experiments. The next step in autophagy that must be examined is autophagosome formation. Attempts to do so by visualization of endogenous LC3 puncta by immunofluorescence microscopy were not successful. Assessing autophagosome formation by expression and microscopic visualization of GFP-LC3 puncta is a widely used tool in autophagy research. However, there are multiple caveats to this assay, and extensive controls are required to ascertain reliable results [235]. Moreover, given that GFP is about twice the size of LC3, it is possible that the trafficking and degradation may not parallel endogenous LC3 in every aspect. The final step in autophagy that enables degradation of autophagic cargoes is fusion of autophagosomes with lysosomes. It has been shown that LAMP1 and LAMP2 are required for this process [236, 237]. Preliminary data indicate that β-arrestin 2 depletion reduces

the expression of LAMP1 and LAMP2. This suggests that β-arrestin 2 might play a role in regulating autophagosome-lysosome fusion. Additionally, the arrestins can interact with small GTPases like the Rab and Arf proteins, which participate in autophagosome maturation and fusion with the lysosomes [55, 238]. This process can be visualized in living cells using expression of RFP-tagged LC3 as the autophagosome marker combined with the use of acidotropic dyes that label lysosomes [239].

Arrestins can also impact gene expression [240]. Many autophagy genes, including p62 and LC3 can be transcriptionally regulated during autophagy [241]. Therefore, it will also be important to investigate the effect of β-arrestin 2 depletion on the mRNA expression of these genes, which can be achieved using qPCR.

The initial motivation behind this study was to determine whether the arrestins could mediate the effects of the heterodimeric, amino acid sensing GPCR T1R1-T1R3 on mTORC1 and autophagy. Although β-arrestin 2 depletion largely phenocopies T1R3 and T1R1 depletion in terms of mTORC1 activity and autophagy, it is possible that these processes are regulated independently by arrestins and by T1R1-T1R3. A surprising finding was the reduction of T1R3 and T1R1 protein expression by β-arrestin 2 knockdown. This raises the possibility that the effects of arrestin depletion might be due, at least in part, to decreased T1R1-T1R3 expression.

How does β-arrestin 2 regulate T1R1-T1R3 expression? After internalization, cell surface receptors can persist in endosomes or undergo degradation due to endo-lysosomal fusion, or they may undergo re-sensitization and be recycled back to the plasma membrane. Little is known about the trafficking of T1R1-T1R3. Therefore, it is unclear which step in trafficking is affected by β-arrestin 2. Generation of cell lines stably expressing tagged T1R1-T1R3 would provide a tool to study the trafficking of this receptor. Immunofluorescence studies in such cell

lines using markers for lysosomes and different endosome populations could shed light on the differences in T1R1-T1R3 trafficking events between normal cells and cells depleted of β-arrestin 2. It is possible that association with β-arrestin 2 might prevent T1R1-T1R3 from associating with factors that target the receptor to lysosomes. If this is correct, then β-arrestin 2-deficient cells would show increased co-localization of lysosomal markers with T1R1-T1R3. Additionally, blocking lysosomal degradation in β-arrestin 2 depleted cells would restore receptor amounts to levels comparable to normal cells.

PROJECT 2: Future perspectives on exploring mechanisms of WNK1 degradation

Which type of autophagy plays a role in WNK1 degradation? Inhibition of lysosomal function can have other cellular consequences that extend beyond autophagy disruption. Therefore, it is important to use other means of inhibiting autophagy to confirm the involvement of this process in the degradation of WNK1. This can be achieved by silencing the expression of key autophagy regulators like ATG7 and Beclin-1. It is not known if macro-autophagy, micro-autophagy or chaperone mediated autophagy is involved in WNK1 degradation. Chaperone mediated autophagy (CMA) can be inhibited in cells by knockdown of its central regulators LAMP2A and Hsc70. However, interpretation of results can be confounded by the fact that CMA inhibition leads to increased macro-autophagy as a compensatory mechanism [242]. Therefore, the most reliable method to determine whether a protein can serve as a substrate for CMA is to measure the uptake of the purified protein by isolated, intact lysosomes [243]. This method could be used to assess the ability of WNK1 to act as a CMA substrate.

How does Hsp90 inhibition by geldanamycin affect WNK1 protein stability? As mentioned in Chapter 3, Hsp90 inhibition has been reported to induce Hsp70 expression. It must first be confirmed that Hsp70 is induced by geldanamycin in HeLa cells for the doses and times used in the experiments described in Chapter 3. If it is found to be induced, the next step would be to examine the effect of silencing Hsp70 on WNK1 protein amount during geldanamycin treatment to determine whether increased Hsp70 accounts for the increased stability of WNK1.

