Novel Upstream Activation of mTORC2 by the Innate Immune Kinase TBK1

by

Aaron Seth Tooley

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Doctoral Committee:

Professor Marina Pasca di Magliano, Chair
Associate Professor Diane C. Fingar
Associate Professor Ken Inoki
Professor Kristen J. Verhey

Aaron Seth Tooley

atooley@umich.edu

ORCID iD: 0000-0002-0874-0391

Dedication

I dedicate this dissertation to my mother, Carla Anita Tooley, who has sacrificed tremendously for me to have the opportunities I've been so fortunate to have. She raised me to love God, always present my best self, and be a decent human being. In spite of adverse circumstances during my youth, she remained steadfast in her moral principles and supported me in the best way she could, even if all she could provide at the time was unconditional love. But were it not for her, I would not exist, nor would the accomplishments I've been so blessed to have achieved at this point in my life. Thank you, mom, for being my persistent and fearless champion, and I hope I've made you proud. I love you more than you will ever know.

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Abstract

The mechanistic target of rapamycin (mTOR) senses diverse intracellular and extracellular cues to control key fundamental cellular processes, including cell growth and proliferation, cell survival, and metabolism. Consequently, aberrant mTOR activity contributes to various pathological diseases, including cancer, type 2 diabetes, and autoimmune diseases. mTOR nucleates the catalytic core of two multimeric complexes: mTOR complex 1 (mTORC1) and mTORC2. mTORC1 integrates nutrient and growth factor cues to promote cell growth, cell cycle progression, and anabolic processes, marked by increases in protein, lipid, and nucleotide biosynthesis. Growth factors and energetic stress activate mTORC2, which promotes cell survival and regulates metabolism. While upstream regulation of mTORC1, including the environmental stimuli and associated signaling intermediates, is far more comprehensively characterized, upstream regulation of mTORC2 remains woefully ill-defined.

The noncanonical IKK-related kinases, Tank-binding kinase 1 (TBK1) and IKKε, initiate the innate immune response to circumvent pathogenic infection by integrating signals from various pattern recognition receptors' detection of microbial-derived antigens. TBK1 and IKKε, in turn, phosphorylate and activate IRF-3/7 transcription factors to drive interferon beta (IFNβ) production to promote eradication of infectious agents. Prior work from our lab identified TBK1 as a novel mTOR S2159 kinase. In macrophages, TBK1-mediated phosphorylation of mTOR S2159 within mTORC1 was

found to promote IRF-3 nuclear translocation and IFNβ production, and EGF-stimulated mTORC1 signaling was reduced in mouse embryonic fibroblasts (MEFs) lacking TBK1 or wild type MEFs treated with the TBK1 inhibitor amlexanox. While studying TBK1-mediated activation of mTORC1, we also observed reduced EGF-stimulated phosphorylation of Akt S473, a well-established mTORC2 substrate. Here we demonstrate that TBK1-mediated phosphorylation of mTOR on S2159 increases mTORC2 intrinsic kinase activity and downstream signaling to Akt S473 in response to EGF, and, more broadly, mTOR S2159 phosphorylation promotes mTORC2 and mTORC1 signaling in response to a broader array of growth factors (EGF, FBS, PDGF, and insulin). Additionally, we demonstrate that mTOR S2159 phosphorylation promotes TLR-3-mediated mTORC2 and mTORC1 signaling in response to the synthetic dsRNA mimetic, poly (I:C). Mechanistically, TBK1 interacts with mTORC2 in intact cells and phosphorylates mTOR S2159 within mTORC2 *in vitro*. These results contribute important mechanistic insight toward our poor understanding of the upstream regulation of mTORC2. More importantly, since aberrant TBK1 and mTORC2 activity have both been implicated in pathological diseases, most notably obesity-driven type 2 diabetes and oncogenesis, the TBK1-mTORC2 axis may represent an attractive therapeutic target for such diseases.

Chapter 1

Introduction

1.1 Historical identification of rapamycin and mTOR

The mechanistic target of rapamycin (mTOR) is an evolutionarily conserved eukaryotic serine/threonine protein kinase. mTOR functions as an environmental sensor by virtue of its ability to monitor fluctuations in environmental conditions and modulate cellular processes, such as cell growth and metabolism, appropriately to maintain homeostasis. The identification of mTOR dates back to the 1960s where researchers in Rapa Nui (Easter Island) happened upon a unique compound produced by the bacterium, *Streptomyces hygroscopicus*, present in their soil isolate (1). Shortly thereafter named rapamycin, this compound was found to possess robust anti-proliferative, anti-fungal, anti-tumor, and immunosuppressive properties (1-4). Unfortunately, nearly three decades would elapse before rapamycin's intracellular target and mechanism of action would be discovered. Rapamycin binds the intracellular receptor and immunophilin, FKBP12, a cis-trans peptidyl-prolyl isomerase, to form a functional ternary complex (5,6). This rapamycin-FKBP12 complex docks to the FKBP12-rapamycin binding (FRB) domain on mammalian mTOR (amnio acids E2015-Q2114) to inhibit its activity and mTOR-dependent cell growth (Fig 1.1) (7-10).

1.2 mTORC1 and mTORC2 partner proteins and structure

mTOR is a member of the phosphoinositide 3 kinase (PI3K)-like kinase family of serine/threonine protein kinases (11,12). In cells, through its association with both shared and unique partner proteins, mTOR comprises the catalytic core of two known multimeric complexes known as mTOR complex 1 (mTORC1) and mTORC2. The partner proteins that are common to both complexes include mammalian lethal with Sec13 protein 8 (mLST8) (alias: G-protein β-subunit like protein (GβL)) and DEP-domain containing mTOR interacting protein (DEPTOR) (12,13). The unique partner proteins conveniently permit the biochemical differentiation, isolation, and analysis of each complex. mTORC1-exclusive partner proteins include Raptor (regulatory protein associated with mTOR) and PRAS40 (proline rich Akt substrate of 40 kDa) (12,14-16). Raptor confers dual functions on mTORC1, first by serving as an obligate scaffold partner to maintain complex stability and bind mLST8, and second, by facilitating mTORC1 recruitment of substrates via their TOR signaling (TOS) motifs, as well as recruitment of mTORC1 itself to distinct subcellular locations for activation (14,17,18). PRAS40 inhibits mTORC1, but this inhibition is relieved following insulin stimulation, which induces Akt-mediated phosphorylation and subsequent dissociation of PRAS40 from the complex (19-21). Cryo-EM approaches indicate that mTORC1 exists in cells as a dimer with an apparent rhombus-like or “logenze” shape, with key binding contacts formed between Raptor, mTOR, and the mTOR HEAT repeats (22,23).

mTORC2-specific partner proteins include Rictor (rapamycin insensitive companion of mTOR), mSin1 (MAPK-interacting protein 1), and Protor 1/2 (protein

associated with rictor 1/2) (24-29). Rictor and mSin1 are obligate scaffolding partners of mTORC2. Genetic ablation of Rictor profoundly destabilizes mTORC2 and reduces its activity in cells (30). mSin1 harbors the lipid-binding pleckstrin homology (PH) domain, which proves important for mTORC2's recruitment to the plasma membrane in response to insulin where it phosphorylates and activates Akt (31). Activation of insulin or IGF-1 receptor induces phosphorylation of specific tyrosines within the receptor's intracellular domains, which enables phosphatidylinositol 3 kinase (PI3K) to dock at the phosphotyrosines either directly, via the regulatory p85 subunit, or through the Src homology 2 (SH2) domains of co-adaptors (Fig 1.2) (32,33). PI3K recruitment permits it to catalyze the conversion of phosphatidylinositol 4,5 bisphosphate (PIP_2) into phosphatidylinositol 3,4,5 triphosphate (PIP_3) via pehosphorylation of the 3' hydroxyl moiety of inositol ring-bearing lipids (Fig 1.2) (32-34). The generation of PIP_3 on the inner leaflet of the plasma membrane facilitates recruitment of mTORC2, via the pleckstrin homology (PH) domain of mSin1, and Akt/protein kinase B (PKB), also via its PH domain, effectively relieving mSin1-mediated inhibition of mTORC2 and enabling it to phosphorylate Akt on its hydrophobic motif on S473 (11,35) (Fig1.2).

mTORC2 is resistant to acute rapamycin treatment; however, chronic administration of rapamycin inhibits mTORC2 activity by disrupting mTORC2 complex integrity, a hypothesized consequence of ubiquitous rapamycin-FKBP12 complexes binding to newly synthesized mTOR and impeding its incorporation into new mTORC2 complexes (12,36). Recent cryo-EM studies reveal incredible insight into mTORC2 assembly and the structural basis underlying mTORC2 rapamycin insensitivity. Similar

to mTORC1, mTORC2 also assumes a rhomboid dimeric structure, with mTOR-mTOR interactions forming a more compact conformation (37,38). In addition, the N-terminal helical rich clusters of Rictor bind mTOR, and mSin1 binds near the FRB domain and active site of mTOR, which imparts the steric hinderance that excludes rapamycin-FKBP12 from the active site (37,38). These unique partner proteins permit differentiation between mTORC1 and mTORC2, play active roles in distinct regulation of both complexes, and confer distinct cellular functions on each complex.

1.3 mTORC1: signaling pathways, substrates, and cellular functions

mTORC1 senses a diverse array of intracellular and extracellular stimuli, including growth factors, hormones, nutrients (i.e. amino acids and glucose), cytokines, hypoxic factors, and high AMP levels (indicative of deficient cellular energy) (57-60). These stimuli activate numerous upstream pathways, all of which converge on mTORC1, through a central signaling node and inhibitor of mTORC1 known as the Tuberous Sclerosis Complex (TSC), to either activate or inhibit mTORC1. Upstream pathways that activate mTORC1 include the Ras/Raf/MAPK and PI3K/Akt pathways, which are activated downstream of growth factor-stimulated receptor tyrosine kinases (RTK) (Fig 1.2) (12,57). Conversely, upstream pathways that attenuate mTORC1 signaling include DNA damage-induced activation of the tumor suppressor p53; energetic stress-induced activation of AMP activated protein kinase (AMPK); and hypoxia-induced activation of REDD1 (61) (12). Whereas mTORC1 activating stimuli

and associated pathways do so by phosphorylating and inhibiting TSC, mTORC1 inhibiting signals do so by phosphorylating and activating TSC.

Growth factors such as epidermal growth factor (EGF) and insulin/insulin-like growth factor 1 (IGF-1) engage their cognate RTK to activate either the mitogenic Ras/Raf/MAPK pathway or the PI3K pathway (Fig 1.2) (32,33,62). In terms of the Ras/Raf/MAPK pathway, p90 ribosomal S6K (p90 RSK) and ERK1/2 phosphorylate TSC2 on inhibitory sites to relieve its suppression of mTORC1 (Fig 1.5) (63,64). In terms of the PI3K pathway, growth factor activated insulin/IGF-1 receptor recruits 3'-phosphoinositide-dependent kinase 1 (PDK1) and Akt/protein kinase B (PKB) via their pleckstrin homology (PH) domains (Fig 1.2) (32,34,65).This coordinated localization enables PDK1 to directly phosphorylate Akt on its activation loop site on T308, and Akt inhibits TSC2 through multi-inhibitory site phosphorylation (Fig 1.2) (66-68).

The TSC is a heterotrimeric complex comprised of three proteins: TSC1 (hamartin), TSC2 (tuberin), and TBC1D7 (69,70). TSC was originally identified as a tumor suppressor after inactivating mutations in TSC1 and TSC2 were found to cause the formation of benign tumors on diverse organs such as the brain and kidneys (70). Akt-mediated phosphorylation of TSC2 (on S939 and T1462 in humans) induces dissociation of the TSC complex from the lysosomal membrane, thereby relieving its antagonistic effect on mTORC1; TSC fulfills this function by exerting its GTPase activating protein (GAP) activity toward the GTPase Rheb (Ras homolog enriched in brain), stimulating the conversion of active GTP-bound Rheb to inactive GDP-bound Rheb (68,71-76). This enables Rheb to directly activate mTORC1 through a still elusive

mechanism that likely involves a large conformational change in mTORC1 induced by Rheb binding near the mTOR kinase domain, effectively positioning critical active site residues for efficient catalysis (20,73,74,77,78).

In addition to growth factors, mTORC1 also senses nutrients (amino acids) via rigorously deciphered intricate mechanisms. Nutrient deprivation of cells confines mTORC1 to the cytoplasm, whereas nutrient stimulation induces translocation of mTORC1 to the lysosome; mTORC1 remains tethered to the lysosome in the presence of amino acids through its interaction with the Rag-GTPases via an emanating claw-like structure on Raptor (79-82). Indeed, irreversible targeting of mTORC1 (via Raptor) to the lysosome uncouples mTORC1 activation from amino acid availability (59,83). The Rag-GTPases exist as obligate heterodimers that are regulated by multiple recently identified nutrient-binding proteins, among them Sestrin2, a direct leucine sensor, and CASTOR1, a direct arginine sensor (59,79,80,84-86) (Figure 1.5). Leucine binds Sestrin2 via a pocket that exploits specific charged residues on leucine to effectively engage a specific hydrophobic group (87). One GTP-bound Rag A or B binds one GDP-bound Rag C or D to yield a competent heterodimer (84). The Rags are essential for mTORC1 responsiveness to acute shifts in amino acid concentrations, as constitutively active Rag alleles irreversibly tether mTORC1 to the lysosome regardless of amino acids depletion (59).

A large five member complex known as the Ragulator or LAMTOR complex consists of LAMTOR1 (p18), LAMTOR2 (p14), LAMTOR3 (MP1), and LAMTOR4 (C7ORF59), and LAMTOR5 (HBXIP) (59,88). The Ragulator/LAMTOR complex and the

vacuolar H^+ adenosine triphosphatase ATPase (v-ATPase) represent a key nexus between amino acid-induced mTORC1 activation. Amino acid starvation promotes engagement between the catalytic V_1 ATPase subunit and the V_0 proton pump subunit, while amino acid repletion disrupts this interaction, and an intralysosomal acidic pH proves essential for v-ATPase complex assembly (89). Also, using an eloquent *in vitro* biochemical reconstitution approach, Zoncu et al demonstrated a requirement for a sufficient intralysosomal amino acid pool in the ability of Raptor to interact with lysosome-associated Rag B/C as well as amino acid-induced activation of mTORC1 (90). Importantly, v-ATPase activity appears to be important for the dynamic changes in interactions observed between the Ragulator/LAMTOR complex and the v-ATPase complex in response to amino acids (90). Specifically, the Ragulator/LAMTOR interaction, via its p18 and p14 subunits, with the v-ATPase V_1 subunit is weakened following amino acid stimulation of amino acid starved cells (90).

Additionally, through its cytoplasmic tail, the transmembrane-spanning intralysosomal arginine sensor SLC38A9 was shown to promote activation of the Rag GTPases by disrupting the lysosomal folliculin complex (LFC) comprised of three constituents: inactive Rag GTPase, Ragulator/LAMTOR, and folliculin (FLCN)-folliculin-interacting protein 2 (FNIP2) (91). Specifically, SLC38A9-mediated disruption of the LFC permits FLCN-FNIP2 to exert its GAP activity toward GTP-bound Rag C, effectively relieving the locked, inactive state of the Rag GTPase (91). Interestingly, Shen et al revealed a uniquely distinct mechanism by which, through a coordinated effort, the Ragulator/LAMTOR complex and SLC38A9 activate the Rag GTPases (92).

Specifically, the Ragulator/LAMTOR exerts atypical guanine nucleotide exchange factor (GEF) activity toward GTP-bound Rag C, while, following arginine engagement, SLC38A9 directs its GEF function toward GDP-bound Rag A to yield an active Rag GTPase heterodimer (92). These studies support a model where cellular sensing of amino acids initiates within the lysosomal lumen, culminating in the activation of mTORC1. However, the report of two different mechanisms underlying the production of active Rag GTPase heterodimers warrants further investigation into this matter.

In addition to, but unlike Sestrin2 and CASTOR1, SLC38A9, through interaction with the Ragulator (via its initial 119 N-terminal residues) and Rag GTPases and in an intralysosomal arginine-dependent manner, senses and exports intralysosomal amino acids obtained from the catabolic degradation of cellular proteins to free up free amino acids for energy and to re-activate mTORC1 (93-96). Note that SLC38A9 is particularly important for the export of leucine, which, with arginine, represent the two most potent activators of mTORC1 (59,96).

Several additional regulatory proteins also compose the current amino acid sensing mTORC1 pathway. The trimeric GATOR1 complex, composed of DEPDC5 (DEP domain containing 5), Npr12 (nitrogen permease regulator 2-like protein), and Npr13, exerts its GAP activity toward Rag A and B to suppress mTORC1 signaling (59,84). The five member GATOR2 complex, comprised of Mios (meiosis regulator for oocyte development), tryptophan-aspartic acid repeat-containing protein 24 (WDR24), WDR59, SEH1-like nucleoporin (Seh1L), and Sec13, inhibits GATOR1 (84,97). In the absence of arginine CASTOR1 directly binds and inhibits GATOR2; this inhibitory effect

is relieved in the presence of arginine where CASTOR1 is proposed to directly engage arginine through its four tandem aspartate kinase chorismite mutase and TyrA (ACT) domains (59,86,98). In the absence of leucine Sestrin1/2 directly bind and inhibit GATOR2 (85), and leucine exclusively binds Sestrin1/2 to induce its dissociation from GATOR2 (99-101).

KICSTOR, a four member complex consisting of ITFG2, KPTN, C12ORF66, and SZT2, localizes GATOR1 to the lysosome, enabling it to suppress mTORC1 in the absence of amino acids by exerting its GTPase activating protein (GAP) function toward the Rag GTPases and GATOR2 (59,102). Also, the heterodimeric FLCN-FNIP1 complex, comprised of folliculin (FLCN) and FLCN-interacting protein 1 (FNIP1), translocates to the lysosome in the absence of amino acids, where it exerts GAP activity toward Rag C and D to maintain them in the active GDP-bound state, and dissociates from the lysosome in the presence of amino acids (59,103). This highlights the eloquently coordinated mechanisms underlying nutrient sensing by mTORC1. Both amino acid induced recruitment of mTORC1 to the lysosome and growth factor-mediated phosphorylation and dislocation of TSC from the lysosome synergize, culminating in the full activation of mTORC1. Therefore, amino acid and growth factor signaling represent the best characterized activating mechanisms for mTORC1.

mTORC1 is a well-established master regulator of cell growth (characterized by a cell autonomous increase in size and mass). Cell growth requires amenable amounts of lipids, nucleotides, and amino acids, and growth conducive signals induce mTORC1, which drives biosynthesis of these key molecular constituents. Thus, mTORC1

functions as a metabolic rheostat by dually promoting anabolic programs and suppressing catabolic processes. Pro-growth conditions (i.e. sufficient levels of nutrients, growth factors, and energy) activate mTORC1, which promotes cell cycle progression (proliferation) and drives anabolic metabolism (12,57,60,104). Thus, mTORC1 is known as the master regulator of cell growth and an anabolic kinase.

mTORC1 promotes cell growth by increasing protein synthesis through direct phosphorylation of two key substrates, p70 S6K1 on T389 and eukaryotic initiation factor 4E binding protein 1 (4EBP1) (on multiple sites including T37, T46, S65, and T70) (105-109). S6K1 phosphorylates elongation initiation factor (eIF) 4B to drive formation of the eIF4F complex, which associates with the 5' cap of mRNA transcripts to drive their translation (12,110). Also, mTORC1-mediated phosphorylation of 4EBP1 induces its dissociation from eIF4E and also drives eIF4F complex assembly (12,105). Thus, mTORC1 promotes translation of 5' cap-dependent mRNA, with a particular preference for transcripts harboring 5' pyrimidine rich TOP-motifs, which encode for ribosomes and other essential translation machinery (12,57,111).

mTORC1 also supports anabolic processes such as nucleotide and lipid synthesis, while it coordinately suppresses catabolic processes such as autophagy. mTORC1-mediated phosphorylation of Lipin 1, an inhibitor of the sterol response element binding protein (SREBP) transcription factors, effectively sequesters it in the cytoplasm, and S6K1-mediated phosphorylation of the SREBPs, both drive the synthesis of fatty acids, cholesterol, and lipids for membrane formation (12,112). Nucleotides are indispensable for DNA replication and serve as key intermediates for

energy production during cellular stress. Thus, mTORC1 also promotes the mitochondrial-associated tetrahydrofolate cycle through activation of methylene tetrahydrofolate dehydrogenase 2 (MTHFD2), which increases synthesis of one carbon intermediates for purine synthesis (57,113). S6K1-mediated phosphorylation of carbamoyl phosphate synthetase (CAD) on S1859 promotes the rate-limiting step of pyrimidine biosynthesis (57,114,115). Autophagy is a survival mechanism employed by cells to maintain homeostasis through the breakdown and recycling of old/damaged organelles and other macromolecules into their monomeric constituents. mTORC1 suppresses autophagy by phosphorylating unc-51-like autophagy activating kinase 1 (ULK1) on S757, thus preventing assembly of the autophagy-initiating complex comprised of ULK1, ATG101, ATG13, and FIP2000 (116,117). mTORC1 co-localization with the transcription factor EB (TFEB) at the lysosome enables mTORC1 to phosphorylate TFEB on S211, impeding its nuclear translocation and ability to drive expression of genes needed for autophagosomal and lysosomal biogenesis (118-120).

Aberrant mTORC1 activation is a common feature of cancer and metabolic diseases. As such, mTORC1 is tightly regulated such that it is only activated in the presence of sufficient energy, pro-growth signals, and low stress. AMP activated protein kinase (AMPK) is a key sensor of cellular energy stress. A couple of indicators of energetic stress are deficient glucose levels and the accumulation of AMP in cells, both of which are sensed by AMPK via allosteric binding of AMP (121). Once activated, AMPK directly phosphorylates Raptor as well as TSC2 (to activate it) to inhibit mTORC1 signaling (51,57,122,123). AMPK and regulated in DNA damage and development

(REDD1) also coordinate to sense deficient oxygen levels and inhibit mTORC1 signaling, also via phosphorylation and activation of the TSC (12,124). These represent clear examples of how cells ensure mTORC1 only becomes active under favorable conditions.

1.4 mTORC2: signaling pathways, substrates, and cellular functions

Growth factors, largely via the insulin/insulin-like growth factor (IGF)/PI3K pathway, activate mTORC2, which promotes cell survival and proliferation and modulates cellular metabolism (11,27,29,30). One group recently delineated interesting mechanistic detail underlying mTORC2's involvement in the regulation of cell migration downstream of G-protein coupled receptor activation by chemoattractants in *Dictyostelium discoideum* (125,126). Intriguingly, and in contrast to accepted dogma that GDP-bound G-proteins possess very low activity and downstream activating potential relative to GTP-bound G-proteins, GDP-bound RacE was shown to interact with mTORC2 and promote mTORC2 signaling to Akt, which facilitated directional migration toward the chemoattractant cyclic adenosine monophosphate (cAMP) (125). Importantly, GTP-RacE was shown to antagonize RacE-GDP-mediated activation of mTORC2 (126). Specifically, in response to cAMP glycogen synthase kinase 3 (GSK-3)-mediated phosphorylation of RacE-GDP on S192 promoted the assimilation of trimolecular complex comprised of RacE-GDP, RasC-GTP, and mTORC2, which increased mTORC2's kinase activity toward Akt *in vitro* and in cells to promote their

migration toward cAMP (125,126). These studies highlight a key role for mTORC2 in Rho- and Ras-mediated directional cell migration.

Through association with ribosomes, mTORC2 also catalyzes the co-translational phosphorylation of Akt on its C-terminal turn motif on T450 as it threads off of the ribosome, which is essential for Akt stability, as it effectively positions the C-tail proximal to the kinase domain the (127-130). Recently, AMPK was shown to directly activate mTORC2 to promote cell survival during acute energetic stress (43). mTORC2 directly phosphorylates members of the AGC kinase family to exert its cellular functions, the best known being Akt/PKB (30). mTORC2 is the prominent kinase for Akt S473 both in intact cells and *in vivo*, and phosphorylation of Akt on S473 boosts its kinase activity to a relative maximum level, while also conferring substrate specificity (12,131). mTORC2 also phosphorylates several conventional protein kinase C isoforms, including protein kinase C alpha (PKCα) on its turn motif on T638 and hydrophobic motif on S657, and PKCβII on its turn motif on T641 and hydrophobic motif on S660 (11,128,129). Similar to mTORC2-mediated phosphorylation of Akt, phosphorylation of these sites seems to stabilize PKC isoforms. mTORC2 also employs the partner protein Protor to phosphorylate the AGC member, serum and glucocorticoid-induced kinase 1 (SGK1) on its hydrophobic motif on S422, which is required for SGK1 phosphorylation of downstream substrates (132,133). Unlike the case for Akt and conventional PKC isoforms, abrogation of mTORC2 does not affect SGK1 expression (11).

While growth factors activate mTORC2, much less is known about how additional stimuli and associated signaling intermediates regulate and converge on mTORC2. However, through activation of AGC family kinases, mTORC2 regulates a plethora of cellular functions, including cell survival and proliferation, metabolism, and actin cytoskeletal organization associated with cell migration (12). Interestingly, mTORC1 also regulates mTORC2 through a negative feedback loop. mTORC1-mediated phosphorylation of Grb10 enhances its inhibitory effect on insulin/PI3K signaling, and S6K1-mediated phosphorylation of IRS-1 induces its degradation (134,135). Both of these mechanisms effectively dampen PI3K flux to mTORC2 and Akt.

1.5 Phosphorylation of mTOR and mTOR complex partner proteins

Phosphopeptide mapping and mass spectrometry based phosphoproteomic approaches have led to the identification of a swath of phosphorylation sites on mTOR and mTOR interacting partner proteins, Raptor, Rictor, and mSin1, most of which remain uncharacterized. In the case of mTOR, the direct mTORC1 substrate S6K1 was identified as a direct mTOR S2448 and T2446 kinase via a feedback mechanism with no known function (Fig 1.1) (39,40). Also, shortly after initial identification of the mTOR S2481 autophosphorylation site (41), Soliman et al demonstrated that this site accurately monitors both mTOR and mTOR complex specific catalytic activity in cells, as S2481 autophosphorylation increased in response to mTOR-activating stimuli (i.e. insulin and amino acids) and is reduced upon withdrawal of such stimuli (Fig 1.1) (42). Importantly, mTOR-S2481 autophosphorylation correlates with increased or decreased

mTORC1 and 2 signaling depending on the cellular context. mTOR S1261 was recently identified as a direct AMPK substrate that is phosphorylated in response to acute energetic stress, which increases mTORC2 activity and signaling to drive a pro-survival phenotype (Fig 1.1) (43). Finally, through an *in vitro* kinome screen of 300 human kinases, mTOR S2159 was identified as a novel TBK1 substrate, and in response to EGF and TBK1-activating innate immune agonists (i.e. poly (I:C) and LPS), TBK1-mediated phosphorylation of mTOR S2159 promoted mTORC1 signaling and innate immune function (Fig 1.1) (44).

In the case of Raptor, the Ras/Raf/MAPK pathway was shown to promote multi-site phosphorylation of Raptor (45,46). Indeed, p90 ribosomal S6 kinase 1 (p90 RSK1) and p90 RSK2 were demonstrated to phosphorylate Raptor following activation of MAPK kinase signaling, and through classic IP-MS, S719, S721, and S722 were identified as the prominent RSK1 and 2 substrates (Fig 1.3) (45). Importantly, Raptor phosphorylation of all three sites was required for MAPK kinase agonists to increase mTORC1 catalytic activity (45). Similarly, ERK2 was found to interact with Raptor, and ERK1/2 phosphorylated Raptor on proline-directed sites S8, S696, and S863, which promoted mTORC1 kinase activity in response to Ras/MAPK agonists (Fig 1.3) (46).

Another study identified mTOR itself as a kinase for both Raptor S859 and S863, but only Raptor P-S863 was found to contribute appreciably to increasing mTORC1 kinase activity and signaling in response to insulin-mediated activation of the PI3K/TSC/Rheb axis (Fig 1.3) (47). Through IP-MS Foster et al identified six phosphorylation sites on Raptor (S696, T706, S855, S859, S863, and S877), including

the above-mentioned sites (48). Raptor S863, in particular, was shown to function as a critical nexus between diverse pro-growth agonists (i.e. insulin, amino acids, and EGF) and mTORC1 activation, and Raptor S863 phosphorylation was a required prerequisite for subsequent phosphorylation on S855 and S859 (Fig 1.3) (48). Also, while mutation of S863 to alanine modestly reduced mTORC1 intrinsic catalytic activity, mutation of all six sites to alanine markedly reduced insulin-stimulated mTORC1 catalytic activity *in vitro* (48). Importantly, the sensitivity of growth factor and nutrient induced Raptor P-S863 to rapamycin indicates that mTOR within mTORC1 is the Raptor S863 kinase in this cellular context. Thus, while all six of the Raptor phosphorylation sites appear to be positive regulators of mTORC1 under pro-growth conditions, it remains possible that a subset of them cooperate with additional uncharacterized or unidentified phosphorylation sites to negatively regulate mTORC1 in different cellular contexts.

In support of this notion, another study demonstrated that Raptor S863 phosphorylation participates in a mechanism to suppress mTORC1 signaling in response to hyperosmotic and oxidative stress (Fig 1.3). Specifically, Nemo-like kinase (NLK)-mediated phosphorylation of Raptor S863 impeded mTORC1 recruitment to the lysosome by disrupting Raptor's interaction with the Rag-GTPase (49). Also, it was demonstrated that Raptor S877 phosphorylation by TBK1 occurs in response to cellular stimulation with TBK1-activating innate immune agonists (i.e. LPS) to attenuate mTORC1 catalytic activity (Fig 1.3) (50). In the context of energetic stress, the AMP activated protein kinase (AMPK), in a manner dependent on the presence of its direct upstream kinase, liver kinase B1 (LKB1), phosphorylates Raptor on S722 and S792,

resulting in binding of the regulatory protein, 14-3-3, which allosterically attenuates mTORC1 intrinsic kinase activity and concomitantly arrests cells in G1/S phase of the cycle (Fig 1.3) (51). Note that a commonality among these studies is that phosphorylation of Raptor on these sites does not affect the critical mTOR-Raptor interaction.

Out of the 21 total Rictor phosphorylation sites originally identified by Dibble et al using LC-MS, Rictor T1135 was identified and characterized as a mTORC2 inhibitory site targeted by mTORC1 (52,53). Specifically, in response to growth factors, S6K1 phosphorylated Rictor on T1135 to inhibit mTORC2 signaling exclusively to Akt S473, concomitant with impaired cell proliferation, without any effects on mTORC2 complex integrity or intrinsic catalytic activity (Fig 1.4) (52,53). Another group demonstrated that oncogenic Ras expression decoupled Rictor T1135 phosphorylation from IGF-1 regulation, rendering it constitutive (54). Intriguingly, IGF-1-stimulated Rictor T1135 phosphorylation was rapamycin and mTOR-dependent, invoking either mTOR, as part of mTORC2, or mTORC1 in modulating Rictor phosphorylation (54). Interestingly, but contrary to Julien et al and Dibble et al, no discernable increase in growth factor stimulated Akt P-S473 was observed, leaving the definitive function of Rictor T1135 an unresolved enigma (54). Collectively, this suggests that, rather than negatively regulating mTORC2 signaling to Akt S473, as reported by Julien et al and Dibble et al, Rictor T1135 phosphorylation exerts an unknown regulatory role, perhaps on other mTORC2 substrates or independently of mTORC2, that is possibly dependent on mTORC1 (54). In support of this, another group also employed genetic and

pharmacological inhibitor approaches to invoke S6K1 as a Rictor T1135 kinase, but Rictor T1135 phosphorylation has no effect on growth factor stimulated mTORC2 signaling (55). Also, S6K1-mediated Rictor phosphorylation promoted binding of a regulatory protein 14-3-3 (55). Thus, while Rictor T1135 appears to serve as an inhibitory site, the differences among the studies highlighted above underscores the importance of future work aimed at characterizing the remaining Rictor phosphorylation sites.