How do calpains affect WNK1 stability? As mentioned in Chapter 1 (Project 1), there are more than a dozen different isoforms of calpains, some of which exhibit tissue specific expression. Knockdown experiments would reveal which calpain family member is involved in regulation of WNK1 stability. Other ways to assess calpain involvement in WNK1 degradation would involve the manipulating calpain activity by altering intracellular calcium concentration or over-expressing the calpain suppressor calpastatin. Demonstrating that WNK1 is a direct substrate for calpains would be supported by in vitro experiments examining proteolysis of purified WNK1.

Further characterization of the UBR5-WNK1 interaction. I attempted to identify the region of UBR5 that is required for interaction with WNK1 by testing the ability of overexpressed UBR5 fragments spanning residues 1-700, 700-1400, 1400-2100, and 2100-2800 to co-immunoprecipitate endogenous WNK1 in cells. I did not observe co-immunoprecipitation of endogenous WNK1 with any of these fragments. This suggests that the binding region might lie within a region whose contiguity or folding is disrupted in the four fragments tested. Constructs for expression of fragments spanning these regions have been made and remain to be tested for endogenous WNK1 co-immunoprecipitation. UBR5 has been reported to form a complex with the adaptor protein VPRBP (Vpr (HIV-1) binding protein), DDB1 (damage-specific DNA binding protein 1) and DYRK2 (dual-specificity tyrosine phosphorylation

regulated kinase 2) in order to bind some of its substrates [244, 245]. Whether these proteins are required for the association of WNK1 with UBR5 is a question that remains to be addressed.

How does UBR5 promote KLHL3 expression? UBR5 has been reported to promote the protein stability of the transcription factor myocardin, but the underlying mechanism remains unknown [246]. UBR5 could either positively regulate KLHL3 mRNA expression, or negatively regulate proteins mediating KLHL3 protein degradation. CUL3 has been reported to promote KLHL3 ubiquitination and degradation. UBR5 knockdown did not appear to affect CUL3 protein amount in cells, but this does not exclude the possibility that UBR5 could suppress CUL3 activity. CUL3 function is regulated by the conjugation of the small protein Nedd8, a process known as neddylation [247, 248]. Perhaps UBR5 regulates the neddylation of CUL3. UBR5 can affect gene transcription by regulating the stability of transcription factors [129, 246]. qPCR analysis would reveal whether KLHL3 gene transcription can be regulated by UBR5.

What sites on WNK1 are ubiquitinated by UBR5 and what are the functions of these modifications? Although both WNK1 and WNK4 have been shown to bind to and be regulated by the KLHL3/CUL3 complex, the number and position of ubiquitination events have only been identified for WNK4 [122]. It is possible to predict the sites on WNK1 based on its homology with WNK4. However, WNK1 is nearly twice the size of WNK4, and it is thought that larger proteins are more likely to undergo multi-monoubiquitination and poly-ubiquitination events than smaller proteins. Therefore, while some ubiquitination events may be conserved between the two WNK isoforms, there may be many that are specific to WNK1. As mentioned in Chapter 1 (Project 2), studies comparing the effects of short-term and long term CUL3 depletion on WNK1 and WNK4 protein amounts clearly demonstrate that WNK1 degradation is more tightly controlled than WNK4, suggesting the existence of multiple mechanisms that can compensate for long-term CUL3 loss [126]. It would be interesting to determine whether UBR5 can act as one such compensatory factor. The study that indentified sites of KLHL3-CUL3-RBX1-

mediated ubiquitination on WNK4 employed over-expression of KLHL3 as the strategy for site enrichment [122]. A similar approach could be adopted for identification of UBR5-mediated ubiquitination sites on WNK1. However, successful overexpression of full length UBR5 is challenging, which may be due to the auto-ubiquitination exhibited by many E3 ligases that promote their own degradation. Overexpression of a fragment of UBR5 that contains the PABC and HECT domains of UBR5 (residues 2100-2800) can be achieved more easily than full length UBR5. However, the utility of this fragment of UBR5 is not clear because of the absence of information on UBR5 binding regions and adaptor proteins required for WNK1 association. If an adaptor protein such as VPRBP is found to be required for UBR5-WNK1 interaction, overexpressing the adaptor might be a better strategy for enrichment of UBR5-mediated WNK1 ubiquitination sites.

What would be the functions of these ubiquitination events? Some of these modifications probably serve as markers for lysosomal (or proteasomal) degradation. Mutational analysis could reveal which sites play a role in degradation. Other sites may play roles in regulating one or more functions of WNK1. Preliminary data suggest that UBR5 knockdown does not affect the ability of WNK1 to phosphorylate OSR1 in response to osmotic stress in cells. Additionally, osmotic stress does not appear to disrupt the interaction of UBR5 with WNK1. However, WNK1 has many kinase-dependent and independent functions, and the possible impact of UBR5 on any of these functions remains to be explored.

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