Liu et al demonstrated that, in response to growth factors (i.e. EGF, PDGF, and IGF-1), S6K1 phosphorylated the mTORC2 partner protein mSIn1 on T86 and T398, effectively inducing its dissociation from the mTORC2 complex and severely attenuating mTORC2 intrinsic catalytic activity and signaling (Fig 1.5) (56). mSin1 phosphorylation at both sites also correlated with impaired mTORC2 engagement of Akt (56). Importantly, interrogation of a neighboring mutation (R81T) in mSin1 identified in an ovarian patient, revealed that phosphorylation of this site was required for T38 phosphorylation in response to oncogenic growth factor receptor signaling, which correlated with increased FOXO3-mediated cell survival in response to the chemotherapeutic agents cisplatin and etoposide and tumor progression *in vivo* (56). Overall, while several of the above-mentioned phosphorylation sites have been linked to important functional outcomes, a largess of them remains uncharacterized or poorly characterized, underscoring how minimal our knowledge remains regarding the function of mTOR and mTOR partner protein phosphorylation in cells and *in vivo*.

1.6 Physiological functions of mTORC1 and mTORC2

The ability of mammals to appropriately regulate diet-induced fluctuations in circulating nutrients represents an evolutionary adaptation critical for both immediate post-natal and adult survival. Conveniently, mTOR orchestrates essential crosstalk between key metabolic target tissues (i.e. liver, skeletal muscle, and adipose tissue), primarily by sensing the post-prandial secretion of the hormone insulin from the pancreas, as well as nutrients (i.e. glucose and amino acids), inflammatory cytokines, and growth factors. Thus, mTOR signaling is critical for maintaining whole-body blood glucose homeostasis and lipid metabolism through both mTORC1 and mTORC2. The important roles of mTOR in metabolic homeostasis is supported by observations made from genetic mouse models.

Evidence that mTORC1 function is important for glucose homeostasis and insulin sensitivity includes the observation that mice harboring a constitutively active allele of Rag A die shortly after birth due to their inability to maintain blood glucose at amenable levels following perinatal fasting (136). Hyperactive mTORC1 signaling (via constitutive Rag A) also induces lethal hypoglycemia in mice, which was linked to impaired activation of autophagy in the liver to yield amino acids for *de novo* glucose production (136). Also, constitutive mTORC1 activation (via TSC2 knockout) in pancreatic beta cells long-term results in reduced beta cell size, insulin secretion, and glucose intolerance (137). Genetic deletion of Raptor in adipose causes lipodystrophy, characteristic of the inability of adipocytes to properly expand and retain morphological

features compatible with triglyceride storage (138,139). This is exacerbated in mice administered a high-fat diet (HFD), and consequently, these mice develop metabolic defects including hepatomegaly (enlarged liver), hepatic steatosis (lipid accumulation in the liver), insulin resistance, and inflammation (139,140). Interestingly, however, these mice also display normal blood glucose levels and resistance to a HDF-induced increase in body mass. Adipocytes devoid of insulin receptor phenocopied mice lacking Raptor in adipocytes, invoking the insulin/IGF-1/PI3K/Akt pathway as a critical upstream mediator of mTORC1-dependent adipocyte maintenance (140).

Not surprisingly, mTORC2 is also crucial for maintenance of glucose homeostasis. Indeed, genetic deletion of Rictor in adipose impairs glucose uptake by adipocytes, increases lipolysis concomitant with reduced *de novo* lipogenesis, reduced adiposity, and promotes insulin resistance (140,141). While slightly less severe, these mice also exhibit hepatomegaly and steatosis, yet they are resistant to HFD-induced weight gain and exhibit normal adipocyte morphology (140,141). Collectively, these *in vivo* studies highlight a crucial role for mTORC1 and mTORC2 in control of lipid and glucose metabolism as well as whole body glucose tolerance and insulin sensitivity.

Given the plethora of cellular processes controlled by mTORC1 and mTORC2, it is not surprising that aberrant activity of both complexes is implicated in cancer initiation and progression. Interestingly however, mutations in mTOR itself are rare; rather it's the effect of collective mutations in components of upstream growth-promoting signaling pathways, most notably Ras/Raf/MAPK and PI3K/Akt, that lead to constitutive mTOR activity in various cancers. In fact, aberrant mTOR activity exists in approximately 80

percent of human cancers (142). Constitutive mTORC1 activation, as a consequence of mutations in amino acid sensing pathway components, has been observed in some cancers. Specifically, mutations in the GATOR1 complex and the Rag C GTPase have been identified in the brain cancer glioblastoma and follicular lymphoma, respectively (97,143). It is suggested that these mutations confer a competitive advantage on cancer cells by enabling them to sustain high levels of proliferation under nutrient poor conditions (57). Mutations in Ras, Raf, the p110 catalytic subunit of PI3K, PTEN, and mTOR exist in a slew of different cancer types ranging from pancreatic to renal (12).

mTORC2 is also implicated in tumorigenesis, as evidenced by the fact that *Pten* null prostate cancer cells lacking Rictor fail to form tumors following subcutaneous injection and successful Pten-induced transformation of normal prostate epithelium requires Rictor (144). mTORC2 has also recently been identified as a direct downstream effector of oncogenic Ras (145). mTORC2 preferentially interacts with oncogenic Ras over wild type Ras, and this interaction promotes a proliferative gene expression profile and tumor oncogenic Ras-driven tumor growth *in vivo* (146). mTORC2 also functions critically in melanoma cancer, as increased Rictor expression in melanoma tumor cells was found to promote Akt activation and tumor cell migration to, and metastasis of, the liver in response to signals excreted from hepatic stellate cells (147). Functional mTORC2 also promotes HER2-positive breast cancer migration and metastasis (148).

Modulation of mTOR, and more specifically mTORC1, activity has been shown to ameliorate the effects of ageing. A central cause of ageing is a deviation from metabolic

homeostasis in a way that adversely affects physiological fitness, leading to a gradual decline in health span and longevity (57,149). mTOR's multifaceted role in metabolic regulation renders it an attractive therapeutic target for improving age-related metabolic decline and reduced lifespan, as evidenced by the fact that reduced dietary caloric consumption reduces mTORC1 activity while extending lifespan (57,149). Indeed, one group demonstrated that genetic ablation of S6K1 increased lifespan, while curbing insulin resistance and immune and muscle decline (150). Importantly, S6K1 loss produced a transcriptional signature similar to one resulting from dietary restriction (150).

Also, reduced mTOR expression significantly increased lifespan in *Caenorhabditis elegans*, and mutations in the *C. elegans* gene encoding Raptor (*daf-15*) extended lifespan by decoupling mTORC1 activation from the insulin/IGF-1 pathway (151,152). Also, in *C. elegans* rapamycin or genetic perturbation of mTOR induced transcriptional upregulation of stress response genes mediated by the transcription factors SKN-1/Nrf and DAF-16/FoxO, while also extending lifespan (153). Also, administration of rapamycin to late-age mice curtailed the effects of aging to extend life span (154). Moreover, rapamycin rendered fruit flies resistant to starvation-induced stress while increasing longevity, which correlated with reduced protein translation and increased autophagy (155). Rapamycin administration to yeast in stationary phase improved longevity, in part by driving a stress protective transcriptional program orchestrated by the transcription factor Msn2 (156). The fact that mTORC1 inhibition offers a profound protective advantage under various stress conditions while deflecting

cellular changes that incur with ageing supports the proposition of targeting mTOR to extend lifespan.

Constitutive mTORC1 activity drives the high energy taxing process of protein synthesis, which likely consumes cellular energy needed for maintenance and protective mechanisms to mitigate ageing. This is supported by that fact that suppressing protein synthesis extends lifespan (157). Thus, mTORC1 inhibition shifts the cellular metabolic program toward a catabolic state, which increases autophagy, forcing the cell to breakdown and recycle old/damaged organelles for energy and macromolecular synthesis rather than through constant utilization of diet-obtained nutrients. Moreover, remaining in a net catabolic state indirectly reduces the accumulation of toxic metabolic by-products associated with heightened anabolism, which inadvertently damage existing organelles. Therefore, aged cells likely benefit from a slight net catabolic rather than anabolic program. Indeed, mice with increased basal autophagy conferred by mutational disruption of the association between beclin 1 and its negative regulator BCL2, exhibited increased longevity and protection against age-related pathological alterations that compromise kidney and heart function (158). Interestingly, mTORC1 inhibition depends on functional autophagy machinery to produce desired anti-ageing benefits, as both the loss of essential autophagy genes and reduced mTORC1 activity fail to recapitulate the improved lifespan benefits that occur with dietary restriction (159,160).

Finally, mTOR also has roles in neurophysiology and neurodevelopment, evidenced by the fact that genetic ablation of Raptor and Rictor in the brain yield similar

adverse outcomes, namely stark decreases in neuron autonomous growth and proliferation (contributors to microcephaly) and differentiation (57). Raptor knockout in the central nervous system progenitor cells results in the onset of microcephaly at approximately E17.5 due to an overall abbreviated cell cycle and apoptosis, as well as acute postnatal death (161). Rictor ablation in both the entire central nervous system and exclusively in Purkinje cells also resulted in decreased brain size attributed to a cell autonomous reduction in neuronal growth rather than increased cell death or decreased proliferation (162). Also, Rictor loss disrupted proper cerebellar morphology and folding and the extension of dendrite processes (57,162).

Constitutive mTOR activation, largely a consequence of mutations that render essential upstream mTOR negative regulators inactive, also contributes to neurophysiological diseases (57,163). These diseases typically exhibit abnormal focal growths, an overall enlarged brain, and tumor growths in critical regions that can cause epileptic seizures. Indeed, inactivating mutations in NPRL2 and NPRL3, constituents of the GATOR1 complex which inhibits mTORC1 in response to amino acid starvation, correlated with the occurrence of cortex-specific focal dysplasia and resulting epileptic seizures (164). Also, conditional knockout of DEPDC5 (another GATOR 1 component) in neurons results in increased neuron size and focal dysplasia specifically within the cortex, concomitant with constitutive mTORC1 signaling, and motor abnormalities and spontaneous seizures (165). The best studied neuropathological disease is TSC syndrome, where inactivating mutations in either TSC1 or TSC2 results in increased neuron growth, the development of large benign tumors within diverse regions of the

brain, and in some cases, epileptic seizures (57). However, administration of rapamycin to mice harboring TSC1 mutations attenuates associated seizures, suggesting that mTORC1 might be an efficacious therapeutic target for ameliorating neuropathological diseases driven my aberrantly high mTORC1 activity (166).

Unfortunately, minimal knowledge exists regarding the function of mTOR in the postnatal normal brain. However, activation of mTORC1 through genetic ablation of the negative regulator *PTEN* in retinal ganglion cells robustly promoted axon repair following artificial injury (167). Also, through PI3K activation, brain-derived neurotrophic factor (BDNF), a neurotrophin growth factor that promotes neuronal growth and differentiation, promotes localized protein synthesis within axons to enhance their migration and repair following injury (57,168,169). Lastly, several studies demonstrate that sufficient levels of mTOR signaling may aid in thwarting cognitive disorders such as autism spectrum disorder. Indeed, increased mTORC1-mediated localized protein synthesis within axons has been shown to correlate with autism-like behaviors in mice (170,171), whereas activation of autophagy through mTORC1 inhibition was shown to ameliorate the effects of autism spectrum disorder (172). These intriguing reports highlight the importance of future worked aimed at delineating the mechanisms through which mTOR contributes to brain development and neuropathological abnormalities associated with cognitive dysfunction.

1.7 mTORC2: the major physiological Akt-S473 kinase

Akt, also known as protein kinase B (PKB), is a member of the AGC (cAMP dependent, cGMP dependent, protein kinase C) family of serine/threonine kinases, and it exists in three isoforms in mammals: Akt1, Akt2, and Akt3 (173). All three isoforms possess the following structural elements: a central kinase domain flanked by an N-terminal pleckstrin homology (PH) domain and a C-terminal hydrophobic motif (173). Through engagement of various effectors, AGC kinases, including Akt, regulate multiple cellular processes downstream of receptor tyrosine kinases, including cell growth, proliferation, and survival, differentiation, and apoptosis (173,174). Phosphorylation of Akt on its activation loop site (T308) is indispensable for its activation (175). Although recent groups have reported additional Akt-T308 kinases, PDK1 remains the accepted prominent Akt-T308 kinase in intact cells and *in vivo* (176-178). PDK1 also phosphorylates the conserved activation loop of several additional AGC kinases including p70 S6K1, p90 ribosomal S6K1 (RSK), and serum and glucocorticoid regulated kinase (SGK) (174). PDK1 regulation is governed by 3 elements: subcellular localization, conformational changes, phosphorylation (174). In response to growth factors (i.e. insulin/IGF-1) PDK1 is recruited, via its C-terminal PH domain, to PIP_3 decorated membrane regions. PH engagement of PIP_3 induces a conformational change that relieves its autoinhibitory action on PDK1, allowing PDK1 to engage and phosphorylate substrates. PDK1 also requires trans-autophosphorylation on S241 in humans (S244 in mice) for growth factor activation. These regulatory mechanisms ensure that PDK1 is only activated under pro-growth conditions.

While PDK1-mediated phosphorylation of Akt-T308 is unequivocally required for Akt activation, phosphorylation of Akt within its hydrophobic motif on S473 is now appreciated as contributing profoundly to maximum Akt activity, stability of the active conformation, and dictating substrate preference (174). X-ray crystals of inactive and active Akt were seminal for elucidating the functional role of Akt-S473 phosphorylation (179,180). In the crystalized unactive Akt, the activation loop assumes a disordered configuration and sterically obscures the active site to restrict ATP and substrate access (174,180). Conversely, crystalized active Akt assumes a starkly different configuration in which Akt-S473 phosphorylation permits the hydrophobic motif to interact with the N-terminal region of the kinase domain (174,179). This configuration not only represents a more stable conformation, but it also fully exposes the active site, effectively expanding the repertoire of substrates that Akt can accommodate (174,179). While mTORC2 represents the major kinase for Akt-S473 in cells and *in vivo* (30), approximately ten additional Akt-S473 kinases have been proposed (discussed below).

Mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK 2 or M2K), activated in response to various cellular stress (i.e. heat shock), was shown to phosphorylate Akt-S473 *in vitro*, and a reduction in Akt-S473 phosphorylation was observed in neutrophils harboring an antagonizing M2K peptide (181,182). However, suppression of M2K activity in fibroblasts left insulin/IG-1- and stress-induced Akt-S473 unperturbed, suggesting that M2K may phosphorylate Akt-S473 *in vitro* but not under normal physiological conditions (183). Integrin-linked kinase (ILK) was also initially postulated to be an Akt-S473 kinase based on *in vitro* kinase assays demonstrating that

recombinant ILK phosphorylates recombinant GST-Akt, as well as experiments employing genetic knockdown/knockout of ILK and expression of kinase dead alleles of ILK, all of which demonstrated a reduction in Akt-S473 phosphorylation in cells (174,184). However, follow up studies from other groups failed to recapitulate ILK-mediated phosphorylation of Akt *in vitro*, and genetic ablation of ILK in fibroblasts or expression of a kinase dead ILK allele left insulin-stimulated Akt-S473 phosphorylation unperturbed (174,185,186). Evidence for the involvement of p38 MAPK in Akt-S473 phosphorylation exists from approaches utilizing pharmacological inhibition of p38 MAPK (with SB-203580) or expression of wild type or dominant negative p38M MAPK alleles or constitutively active alleles of the p38 MAPK upstream kinase MKK6 (174). Genetic or pharmacological inhibition of p38 MAPK reduced Akt-S473 phosphorylation in response to PIP_3 (in neutrophils), PDGF, and EGF, but not insulin, and during muscle tissue development (181,187,188). Also, a constitutively active MKK6 allele drives Akt-S473 phosphorylation (174). Conversely, cellular stress (i.e. reactive oxygen species) and insulin have also been shown to promote Akt-S473 phosphorylation independently of p38 MAPK (174,183,187).

The protein kinase C (PKC) isoform PKCα was shown bind IGF-1-generated PIP_3 on the plasma membrane and promote IGF-1-stimulated Akt-S473 phosphorylation in rat fat pad endothelial cells, and genetic perturbation of PKCα inhibited Akt-S473 phosphorylation (174,189). Also, PKCα was shown to directly phosphorylate Akt-S473 *in vitro* (189). Reconstitution of PKCβII devoid mast cells with PKCβII specifically was shown to promote Akt S473 phosphorylation in response to Immunoglobulin E (190).

However, contrasting evidence challenges the postulation that conventional PKC isoforms (including PKCα and PKCβII) increase Akt S473 phosphorylation *in vivo*, as these isoforms do not require PI3K for localization to membrane regions decorated with phospholipase $C_{\gamma 1/2}$-generated diacylglycerol, and PKC's generally recognize a basic consensus sequence, which differs from the typical hydrophobic motif consensus sequence encompassing S473 (174,191,192). Despite the inability of PKCζ to phosphorylate the Akt3 isoform on its hydrophobic motif (S472) *in vitro*, it did interact with and promote phosphorylation of Akt3 in cells (193). Evidence for NIMA-related kinase 6 (NEK6) as a putative Akt-S473 kinase is woefully scant, particularly considering that NEK6 is known to support cellular progression through mitosis in a kinase-dependent manner (174,194,195). The only evidence that supports a role for NEK6 in Akt S473 phosphorylation is that PI3K inhibition with wortmannin or L-294002 marginally reduced NEK6 kinase activity (174,196).

DNA-dependent protein kinase catalytic subunit (DNA-PKcs), isolated from membranes displaying constitutively active Akt S473 kinase activity (186), was shown to phosphorylate Akt-S473 *in vitro*, and although genetic ablation of DNA-PKcs or PI3K inhibition significantly reduced Akt S473 phosphorylation, residual phosphorylation remained (197). However, this contrasts with canonical roles of DNA-PK in the repair of dsDNA breaks and DNA replication (174). Also, DNA-PK preferentially recognizes ST/Q consensus motifs rather than the typical hydrophobic motif consensus sequence, and DNA-PK primarily engages its substrates by binding to DNA (174). Ataxia telangiectasia mutated (ATM) is also a proposed Akt S473 kinase, as overexpression of ATM

increased Akt S473, and not T308, phosphorylation in intact cells, but not *in vitro* (198). Also, ATM-induced Akt S473 phosphorylation was blunted following genetic depletion of ATM or inhibition of PI3K (198). Recently, the non-canonical Ikb kinase-related kinases TBK1 and IKKε, best known for their role coordinating pathogen detection with initiation of the innate immune response, have also been identified as Akt S473 (and Akt T308) kinases (177,199). None of the abovementioned studies provide compelling evidence in support of any of these kinases as bona fide Akt-S473 kinases for a few reasons. A majority of the studies employed overexpression and/or *in vitro* kinase assays, which tend to promote non-physiological phosphorylation events due to dysregulated kinase and substrate localization or other regulatory mechanisms. In addition, the cellular or physiological contexts differed quite drastically between the studies, with some studies investigating growth factor-mediated activation of Akt and others looking in specific immune cells (i.e. neutrophils). It is possible that in unique cellular context that one or a few of these kinases might acquire the ability through perhaps a compensatory mechanism to phosphorylate Akt S473, but under normal physiological conditions it doesn't appear to be the case. Thus, mTORC2 remains the physiologically relevant Akt-S473 kinase in intact cells and *in vivo* (30).

1.8 TBK1 and IKKε: identification, structure, and mechanisms of activation

The noncanonical IKK-related kinases, Tank-binding kinase 1 (TBK1), also known under the aliases TRAF2-associated kinase (T2K) and NF-kB-activating kinase (NAK), and IkB kinase epsilon (IKKε) (also known as inducible IKK (i-IKK)), function

distinctly from the canonical IKKs (i.e. IKKa and IKKb). TBK1 is a 729 residue 84 kD protein, and IKKε is 716 residues and 80 kD (200) (Fig 1.6). TBK1 was originally identified and cloned through its interaction with TANK (201,202), and IKKε was originally identified as a gene whose expression is induced following stimulation of macrophages with bacterial lipopolysaccharide (LPS) (203). TBK1 and IKKε share 49% identity and 64% similarity (202,204), and both harbor the following structural domains: a N-terminal kinase domain, a ubiquitin-like domain (ULD), a C-terminal leucine zipper (also known as the scaffold/dimerization domain (SDD)), and a C-terminal helix loop helix (HLH) (FIG) (204). While TBK1 enjoys ubiquitous expression across virtually all tissue types, IKKε expression is more restricted and includes peripheral blood lymphocytes and epithelial cells (204). The ULD of TBK1 and IKKε is crucial for their function in inducing the antiviral interferon response, and K63-linked polyubiqutination of lysines 30 and 401 within the ULD of TBK1 and IKKε and subsequent phosphorylation of both on serine 172 are both required for their activation (205). The SDD domain enables TBK1 and IKKε to homodimerize and form higher order oligomeric structures. Most of the cellular pool of TBK1 exists as stable homodimers, where contacts between the KD, ULD, and SDD culminates in a zipper-like backbone structure that effectively positions the kinase domains in directional opposition to each other (200). This is believed to be an inherent regulatory mechanism to discourage aberrant TBK1 activation, which would likely contribute to pathological inflammation. Two mechanisms have been proposed to describe activation of TBK1. The first describes adaptor-mediated recruitment of TBK1 to specific subcellular locations where an unknown

upstream kinase co-localizes to directly phosphorylate and activate TBK1 (206). The second also describes adaptor-mediated recruitment of TBK1 where instead, local high concentrations of TBK1 dimers enable proximal-mediated phosphorylation on S172, termed transautophosphorylation (207).

1.9 Innate immunity: Roles for the noncanonical IKK kinases (TBK1 and IKKε) and NF-kb

TBK1 and IKKε constitute key signaling nodes of the host's first line of defense against pathogenic infections. The innate immune system comprises various types of pattern recognition receptors (PRR) that detect microbial-derived antigens, collectively known as pathogen associated molecular patterns (PAMPs) (200). PRRs that activate TBK1 and IKKε include the membrane associated Toll-like receptors (TLRs) and the cytosolic DNA sensing receptor cyclic GTP-ATP Synthetase (cGAS) (208-211). Ligand engagement of TLR-3 or TLR-4 specifically leads to the activation of TBK1 and IKKε TLR-3 resides primarily on endosomal membranes, and in certain cell types on the plasma membrane, whereas TLR-4 exclusively occupies the plasma membrane (209,212) (Fig 1.7) . TLR-3 recognizes viral dsRNA exposed during lysosomal degradation of viral particles and during viral genome replication, as well as the synthetic dsRNA mimetic, polyribinosinic polyribocytidylic acid (poly I:C). TLR-3 has also been shown to recognize a subset of ssRNA and dsDNA viruses (212-214).TLR-4 recognizes lipopolysaccharide (LPS), a constituent of the outer membrane of gram-negative bacteria (210). Following cognate ligand binding, TLR-3 and TLR-4

homodimerize, which causes a conformational change in the cytoplasmic Toll-Interleukin 1 receptor (TIR) domain, initiating the recruitment of key adaptors (210). Both TLR-3 and TLR-4 employ the adaptor TRIF, while TLR-4 also accommodates TRAM, to transduces their signals (210). TRIF and/or TRAM recruitment precedes recruitment of TBK1 and IKKε, where they are activated by transautophosphorylation on their activation loop site on serine 172 (Fig 1.7). TBK1 and IKKε then phosphorylate interferon regulatory factor (IRF) 3/7, which induces their homodimerization and nuclear translocation where they transcriptionally induce the interferon response (210,215) (Fig 1.7).

cGAS-cGAMP (cGMP AMP)-STING (stimulator of interferon genes) represents another conduit through which certain PAMPs activate TBK1 (211). cGAS is an intracellular receptor that senses and binds bacterial and viral derived dsDNA (211,216) This binding induces a conformational change that promotes cGAS oligomerization and activation, allowing it to catalyze the synthesis of cGAMP from ATP and GTP (211,217,218). cGAMP binds to ER-localized STING molecules, which induces STING dimerization and subsequent oligomerization as these complexes translocate to the perinuclear region (211,219). In route to the perinuclear regions TBK1 is recruited and activated via transautophosphorylation (211). TBK1 then phosphorylates and activates IRF-3/7 to promote the antiviral INFβ response (211,215). It is important to note that TBK1 deletion in mice is embryonic lethal at E14.5 as a consequence of hepatic apoptosis and liver degeneration, underscoring the importance of TBK1 in both innate immunity and embryonic development (220).

TBK1 function also participates in autophagy-mediated sequestering and clearance of invading bacteria from mammalian cells. Classic participation of TBK1 in autophagy involves its interaction with and phosphorylation of the cargo receptor optineurin through key adaptors, including NDP52 (221) (222). These general mechanisms link TBK1 to autophagosome maturation and the eventual autophagic elimination of pathogenic bacteria (222-224), as well as the selective recycling of old/damaged mitochondria (225,226). In response to poly (I:C) and viral infection, optineurin also recruited TBK1, in a K63 polyubiquitin-dependent manner, to the golgi apparatus to promote INF-b production (227). Additionally, interaction with the endosomal marker Rab8 promotes TBK1-mediated phosphorylation of the core autophagy adaptor p62/sequestosome on S403, which promotes the macrophage eradication of *Mycobacterium tuberculosis* (223). Also, adaptor-mediated recruitment of kinase-active TBK1, via the cargo receptor NDP52 and independently of optineurin, to the autophagy proteins WIPI2 and WIPI1 was found to be essential for TBK1 to thwart *Salmonella typhimurium* infection by impeding its replication (228).

The NF-kB pathway responds to a plethora of cellular stimuli (pro-inflammatory cytokines such as TNF-α and IL-1; DNA damage (i.e. dsDNA breaks); oxidative stress; and pathogenic constituents such as bacterial LPS) and responds through transcriptional regulation of a myriad of genes involved in innate and adaptive immunity, inflammation, cell survival and proliferation (202,229). The mammalian NF-kB family consists of five members: NF-kB1/p50 (obtained via proteolytic cleavage of the p105 precursor); NF-kB2/p52 (obtained via proteolytic cleavage of the p100 precursor); c-Rel;

RelA/p65; and RelB (230). These proteins associate in a combinatorial fashion via their conserved Rel homology domain (RHD) to yield various NF-kB heterodimeric transcriptional activators (230). Importantly, the RHD also confers DNA binding capabilities and provides a contact interface for the NF-kB negative regulators, the inhibitor of kB (IkB) proteins (230). The RHD also harbors a nuclear localization signal (NLS) critical for NF-kB entry into the nucleus (202).

The IkB proteins prevent aberrant NF-kB activation and ensure it is only activated when when appropriate. In the absence of NF-kB activating stimuli (i.e. TNF-α), through its stacked helical-rich ankyrin repeats, IkBα binds to NF-kB dimers to obscure their nuclear localization signal (NLS). This effectively impedes NF-kB function by sequestering it in the cytoplasm (202,230).

Two pathways are known to activate NF-kB: the classical pathway and the alternative pathway. The classical pathway is governed by IKKβ, a catalytic subunit of the trimolecular canonical IkB kinase (IKK) complex. The canonical IKK complex comprises another catalytic subunit, IKKα, and the regulatory subunit IKKγ (also known as NF-kB essential modulator, NEMO) (202,231). Following cytokine stimulation, IKKβ, as part of the IKK complex, phosphorylates IkBα on serine 32 and 36 (232), flagging it for recognition by the β-TrCP F-box component of the E3 ubiquitin ligase complex Skp-1-Cullin-F-box (SCF), referred to as $SCF^{\beta TrCP}$ (233,234). $SCF^{\beta TrCP}$ then polyubiquitinates IkBα on lysine 21 and 22, which targets it for recognition and degradation by the 26S proteasome (202,234-237). IkBα degradation liberates NF-kB (RelA(p65):p50) dimers, enabling their nuclear translocation where they transcriptionally induce a myriad of

target genes involved in proliferation (i.e. cyclin D1), apoptosis suppression (i.e. BCL-X_L), inflammation (i.e. the inflammatory enzyme, cyclooxygenase 2 (Cox-2), and the pro-inflammatory cytokine, IL-6) (229).

The alternative pathway is uniquely governed by IKKα, where, rather than phosphorylating IkBs, it phosphorylates NF-kB2(p100) precursor (202). NF-kB2(p100) phosphorylation leads to recruitment of and polyubiquitination by the $SCF^{\beta TrCP}$ complex, which mediates proteasomal degradation of the C-terminal IkB region of NF-kB2(p100) (202,238). This yields the mature p52 form, which, as a dimer with RelB, translocates to the nucleus to exert its transcriptional activity (239). While IKKγ is required for IKK complex integrity and NF-kB activation via the classical pathway, it is dispensable for NF-kB activation via the alternative pathway. Also, while not required for classical NF-kB activation in response to pro-inflammatory cytokines, alternative NF-kB activation by IKKα in response to certain stimuli (i.e. receptor activator of NF-kB (RANK) ligand) and in certain cell types requires activation loop phosphorylation on serine 176 and 180 (202,240-242).

Constitutive NF-kB signaling can lead to pathological levels of inflammation. The adverse effects of constitutive NF-kB activity have been observed in mice genetically devoid of IkBα (*IkB*$\alpha^{-/-}$), which die approximately one week post-birth due to complications from an inflammatory skin condition (243). Therefore, regulatory mechanisms exist to shut down NF-kB signaling after it has exerted its functions. One example involves IkBα directly. IkBα is uniquely a transcriptional target of NF-kB. Cytokine-induced degradation of IkBα and activation of NF-kB drives expression of new

IkBα proteins, which enter the nucleus, bind to NF-kB to disrupt its association with IkB gene elements, and escort NF-kB back into the cytoplasm using its nuclear export sequence (230,244).

1.10 Toll-like receptor (TLR) 3 and 4

The toll-like receptor family consists of 10 total functional members in humans (TLR 1-9) and 13 in mice (TLR 1-9 and 11-13; TLR-10 is non-functional) (208). TLRs recognize a diverse array of PAMPs that occur both intra- and extra-cellularly, including lipoproteins and bacterial/viral nucleic acid (ie ssRNA) (208). All TLRs harbor an N-terminal leucine rich repeat (LRR)-containing ectodomain, a transmembrane domain, and a C-terminal Toll-interleukin1 receptor (TIR) domain (245,246). The ectodomain assumes a globular, horseshoe shape and is essential for TLR recognition of appropriate PAMPs (245). Following PAMP binding, TLRs associate with various adaptor molecules in a stimulus-specific manner to transduce signals downstream (208). These adaptors contain a Toll/Interleukin 1 receptor (TIR) domain, which enables them to interact with the TIR domains of TLR molecules (208). It is now appreciated that TLRs are co-translationally translocated into the endoplasmic reticulum (ER) and subsequently trafficked through the secretory pathway, where they undergo processing before being translocated to their appropriate membrane platform (208). Dysregulated localization of TLRs is linked to chronic inflammatory responses and autoimmune disorders (208).

The TLR family is bifurcated into two broad groups, one which resides on the plasma membrane and another which resides on internal membrane organelles. One of the earliest TLR's to be discovered and characterized, TLR-4 traffics to the plasma membrane with the assistance of the required ER-resident proteins, PRAT4A, and the heat shock protein 90 (hsp90) family member gp96 (209,247). where it forms a functional heterodimer with myeloid differentiation factor 2 (MD-2) (248,249). The six lipid chains of LPS intricately engage the TLR-4/MD-2 complex, with five of the lipid chains interacting strongly with the hydrophobic pocket of MD-2 and the remaining lipid chain bridging the complex by interacting with a phenylalanine residue on TLR-4 (248,249). In addition to LPS, TLR-4 has been reported to recognize an envelope protein of murine mammary tumor viruses as well as fusion proteins of respiratory syncytial virus (208,250). Following LPS binding, TLR-4 recruits the adaptor molecules, TIRAP and myeloid differentiation factor 88 (MyD88) to activate MAPK signaling and initiate NF-kB-mediated pro-inflammatory cytokine production (208,212). MyD88 is critical for the recruitment of several key IL-1 receptor associated kinases (IRAKs): IRAK4, IRAK1, and IRAK2 (251,252). Upon activation of the IRAKs, TRAF6 is recruited where it catalyzes the K63-linked polyubiquitination of itself and IRAK1 (208,253). These polyubiquitin chains function to tether several proteins for proximal activation. The polyubiquitin chains of TRAF6 engage the kinase, TAK1, through the ubiquitin binding domain (UBD) its regulatory subunits TAB2 and TAB3, as well as the canonical IKK complex (a tri-molecular complex comprised of two catalytic subunits IKKα and IKKβ and a regulatory subunit IKKγ) through the UBD of IKKγ (208,253). This enables

proximal phosphorylation of IKKβ by TAK1, which activates and primes NF-kB for pro-inflammatory gene expression.

TLR-4 also interacts with the adaptors Toll-IL-1-receptor domain-containing adaptor inducing interferon beta (TRIF) and TRIF-related adaptor molecule (TRAM) to transmit signals through both MyD88 and TRIF, culminating in NF-kB and interferon responses (254). Shortly after LPS binding and together with the co-receptor CD14 (255), TLR-4 undergoes endocytosis, which vitiates the MyD88 interaction to permit TRIF and TRAM to bind and recruit and activate TBK1/ IKKε, which activates IRF3/7 and the interferon response (208,212,256). Interestingly, TLR-4 represents the only TLR for which engagement by both MyD88 and TRIF/TRAM are required for a sufficient pro-inflammatory cytokine response (208). This mechanism highlights the degree to which specific adaptors fine tune TLR-mediated innate immune responses.

TLR-3 localizes to endosomal membranes where it recognizes viral-derived dsRNA, which arises either from viral degradation or exposure during viral genome replication (212). Evidence suggests that the ER-resident 12-membrane pass protein, UNC93B1, is involved in trafficking of TLR-3 to endosomes, because it directly binds the transmembrane domain of TLR-3 and a specific missense mutation in UNC93B1 impairs TLR-3-mediated immune responses (257). TLR-3 Initial studies identified TLR-3 by its ability to bind the synthetic viral dsRNA mimetic polyriboinosinic polyribocytidylic acid (poly I:C) (208). TLR-3 has also been shown to bind the dsRNA intermediate that occurs during the replication of ssRNA viruses as well as small interfering RNA (208). dsRNA binding induces TLR-3 homodimerization and subsequent activation. Structural

analysis of TLR-3 reveals a solenoid conformation with a horseshoe configuration (245,258). The characteristic LRR within the ectodomain contribute to the solenoid conformation, and several key asparagine residues provide stabilizing hydrogen bonding (258). The importance of TLR-3 function in coordinating the antiviral interferon response is underscored by the fact that humans lacking sufficient TLR-3 exhibit increased susceptibility to herpes simplex virus (HSV) infection, and mice lacking TLR-3 die following infection with murine cytomegalovirus (213,259).

TLR-3 is the only TLR member that does not employ MyD88, yet it is still able to activate NF-kB inflammatory gene expression and interferon beta production. TLR-3 interacts with the adaptor TRIF via its TIR domain, and TRIF-mediated recruitment of the E3 ubiquitin ligases, TRAF6 or Pellino-1, RIP1, and TRADD, enables crosstalk with the MyD88 pathway via activation of TAK1, which activates the NF-kB axis (208). TRADD and Pellino-1 interaction with RIP1 seems to mediate K63-linked polyubiquitination of RIP1, which is essential for TLR-3-mediated NF-kB activation (260,261). TRIF-mediated recruitment of the adaptor TRAF3 contributes profoundly to the induction of interferon beta production. TRAF3 subsequently recruits TBK1 and IKKε leading to their phosphorylation on S172 and activation, and TBK1/ IKKε phosphorylate and activate IRF3, culminating in the transcriptional and translational up-regulation of type 1 interferons. The fact that genetic ablation of TRAF3 curtails activation of TBK1 and IKKε activation underscores the importance of TRAF3 in orchestrating the interferon beta response (208).

Aberrant TLR signaling is implicated in pathophysiological conditions associated with chronic inflammation, including cancer and autoimmune disorders. Therefore, cells leverage multiple tiers of regulation to minimize this potential outcome. Numerous molecules have been identified as suppressors of both MyD88 and TRIF signaling. TAG (TRAM adaptor with Gold domain) negatively regulates TLR-4/TRIF-mediated IRF3 activation by disrupting the TRIF/TRAM interaction at the TLR-4 cytoplasmic interface (262). The integrin CD11b seems to generally antagonize MyD88 and TRIF responses downstream of TLRs by promoting the ubiquitin-mediated degradation of both in coordination with the E3 ubiquitin ligase, Cbl-b (263). TRAF3-mediated proximal ubiquitination of TBK1 is required for TBK1 activation. The deubiquitinating enzyme A (DUBA) blunts type 1 interferon production by binding to and cleaving the polyubiquitin chains on TRAF3, effectively dislocating it from TBK1 to prevent TBK1 activation and type 1 interferon induction (264). The peptidyl prolyl isomerase Pin1 also negatively regulates type 1 interferon production by interacting with and promoting the proteasomal degradation of phosphorylated IRF3 and (265). Negative regulation of the NF-kB/MAPK inflammatory response is exemplified by the report that TRIM30α interacts with and promotes degradation of the ubiquitin-binding proteins, TAB2 and TAB3, which functions to bring TAK1 in proximity to the canonical IKK complex for NF-kB activation (266). Collectively, these reports highlight the importance of a regulatory check on TLR signaling either through the degradation of or disruption of interactions between key effectors.

1.11 TBK1 and IKKε in oncogenesis

Roles for TBK1 and IKKε in tumorigenesis have also recently been described. TBK1 and IKKε have also been implicated in the promotion of cancer cell survival and proliferation (267). IKKε functions as a breast cancer oncogene where, upon K63-linked polyubiquitination on lysines 30 and 401, orchestrated by the cIAP1/cIAP2/TRAF2 E3 ubiquitin ligase complex, it synergizes in parallel with NF-kB pro-inflammatory signaling to exert its cellular transformation and oncogenic functions (268). IKKε gene amplifications and overexpression also exist in approximately 30 percent of breast cancers (269). Significant transcriptional and translational up-regulation of IKKε expression has been reported in glioma cancers, where functionally it seems to leverage the NF-kB-mediated production of the anti-apoptotic factor, Bcl-2, to promote glioma cancer cell survival (270). Interrogation of ovarian cancer patient samples revealed that IKKε expression was noticeably increased in metastatic samples relative to primary tumors samples, and IKKε seems to drive a cell motility and pro-inflammatory gene signature, which correlated with increased metastasis of a less aggressive ovarian cancer (271). IKKε is also critical for oncogenic Ras-driven pancreatic tumorigenesis and progression through its ability to activate the survival kinase, Akt, in a mTOR-independent manner through activation of the Gli1 transcription factor (272). Indeed, IKKε expression is appreciably increased in patient pancreatic ductal adenocarcinoma samples (273). IKKε also promotes non-small cell lung tumorigenesis through an interaction with mutant EGFR, where EGFR phosphorylates IKKε (on Y153 and Y179)

to increase its kinase activity and IKKε promotes pro-proliferative, growth, and survival pathways via activation of Akt and MAPK (274).

Interestingly, elevated TBK1 expression has also been identified in ER-positive breast cancer, which correlates with resistance to the common treatment regimen tamoxifen, and specifically, TBK1-mediated phosphorylation of ERα on S305 was found to promote tamoxifen-resistance in these ERα positive breast cancers (275). TBK1 was also identified in an shRNA kinome screen as essential for HER2 positive breast cancer growth and proliferation, and pharmacological inhibition of TBK1 induced senescence through reduced canonical NF-kB signaling (276). TBK1 is also critical for the survival and proliferation of adult T-cell leukemias (ATL) driven by constitutive NF-kB activity, where TBK1 (and IKKε) promotes pro-growth/survival STAT3 signaling (277). TBK1 was recently identified in an siRNA screen as a critical factor that promotes the survival and proliferation of cancer cells harboring oncogenic KRas mutations, presumably through coordination with NF-kB antiapoptotic signaling (278). TBK1 (and IKKε) were also shown to promote oncogenic KRas-driven lung adenocarcinoma growth and survival *in vivo* through the activation of the chemokine chemokine ligand 5 (CCL5) and the cytokine IL-6 (279). TBK1 also suppresses apoptosis in MEFs transformed with oncogenic KRas through the exocyst complex (specifically exocyst member Sec5) and activation of the RalB GTPase (280). TBK1 also regulates autophagy to promote cancer cell survival and proliferation. Similar to its role in promoting autophagosome formation to eradicate invading bacteria, TBK1 selectively sequesters the autophagy cargo receptors Ndp52 and Tax1bp1 into autophagosomes to drive noncanonical NF-kB

signaling and promote survival and proliferation of cancer cells harboring oncogenic KRas (223,281). Moreover, both TBK1 and IKKε have been shown to directly phosphorylate and activate the survival kinase Akt on T308 and S473 to support oncogenic transformation (177,199). Collectively, these studies demonstrate that TBK1 and IKKε support oncogenesis through a plethora of mechanisms depending on the context and specific cancer type/subtype.

1.12 TBK1 and IKKε in metabolism and metabolic diseases

Several studies also report roles for TBK1 and IKKε in the regulation of metabolism as well as their implication in diet-induced metabolic diseases. TBK1 has been linked to insulin-mediated glucose homeostasis based on its ability to control glucose uptake in cells. Specifically, TBK1 promotes glucose uptake by regulating GLUT4 trafficking to the plasma membrane through engagement of the exocyst complex and phosphorylation of Exo84 (282). Initial reports demonstrated that global knockout of IKKε in mice confers protection against the untoward effects of diet-induced obesity, specifically by reducing chronic inflammation in adipose and liver, reducing lipid accumulation in the liver, and improving global insulin sensitivity (283). Similarly, an independent study demonstrated that administration of the TBK1/ IKKε inhibitor amlexanox to mice challenged with a high fat diet also improved insulin sensitivity and protected against weight gain, through increased calorie burning, and hepatic steatosis (284). Also, these studies reported elevated TBK1 and IKKε expression and activity in obese adipose tissue, and IKKε expression is uniquely elevated in obese hepatocytes,

which strengthened the idea that TBK1/ IKKε might contribute to the metabolic defects incurred with diet-induced obesity (283,284). Unfortunately, it is difficult to discern the precise physiological role of IKKε (or TBK1) to diet-induced obesity via global knockout or administration of a pharmacological agent. Therefore, it remains unclear how exactly IKKε improved insulin sensitivity in these studies.

Interestingly, the same group later discovered that knockout of TBK1 in adipocytes exacerbates inflammation, promotes insulin resistance, and disrupts glucose homeostasis (i.e. hyperglycemia) (285), instead supporting a physiological role for TBK1 in ameliorating obesity-linked metabolic dysregulation by reducing inflammation and promoting whole body insulin sensitivity and glucose homeostasis. Intriguingly, these mice maintain normal body weight and fat biomass. Zhao proposes that TBK1 curbs the inflammation by inhibiting NF-kB pro-inflammatory signaling through phosphorylation-mediated degradation of NF-kb inducing kinase (NIK) (285).

Also, while whole body knockout of TBK1 ameliorated diet-induced obesity-driven fatty liver, liver specific knockout of TBK1 actually exacerbated fatty liver, which was mechanistically explicated by TBK1's function in the regulation of fatty acid β-oxidation (286). Interestingly, TBK1's role in circumventing lipid accumulation in the liver was attributed to its scaffolding function rather than its kinase activity. Specifically, inactive TBK1 sequestered acyl CoA synthetase long-chain family member 1 (ACSL1) in the mitochondria, effectively impeding its ability to translocate to the endoplasmic reticulum (ER) during fasting to produce acyl CoA that, upon esterification, yields fatty acids (286). During obesity TBK1 becomes activated, which liberates ACSL1 from the

mitochondria to drive aberrant fatty acid β-oxidation in the ER, consequently leading to fatty acid build up in the liver (286). Recently, Reilly et al elucidated a mechanism to explain the metabolic benefits conferred upon high fat diet-induced obese mice by amlexanox, namely sustained long-term weight loss through increased energy expenditure and improved insulin sensitivity. Specifically, amlexanox promoted browning of white adipose by increasing fibroblast growth factor 21 (FGF21) expression specifically in adipocytes, and in an autocrine fashion, FGF12 induced the transcriptional upregulation of genes involved in white adipose browning, namely *Ucp1* and *Dio2* (287). Importantly, amlexanox improved overall metabolic profiles of these obese mice in two key ways. First, by curbing fasting blood glucose levels through adipocyte-liver crosstalk, where amlexanox-induced secretion of IL-6 from adipocytes inhibited signal transducer and activator of transcription (STAT3) signaling in the liver to quell hepatic glucose production and secretion into the bloodstream. Second, and more long-term, the increased energy expenditure conferred on these mice by amlexanox also resulted in improved insulin sensitivity (287). This study mechanistically characterizes TBK1 and IKKε as reconcilers of high fat diet (HFD)-induced obese metabolic abnormalities, notably HFD-induced weight gain and insulin resistance. However, the exact role of inflammation in obesity-linked insulin resistance remains poorly understood. Nonetheless, these independent studies describe important roles for TBK1 (and IKKε) in integrating inflammatory and metabolic cues within metabolic target tissues to restore metabolic homeostasis.

1.13 Introduction to dissertation research

Growth of eukaryotic organisms requires both cell autonomous and non-autonomous coordination through the sensing of pro-growth cues. mTOR represents an evolutionarily conserved serine/threonine protein kinase that functions as a critical conduit between environmental cellular conditions and intracellular responses, aimed at maintaining homeostasis. mTOR exists as the core kinase of two multisubunit complexes known as mTOR complex 1 (mTORC1) and mTORC2. Known as the master regulator of cell growth, mTORC1 also mobilizes an anabolic cellular program to support cell growth and cell cycle progression. mTORC2 senses growth factors and energetic cellular stress and responds by promoting cell survival and proliferation through the survival kinase, Akt. The environmental cues and signaling mechanisms/intermediates governing mTORC1 remain far more extensively characterized than for mTORC2. Therefore, the research presented here contributes important mechanistic insight to the woefully minimal body of knowledge regarding upstream regulation of mTORC2.

Previous work from our lab identified mTOR serine 2159 as a site that promotes mTORC1 signaling (Figure 1.1) . Mutation of this site to a nonphosphorylatable alanine was found to reduce mTORC1 signaling (104). An *in vitro* kinome screen in search of the mTOR-S2159 kinase identified TBK1 and IKKε as strong candidates (44). Studying TBK1 regulation of mTOR revealed that TBK1 is essential for EGF to activate promote mTORC1 signaling. MEFs genetically lacking TBK1 displayed profoundly reduced EGF-stimulated, and inhibition of TBK1 with amlexanox reduced EGF-stimulated mTORC1

signaling. Importantly, wild type but not kinase dead TBK1 rescued EGF-stimulated mTORC1 signaling in TBK1 knockout MEFs. Notably, TBK1-mediated mTOR-S2159 phosphorylation was found to contribute to canonical innate immune functions of TBK1. In response to the detection of pathogen-derived molecular features, TBK1 initiates the innate immune response by phosphorylating the transcription factors IRF-3/7 to induce their homodimerization and nuclear translocation, culminating in the production of IFN-a/B (Figure 1.4). Indeed, $mTOR^{A/A}$ primary bone marrow derived macrophages (BMDMs), isolated from mice genetically modified to bear mTOR-S2159 alanine substitutions at both mTOR alleles, exhibited impaired IRF-3 nuclear translocation and IFN-b production. This novel function for site-specific mTOR phosphorylation in innate immunity revealed that TBK1 crosstalk with mTOR also modulates key cellular processes.

While studying TBK1-mediated activation of mTORC1 we also observed reduced EGF-stimulated Akt P-S473 in MEFs devoid of TBK1, a key piece of preliminary data suggesting that TBK1 may also be important for EGF-stimulated mTORC2 signaling. The data here clearly demonstrate that TBK1 represents a novel direct upstream activator of mTORC2 and delineates the mechanistic details underlying TBK1 activation of mTORC2. Most importantly, results from this study provide targetable therapeutic options for diseases driven by aberrant TBK1 and mTORC2 activity such as cancer and metabolically linked diseases (i.e. type 2 diabetes).

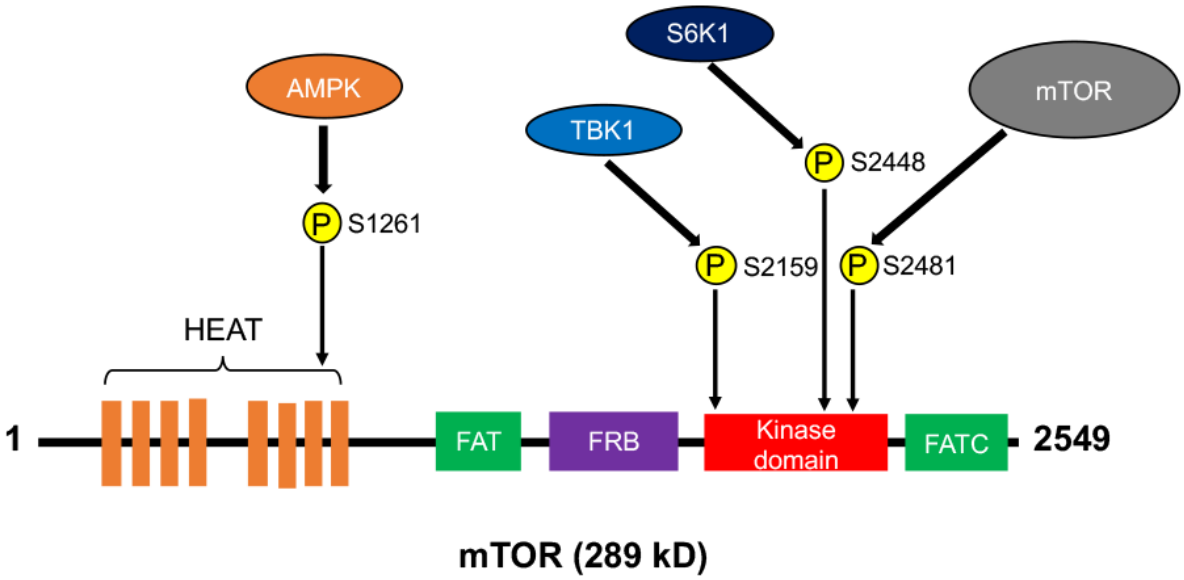

Figure 1.1: mTOR domain structure. mTOR contains N-terminal tandem HEAT repeats, which mediate mTOR complex dimer formation via protein-protein interactions; a FAT domain; FKBP12 rapamycin binding domain (RBD) to which the rapamycin-FKBP12 complex docks to inhibit mTORC1; a kinase domain; and a FATC domain. mTOR S1261 is phosphorylated by AMPK in response to acute energetic stress (43). mTOR S2159 is phosphorylated by TBK1, which promotes EGF stimulated mTORC1 signaling and INFβ production and IFR-3 nuclear translocation in response to innate immune agonists (44). mTOR S2448 is phosphorylated by S6K1 via a feedback mechanism with no known function (39). mTOR S2481 is an autophosphorylation site that serves as a surrogate readout for mTOR catalytic activity (41,42).

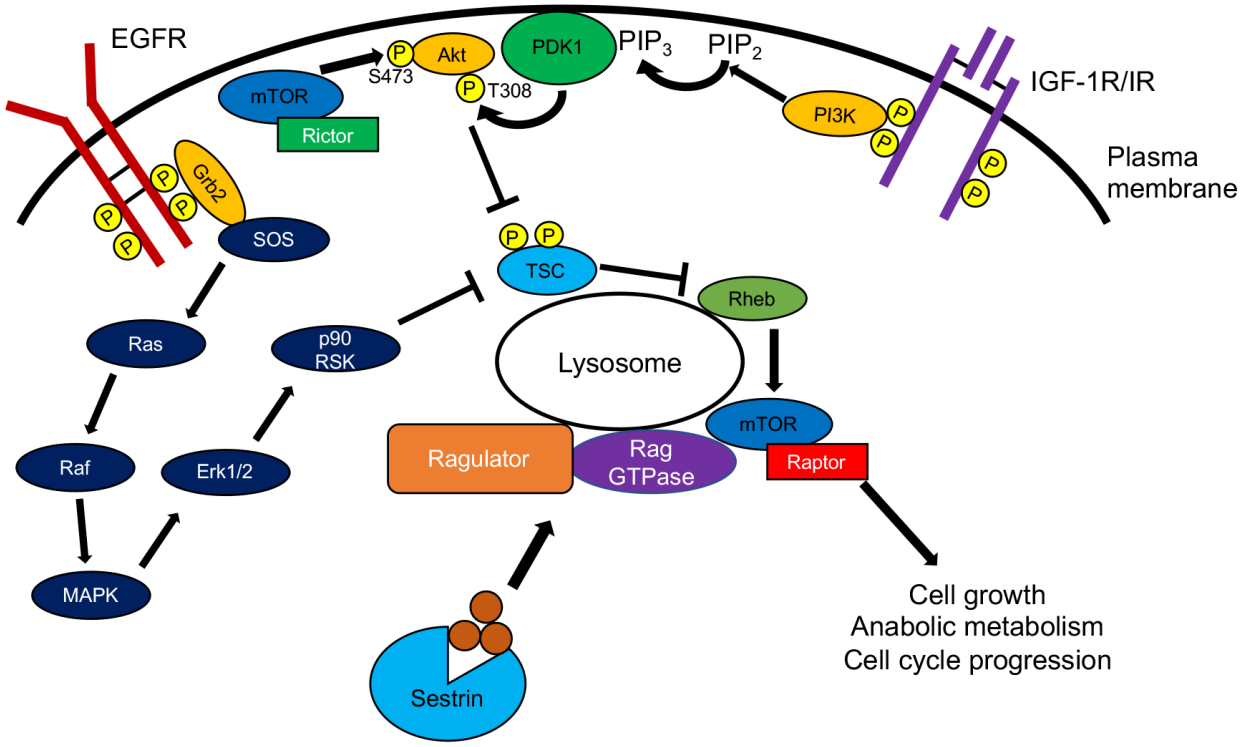

Figure 1.2: Activation of mTORC1 and mTORC2 by stimuli. EGF activates EGF receptor (EGFR) leading to phosphorylation of specific tyrosine residues on the intracellular cytoplasmic domain of the receptor. Growth factor receptor bound protein 2 (Grb2) and SOS dock to phospo-tyrosines and induces the phosphorylation cascade of the Ras/Raf/MAPK/ERK pathway, culminating in activation of p90 ribosomal S6K1 (p90 RSK), which phosphorylates and inhibits TSC. Insulin or IGF-1 binding to insulin/IGF-1 receptor (IR/IGF-1R) recruits PI3K (either directly through p85 regulatory domain SH2 domains or through insulin receptor substrate 1 (IRS-1). PI3K catalyzes the conversion of PIP_2 to PIP_3, which recruits PDK1 and Akt via their pleckstrin homology (PH) domains. PDK1 phosphorylates Akt on T308, and Akt phosphorylates TSC on inhibitory sites. Phosphorylation of TSC disrupts TSC complex integrity and association with the lysosomal membrane, allowing Rheb to activate mTORC1 on the lysosomal surface. Activation of EGFR and IR/IGF-1R leads to recruitment of mTORC2 to the plasma membrane via the PH domain of the mTORC2 partner protein mSin1. mTORC2 phosphorylates Akt on S473 at the plasma membrane to boost Akt activity to a maximum level. The cytosolic leucine sensor, Sestrin1, activates mTORC1 through the Ragulator and Rag GTPases, which recruits mTORC1 (via Raptor) to the lysosomal surface.

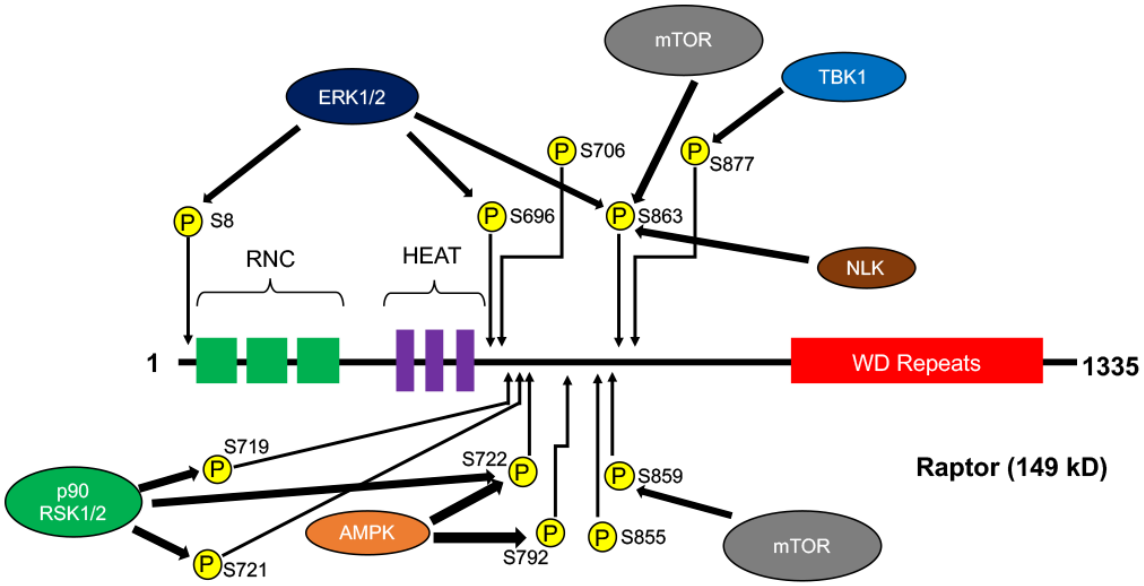

Figure 1.3: Raptor domain structure. Raptor contains a N-terminal raptor N-terminus conserved (RNC) domain, short tandem HEAT repeats, and C-terminal tandem WD40 repeats. ERK1/2 phosphorylates Raptor on S8, S696, and S863 (46), and p90 RSK1/2 phosphorylates S719, S721, and S722 (45) to activate mTORC1, both in response to MAPK pathway activation. mTOR phosphorylates Raptor S863, priming Raptor for subsequent phosphorylation on S855 and S859 by unknown kinases to activate mTORC1 (48). mTOR also phosphorylates Raptor S859 with no defined function (47). AMPK phosphorylates Raptor S722 and S792 to suppress mTORC1 during energetic stress (51). TBK1 phosphorylates Raptor S877 to inhibit mTORC1 following cellular stimulation with TBK1-activating innate immune agonists (50). NLK phosphorylates Raptor S863 to suppress mTORC1 during cellular stress (49).

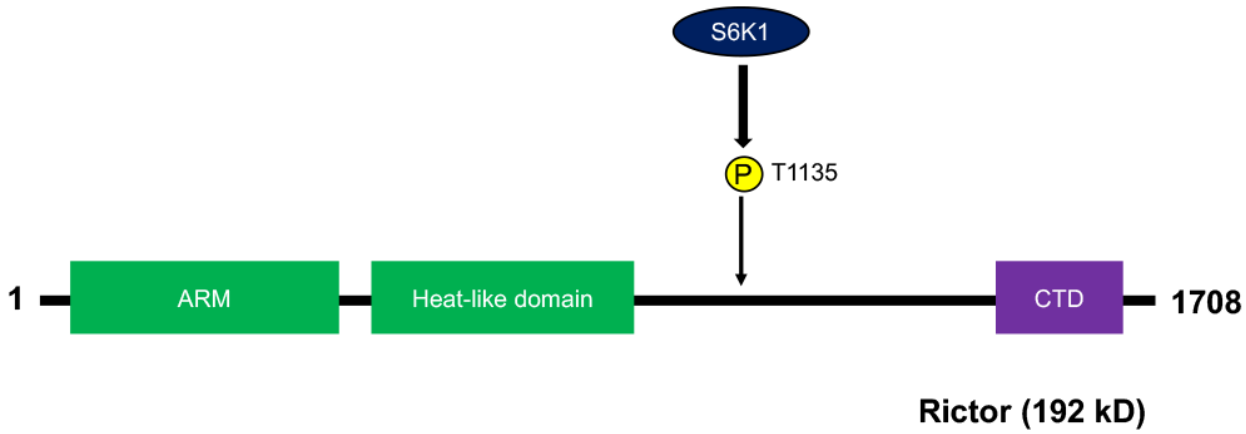

Figure 1.4: Rictor domain structure. Rictor is comprised of a N-terminal armadillo (ARM) repeats domain, HEAT repeats-like domain, and a C-terminal domain. S6K1 phosphorylates Rictor on T1135, which is believed to curtail mTORC2 activity (52-55).

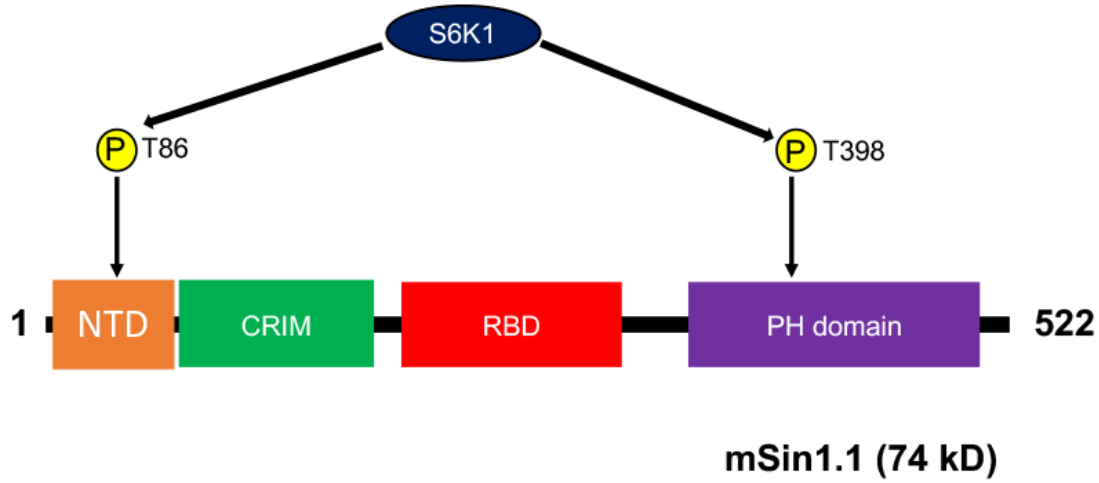

Figure 1.5: mSin1 domain structure. The mSin1.1 isoform harbors a N-terminal domain (NTD), a conserved region in the middle (CRIM) domain, which mediates protein-protein interactions, a Ras-binding domain (RBD), and a pleckstrin homology (PH) domain. S6K1 phosphorylates mSin1 on T86 and T398 to induce mSin1 dissociation from mTORC2.

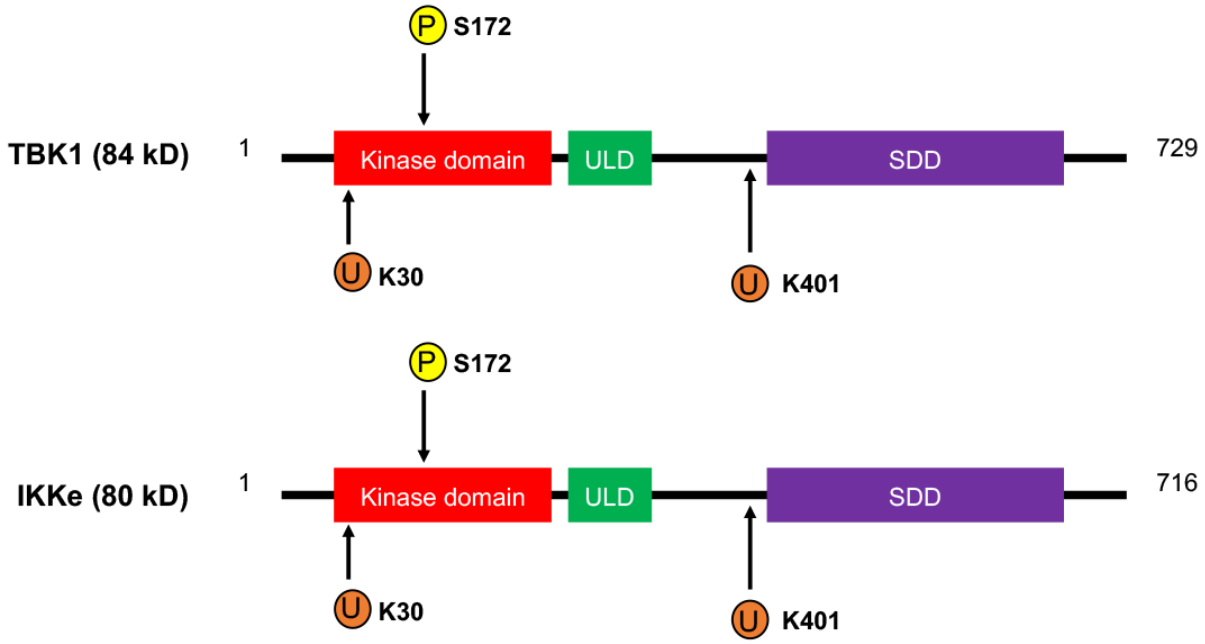

Figure 1.6: Structural domain elements of TBK1 and IKKε. TBK1 (729 amino acids) and its homolog, IKKε (716 amino acids), are comprised of an N-terminal kinase domain, a ubiquitin-like domain (ULD), and a C-terminal scaffold/dimerization domain (SDD). TBK1 and IKKε require K63-linked polyubiquitination at lysine 30 and 401 to precede activating phosphorylation of the activation loop site on serine 172.

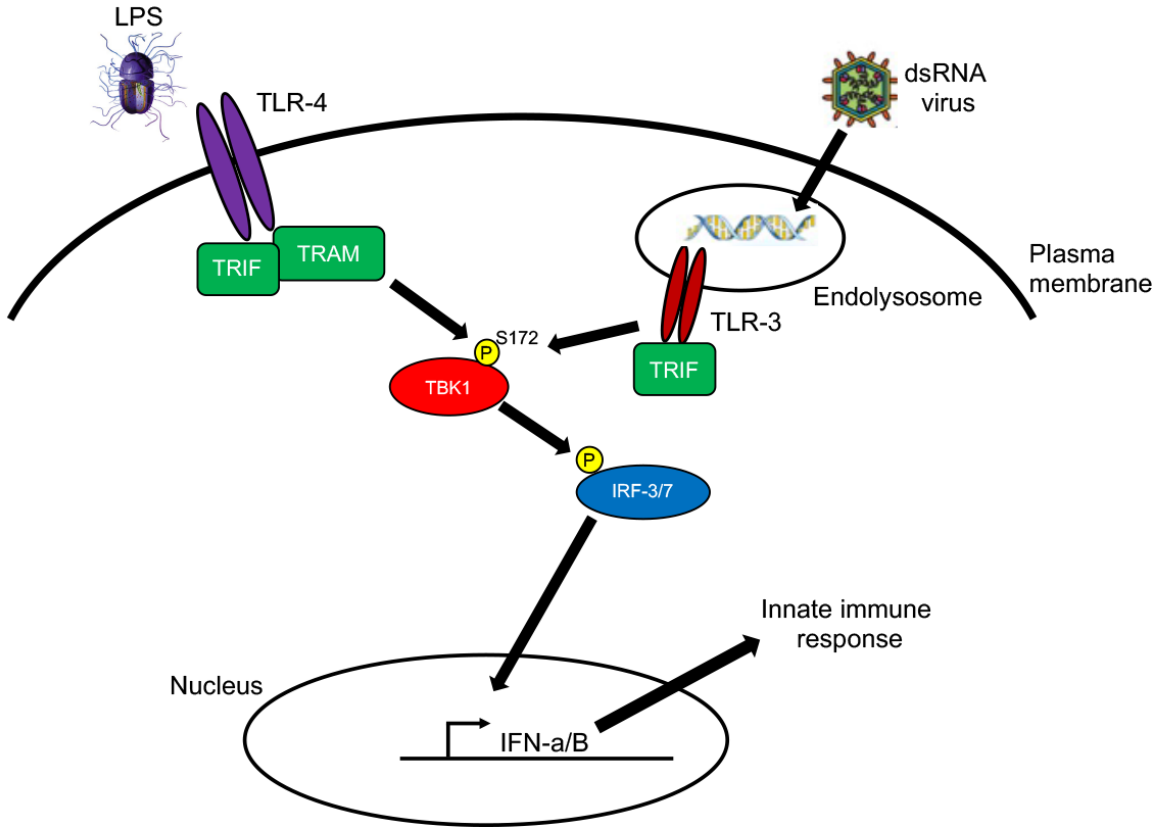

Figure 1.7: TLR-mediated activation of TBK1. Engagement of TLR-4 by bacterial lipopolysaccharide (LPS) induces receptor dimerization and recruitment of the adaptors TRIF and TRAM. Double-stranded RNA (dsRNA) viruses are endocytosed and detected by TLR-3 located on endolysosomal membranes, leading to the recruitment of TRIF. Adaptor recruitment to TLR-4 and TLR-3 leads to the recruitment and activation of TBK1 (via phosphorylation on S172). TBK1 phosphorylates IRF-3/7 on multiple regulatory sites to induce their homodimerization and nuclear translation, where they upregulate the transcription of INF-a/b and interferon stimulated genes, culminating in the innate immune response.

Chapter 2

The Innate Immune Kinase TBK1 Directly Increases mTORC2 Activity and Downstream Signaling to Akt

2.1 Abstract

TBK1 (TANK-binding kinase 1) responds to microbial pathogens to initiate cellular responses critical for host innate immune defense. We found previously that TBK1 phosphorylates mTOR (mechanistic target of rapamycin) (on S2159) to increase mTOR complex 1 (mTORC1) activity and signaling in response to the growth factor EGF and the viral dsRNA mimetic poly (I:C). mTORC1 and the less well studied mTORC2 respond to diverse cues to control cellular metabolism, proliferation, and survival. Here we demonstrate that TBK1 activates mTOR complex 2 (mTORC2) directly to increase Akt phosphorylation at physiological levels of protein expression. We find that TBK1 phosphorylates mTOR S2159 within mTORC2 *in vitro*, phosphorylates mTOR S2159 in cells, and interacts with mTORC2 in cells. By studying MEFs lacking TBK1, as well as MEFs, macrophages, and mice bearing an *Mtor S2159A* knock-in allele ($Mtor^{A/A}$), we show that TBK1 and mTOR S2159 phosphorylation increase mTORC2 catalytic activity and promote mTOR-dependent downstream signaling to Akt in response to several growth factors and poly (I:C). While microbial-derived stimuli activate TBK1, we find that growth factors fail to activate TBK1 or increase mTOR S2159 phosphorylation in MEFs. Thus, we propose that basal TBK1 activity cooperates

with growth factors in parallel to increase mTORC2 (and mTORC1) signaling. Collectively, these results reveal crosstalk between TBK1 and mTOR complexes (mTORCs), key nodes within two major signaling systems. As TBK1 and mTORCs have each been linked to tumorigenesis and metabolic disorders, these kinases may work together in a direct manner in a variety of physiological and pathological settings.

2.2 Introduction

TBK1 and its tissue-restricted orthologue IKKε mediate innate immunity against pathogenic viruses and bacteria in response to microbial-derived stimuli (202,288-291). Viral dsRNA and bacterial LPS bind to and activate the pathogen recognition receptors (PRRs) toll-like receptor 3 (TLR3) and TLR4, respectively (202,288-291). TLR3 and 4 activate the kinases TBK1 and IKKε through phosphorylation of their activation loop sites on S172 by an unknown upstream kinase or through oligomerization and activation loop site auto-phosphorylation on S172 (206,207,292,293). TBK1/IKKε in turn phosphorylate the transcription factors IRF3 and 7, resulting in their translocation into the nucleus where they induce expression of type I interferons (i.e. IFNα/β), multi-functional cytokines that initiate host defense responses while limiting tissue damage (215,294).

In prior work, we found that TBK1 phosphorylates mTOR on S2159, which increases mTOR complex 1 (mTORC1) signaling, mTORC1-mediated cell growth (i.e. cell size/mass) and cell cycle progression, and the production of IFNβ (44,104). This positive role for TBK1 in mTORC1 signaling has been confirmed in other studies (295,296). While studying TBK1 and its regulation of mTORC1, we noted that cellular

treatment with the TBK1/IKKε inhibitor amlexanox or TBK1 knockout in MEFs reduced phosphorylation of Akt on S473, an important metabolic kinase and target of PI3K (44). This observation agrees with other studies (177,199,295-297), several of which reported that TBK1 phosphorylates Akt S473 directly (177,199,297). As Akt S473 represents an established target of mTOR complex 2 (mTORC2) (30,57,149,298), we investigated more fully, the mechanism by which TBK1 promotes Akt phosphorylation, testing the hypothesis that TBK1 directly activates mTORC2 and its downstream signaling to Akt.

The mechanistic target of rapamycin (mTOR) comprises the catalytic kinase core of two known multi-subunit mTOR complexes (mTORCs) (12,57,149). The scaffolding protein Raptor defines mTORC1 (14,15), while the scaffolding protein Rictor defines mTORC2 (26,299). These mTORCs integrate a diverse array of environmental cues to control cell physiology appropriate for cell type and context. Indeed, aberrant mTORC function contributes to pathologic states including oncogenesis and obesity-linked metabolic disorders (12,57,140,149). Despite the physiological importance of mTOR, our knowledge of the upstream regulation of mTORCs remain incompletely defined, in particular mTORC2. mTORC1 drives anabolic cellular processes (i.e. protein, lipid, and nucleotide synthesis) in response to the coordinated action of nutrients (i.e. amino acids and glucose), growth factors (i.e. EGF and IGF-1), and hormones (i.e insulin) to control cell metabolism and promote cell growth (i.e. mass/size) and proliferation (12,57,60,149). The insulin/IGF-1 pathway represents the best-characterized activator of mTORC1, which utilizes PI3K signaling to Akt, TSC, and Rheb to activate mTORC1 on the surface of lysosomes during nutrient sufficiency (60,300,301). mTORC1 in turn

phosphorylates a diverse set of targets (134,135), with S6K1 T389 phosphorylation serving as a widely employed readout of mTORC1 activity in intact cells.

Growth factors and hormones also activate mTORC2 in a manner that requires PI3K. It is important to note that the upstream regulation of mTORC2 remains significantly less well defined than mTORC1 (12,35,57,149,301). Recently, the energy sensing kinase AMPK was shown to activate mTORC2 directly to promote cell survival during energetic stress (43). In addition, the stress inducible protein Sestrin2 was shown to activate mTORC2 (302). mTORC2 phosphorylates Akt on S473, a widely employed readout of mTORC2 activity in intact cells (30,149,298). Akt functions as a key mediator of PI3K signaling that controls diverse aspects of cell physiology (301,303). Akt activation absolutely requires phosphorylation of its activation loop site on T308 by PDK1. Phosphorylation of its hydrophobic motif site on S473 by mTORC2 activates Akt further to a maximal level and controls substrate preference (28). While not well understood, Akt S473 phosphorylation promotes and/or stabilizes Akt T308 phosphorylation, as increases or decreases in Akt P-S473 often result in correspondingly similar changes in Akt P-T308 (30,43,304). It is important to note that in addition to mTORC2, several other kinases have been identified as Akt S473 kinases, including DNA-PK, ATM, and ILK, and more recently TBK1 and IKKε (177,199,297,304). Functionally, mTORC2 controls cell metabolism, modulates the actin cytoskeleton, and promotes cell survival (12,26,57,149,299,305).

Beyond its well-known role in innate immunity, TBK1 has been implicated in oncogenesis and metabolic disorders linked to obesity such as type 2 diabetes, similar to mTOR and Akt (140,199,267,278,279,282-286,295,296,306-309). In oncogenic

KRas transformed cells, TBK1 promotes cell proliferation and survival and the growth of tumor explants *in vivo*, with either mTORC1 or Akt suggested as downstream mediators of TBK1 action (177,199,267,278,279,295,296,306,307). In addition, elevated expression of the TBK1 orthologue IKKε contributes to breast cancer oncogenesis (269,306). In diet-induced obese mice, adipocyte specific knockout of TBK1 decreases Akt S473 phosphorylation in white adipose tissue, increases insulin resistance and pro-inflammation, and impairs glucose homeostasis (285,310), a phenotype that overlaps with those resulting from adipocyte-specific knockout of Raptor (mTORC1) or Rictor (mTORC2) (138,140,141). Moreover, treatment of obese mice or human patients with amlexanox, or knockout of TBK1, Raptor (mTORC1 partner protein), or S6K1 (mTORC1 substrate) in mouse adipocytes, reduces adiposity and body mass, in part due to increased energy expenditure (285,309-311).

To better understand how TBK1 contributes to health and disease, we investigated the molecular mechanism by which TBK1 controls the phosphorylation of Akt. We find that TBK1 phosphorylates mTOR to activate mTORC2 directly, resulting in increased Akt phosphorylation during cellular treatment with growth factors and the innate immune agonist poly(I:C). This work not only elucidates the poorly defined upstream activation of mTORC2, but it improves our understanding of the contribution of TBK1 and mTORCs to physiology and pathologic conditions such as tumorigenesis and obesity-linked metabolic disorders.

2.3 Results

TBK1 increases mTOR dependent Akt phosphorylation in response to EGF

To elucidate the mechanism by which TBK1 positively controls Akt phosphorylation, we first analyzed TBK1 wild type ($TBK1^{+/+}$) and knockout ($TBK1^{-/-}$) MEFs. $TBK1^{-/-}$ MEFs displayed significantly reduced phosphorylation of Akt S473 (Fig 2.1) across an EGF time course, consistent with prior work (44,177,199). $TBK1^{-/-}$ MEFs also displayed reduced Akt T308 phosphorylation in response to EGF (Fig 2.2), and the active-site mTOR inhibitor Torin1 ablated both Akt P-S473 and P-T308 (Fig 2.1, 2.2). These data are consistent with mTORC2 functioning as a major Akt S473 kinase (30,298) and with many reports that Akt S473 phosphorylation promotes Akt T308 phosphorylation (30,43,304). Consistent with our prior work (44), $TBK^{-/-}$ MEFs displayed reduced S6K1 T389 phosphorylation, confirming that TBK1 promotes mTORC1 signaling (Fig 2.1). To confirm that reduced EGF-stimulated Akt phosphorylation in $TBK1^{-/-}$ MEFs results from loss of TBK1, we stably expressed vector control or Flag-tagged TBK1 in $TBK1^{-/-}$ MEFs by lentiviral transduction followed by puromycin selection. We selected several independent clones in which expression of exogenous Flag-TBK1 matched endogenous TBK1, as our prior work found that overexpression of TBK1 functions in a dominant negative manner to inhibit mTORC1 signaling (44), similar to overexpression of the mTORC1 subunit Raptor, an artifact common for proteins with scaffolding function. Expression of Flag-TBK1 rescued the reduced Akt S473 phosphorylation displayed in $TBK1^{-/-}$ MEFs stimulated with EGF (Fig 2.3). Moreover, stable expression of kinase-dead Flag-TBK1 failed to rescue P-Akt S473 (Fig 2.4). These results indicate that the kinase activity of TBK1 rather than its scaffolding function promotes Akt phosphorylation. Consistent with this conclusion, the TBK1/IKKε inhibitor amlexanox significantly reduced Akt P-S473 in response to EGF in MEFs (Fig 2.5) and

HEK293 cells (Fig 2.6). Taken together, these results indicate that TBK1 kinase activity promotes mTOR dependent phosphorylation of Akt S473 and T308 during EGF stimulation.

mTORC2 serves as a major link between TBK1 and Akt S473 phosphorylation at endogenous protein levels

Subsequent to the identification of mTORC2 as the major Akt S473 kinase (30,149,298), several groups demonstrated that TBK1 and IKKε phosphorylate Akt S473 and T308 directly (177,199,297). As we found that TBK1 phosphorylates mTOR to increase mTORC1 activity and signaling (44), we sought to clarify roles for TBK1 vs. mTOR in phosphorylation of Akt. We therefore assessed effects of mTOR inhibition across an EGF time course. We found that Torin1 ablated Akt S473 phosphorylation and reduced Akt T308 phosphorylation at each time point (1-30 minutes) following EGF stimulation of $TBK1^{+/+}$ and $TBK1^{-/-}$ MEFs (Fig 2.7). Consistent with our prior work, TBK1 knockout reduced S6K1 P-T389 (i.e. mTORC1 signaling) and mTOR auto-phosphorylation on S2481 (44). It is important to note that mTOR S2481 auto-phosphorylation represents a simple method to monitor total mTOR or mTORC specific catalytic activity in intact cells (42). These results indicate that in this setting, TBK1 plays a negligible role in Akt S473 phosphorylation, and thus mTOR represents the major Akt S473 kinase in MEFs.

We next investigated whether elevated levels of TBK1 and/or Akt enables TBK1 to directly engage and phosphorylate Akt. We therefore overexpressed increasing amounts of Flag-TBK1 in HEK293 cells. Consistent with prior work (177,199,297),

exogenous Flag-TBK1 WT increased Akt P-S473 (Fig 2.8). Torin1 reduced Akt P-S473 at low to mid doses of Flag-TBK1, indicating mTOR dependency, while higher doses displayed less Torin1 sensitivity. We next co-transfected Flag-TBK1 together with Flag-HA-Akt. The double Flag-HA tag allowed resolution of exogenous Akt (Flag-HA tagged) from endogenous Akt, enabling distinct assessment of phosphorylation on each of these two Akt populations. As before, TBK1 overexpression increased phosphorylation of endogenous Akt in a Torin1 sensitive manner at low doses. Upon co-expression of Flag-TBK1 with Flag-HA-Akt, however, Akt P-S473 became Torin1 resistant at even the lowest dose of Flag-TBK1 (Fig 2.9). These data indicate that, in the context of TBK1 and Akt over-expression, TBK1 phosphorylates Akt S473 independently of mTOR activity, as reported in prior work (177,199). At physiological levels of protein, however, TBK1 requires mTOR activity to mediate Akt S473 phosphorylation. By *in vitro* kinase assay, we confirmed that TBK1 phosphorylates recombinant Akt1 on S473 (Fig 2.10), consistent with other groups (177,199,297), and TBK1 phosphorylates a recombinant GST-mTOR fragment on S2159 (Fig 2.11), consistent with our prior work (44). Importantly, we confirmed that TBK1 phosphorylates wild type but not S2159A Myc-mTOR immunoprecipitated from transfected cells (Fig 2.12). Thus, TBK1 phosphorylates diverse substrates with dissimilar consensus phosphorylation motifs, particularly at elevated levels of expression of kinase and/or substrate.

To examine a potential role for TBK1 in the phosphorylation of Akt S473 in the absence of confounding mTORC2 activity, we analyzed MEFs lacking the critical mTORC2 partner protein Rictor. As expected, Rictor$^{-/-}$ MEFs expressing vector control displayed extremely low Akt S473 phosphorylation in response to EGF, while

exogenous re-expression of HA-Rictor rescued this phenotype in a manner sensitive to the mTOR inhibitor Ku-0063794 (Fig 2.13). Consistent with Fig 2.5 and 2.6, amlexanox inhibited Akt P-S473 in the rescued Rictor$^{-/-}$ MEFs, indicating dependence on TBK1 activity (Fig 2.13). With long blot exposure time, however, EGF increased Akt P-S473 in the Rictor$^{-/-}$ MEFs in a Ku-0063794 sensitive, but amlexanox resistant, manner (Fig 2.13, 2.14). While somewhat unexpected, the Ku-0063794 sensitivity reveals that MEFs lacking Rictor express crippled mTORC2 that still retains a low level of activity toward Akt. In agreement, Xie *et al.* found that mTOR inhibition with Torin1 reduced Akt P-S473 in response to PDGF in Rictor$^{-/-}$ MEFs (177). Therefore, these data indicate that mTOR rather than another kinase (i.e. TBK1) mediates Akt S473 phosphorylation in Rictor$^{-/-}$ MEFs. The amlexanox resistance of Rictor$^{-/-}$ MEFs suggests that TBK1 activity contributes negligibly to Akt phosphorylation in the context of crippled mTORC2. Curiously, shRNA-mediated knockdown of TBK1 in Rictor$^{-/-}$ MEFs reduced Akt P-S473 (Fig 2.14), consistent with Xie *et al.* (177). This finding suggests that the scaffolding function of TBK1 may contribute to mTORC2-mediated phosphorylation of Akt S473, at least in cells lacking physiologically intact mTORC2. Taken together, these data indicate that mTORC2 represents a critical link between TBK1 and Akt S473 phosphorylation at physiological levels of protein expression.

mTOR S2159 phosphorylation promotes mTORC2 signaling in response to EGF

In prior work we generated genome edited mice bearing an alanine knock-in substitution at *Mtor S2159* using CRISPR/Cas9 technology (44). By studying bone marrow derived macrophages (BMDMs) in culture isolated from wild type (*Mtor*$^{+/+}$) and

S2159A knock-in mice (*Mtor*$^{A/A}$), we demonstrated that mTOR S2159 phosphorylation is required for mTORC1 signaling and IFNβ production in macrophages stimulated with innate immune agonists (i.e. poly(I:C) and LPS) (44). Thus, we next investigated a potential direct link between TBK1 and mTORC2 by studying the role of mTOR S2159 phosphorylation in control of mTORC2 signaling. To do so, we isolated littermate matched MEFs from *Mtor*$^{+/+}$ and *Mtor*$^{A/A}$ mice (pair #1 MEFs), subjected them to spontaneous immortalization, and analyzed their response to EGF following serum deprivation. Relative to MEFs from *Mtor*$^{+/+}$ mice (i.e. mTOR$^{+/+}$ MEFs), MEFs from *Mtor*$^{A/A}$ mice (i.e. mTOR$^{A/A}$ MEFs) displayed significantly reduced Akt P-S473 (Fig 2.15) and Akt P-T308 (Fig 2.16) across an EGF time course. mTOR$^{A/A}$ MEFs also displayed reduced S6K1 P-T389 and mTOR S2481 auto- phosphorylation (Fig 2.15). Activation of the MAPK/ERK pathway in response to EGF remained unperturbed in the mTOR$^{A/A}$ MEFs (as monitored by the phosphorylation of ERK1 and 2 on T202/Y204), indicating intact activation of EGF-receptor signaling to MAPK/ERK in mTOR$^{A/A}$ MEFs (Fig 2.15). Importantly, we observed reduced Akt P-S473 and S6K1 P-T389 in response to EGF in a second, independently derived pair of mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs (pair #2 MEFs) (Fig 2.17). These data demonstrate that mTOR S2159 phosphorylation increases mTOR catalytic activity and promotes mTORC2 and 1 signaling upon cellular stimulation with EGF.

We next asked whether EGF activates TBK1 by monitoring TBK1 phosphorylation on its activation loop site (S172). We found that EGF failed to increase TBK1 P-S172 in either mTOR MEFs (Fig 2.18) or TBK1 MEFs (Fig 2.19); as expected, LPS treatment of RAW264.7 macrophages strongly increased TBK1 P-S172 (Fig 2.18,

2.19). Note that EGF also failed to increase TBK1 P-S172 in HEK293 cells (see (44)). These results demonstrate that EGF does not activate TBK1, at least in MEFs and HEK293 cells, and thus basal rather than EGF stimulated TBK1 activity supports mTORC1/2 signaling.

TBK1 phosphorylates mTOR within mTORC2, interacts with mTORC2, and increases mTORC2 intrinsic catalytic activity

To further define the mechanism by which TBK1 promotes mTORC2 signaling, we asked whether recombinant TBK1 (re-TBK1) phosphorylates mTOR S2159 within mTORC2 directly. It is important to note that our prior work demonstrated that TBK1 phosphorylates mTOR S2159 within mTORC1 (44). By *in vitro* kinase assay, we found that re-TBK1 increased mTOR P-S2159 on Rictor-associated mTOR immunoprecipitated from cells, and inclusion of the TBK1/IKKε inhibitor BX-795 *in vitro* blocked this increase (Fig 2.20). We next asked whether TBK1 and mTORC2 interact in cells. By co-immunoprecipitating endogenous proteins, we found that Rictor immunoprecipitates pulled down TBK1 in $TBK1^{+/+}$, but not $TBK1^{-/-}$ MEFs (Fig 2.21).

Together, these data support a mechanism whereby TBK1 interacts with mTORC2 and subsequently mediates the direct phosphorylation of mTOR S2159 within mTORC2. We next asked whether EGF increases mTOR S2159 phosphorylation and whether TBK1 promotes mTOR S2159 phosphorylation in intact cells. EGF failed to increase P-S2159 on mTOR immunoprecipitated from $TBK1^{+/+}$ or $mTOR^{+/+}$ MEFs (Fig 2.22, 2.23). Importantly, TBK1 knockout from MEFs reduced mTOR P-S2159 (Fig 2.22), and mTOR P-S2159 was undetectable in $mTOR^{A/A}$ MEFs (Fig 2.23), confirming *Mtor*

S2159A knock-in. We speculate that the remaining mTOR P-S2159 found in TBK1$^{-/-}$ MEFs results from IKKε-mediated phosphorylation of mTOR. Indeed, while IKKε expression is generally tissue-restricted and extremely low in non-immune cells, MEFs indeed express detectable levels of IKKε (Fig 2.22, 2.23). These data demonstrate that TBK1 mediates mTOR S2159 phosphorylation *in vitro* and in intact cells and support the conclusion that basal rather than EGF stimulated TBK1 kinase activity mediates mTOR P-S2159 to promote mTORC2 signaling.

We next asked whether TBK1 and mTOR S2159 phosphorylation increase the intrinsic catalytic activity of mTORC2 by *in vitro* kinase assay. To do so, we immunoprecipitated Rictor from TBK1$^{+/+}$ vs. TBK1$^{-/-}$ MEFs and from mTOR$^{+/+}$ vs. mTOR$^{A/A}$ MEFs after EGF stimulation of serum deprived cells. The Rictor immunoprecipitates were washed, incubated in kinase buffer with ATP and recombinant His-Akt1 as substrate, and the ability of Rictor-associated mTOR to phosphorylate Akt S473 *in vitro* was monitored by western blot. In both TBK1$^{+/+}$ MEFs (Fig 2.24) and mTOR$^{+/+}$ MEFs (Fig 2.25), EGF increased mTORC2 catalytic activity in a Torin1-sensitive manner, as expected. The fold increase in mTORC2 catalytic activity mediated by EGF, however, was reduced in TBK1$^{-/-}$ MEFs (Fig 2.24) and mTOR$^{A/A}$ MEFs (Fig 2.25). To assess the role of TBK1 and mTOR S2159 phosphorylation in control of mTORC2 catalytic activity by an independent approach, we monitored S2481 auto-phosphorylation on Rictor associated mTOR (i.e. mTORC2). Similar to results obtained with mTORC2 *in vitro* kinase assays, the fold increase in mTOR S2481 auto-phosphorylation mediated by EGF was reduced in TBK1$^{-/-}$ (Fig 2.26) and mTOR$^{A/A}$ MEFs (Fig 2.27). Taken together, these results indicate that TBK1 and mTOR S2159

phosphorylation increase mTORC2 catalytic activity.

TBK1 and mTOR S2159 phosphorylation increase mTORC2 (and mTORC1) signaling inresponse to diverse growth factors

We next investigated whether the positive role of mTOR S2159 phosphorylation in mTORC2 signaling extends to a broader set of growth factors. We thus interrogated mTORC2 signaling to Akt, as well as mTORC1 signaling to S6K1, in $mTOR^{+/+}$ vs. $mTOR^{A/A}$ MEFs in response to several growth factors known to activate mTORC2 and 1 signaling. We first assessed the role of mTOR P-S2159 in control of mTORC2 and 1 signaling in MEFs cultured in complete media (i.e. DMEM/FBS [10%]) containing serum growth factors. $mTOR^{A/A}$ MEFs as well as $TBK1^{-/-}$ MEFs displayed reduced Akt S473 and S6K1 T389 phosphorylation (Fig 2.28). Treatment of cells with Torin1 ablated these phosphorylation events, indicating that mTOR activity is required for mTORC2 and 1 signaling in complete media (Fig 2.28). We next assessed the role of mTOR P-S2159 in control of mTORC2 and 1 signaling in response to stimulation of MEFs with fetal bovine serum (FBS), platelet-derived growth factor (PDGF) (a major constituent of FBS), and insulin (which acts through IGF-1 receptors in MEFs) following serum deprivation. Similar to their response to EGF, $mTOR^{A/A}$ MEFs displayed reduced Akt P-S473 and S6K1 P-T389 in response to all three growth factors with varying dynamics across a time course (Fig 2.29, 2.30, 2.31). In addition, all three growth factors failed to increase TBK1 P-S172 phosphorylation (Fig 2.32), suggesting that growth factor receptor signaling does not activate TBK1, at least in MEFs. These results indicate that mTOR S2159 phosphorylation increases mTORC2 and 1 activity in parallel to growth factor

signaling.

As TBK1 knockout MEFs displayed reduced Akt S473 and S6K1 T389 phosphorylation upon amino acid stimulation following amino acid deprivation (295), we next examined a role for mTOR P-S2159 in control of these phosphorylation events in the absence or presence of amino acids. It is important to note that mTORC1 localization and activity change dynamically in response to amino acids levels, with the majority of studies indicating that mTORC2 does not function in amino acid sensing (58,300,312-316). We cultured $mTOR^{+/+}$ vs. $mTOR^{A/A}$ MEFs, as well as $TBK1^{+/+}$ vs. $TBK1^{-/-}$ MEFs, in DMEM lacking amino acids, but supplemented with dialyzed FBS for 50 minutes; we then added back a 1x mixture of total amino acids (pH normalized to 7.4) for 10 minutes. Amino acids increased S6K1 P-T389, but not Akt P-S473, in wild type MEFs, as expected (Fig 2.33). $mTOR^{A/A}$ and $TBK1^{-/-}$ MEFs displayed reduced Akt P-S473 and S6K1 P-T389 in both the absence and presence of amino acids (Fig 2.33). Similar to wild type MEFs, amino acids increased S6K1 P-T389 but not Akt P-S473 in the mutant MEFs. Taken together, these results indicate that amino acids activate mTORC1, but not mTORC2 signaling, and TBK1 and mTOR P-S2159 support both mTORC1 and 2 signaling in parallel to growth factor and amino acid signaling pathways.

TBK1 activity and mTOR S2159 phosphorylation increase TLR3-mediated mTORC2 signaling in macrophages

Our prior work demonstrated that TBK1 and mTOR S2159 phosphorylation promote mTORC1 signaling in macrophages upon activation of TLR-3 and 4, pathogen recognition receptors that activate TBK1 and IKKε (44). To investigate a role for TBK1

and mTOR P-S2159 in positive control of mTORC2 signaling in macrophages, we assayed how TBK1/IKKε inhibitors or *Mtor S2159A* knock-in modulated mTORC2 signaling to Akt in response to TLR-3 activation with poly (I:C) (a viral dsRNA mimetic). In cultured RAW264.7 macrophages, poly(I:C) activated TBK1 (as monitored by increased TBK1 P-S172) and PI3K-dependent mTORC2 signaling (as monitored by the sensitivity of Akt P-S473 to the class I PI3K inhibitor BYL-719 and the mTOR inhibitor Ku-0063794 (Fig 2.34, 2.35). Inhibition of TBK1/IKKε with two different small molecules, amlexanox (Fig 2.34, 2.35) or BX-795, (Fig 2.35) (207) also reduced poly (I:C) induced Akt P-S473, demonstrating that TBK1/IKKε activity positively controls mTORC2 signaling to Akt in RAW24.7 macrophages.

We next isolated primary bone marrow-derived macrophages (BMDMs) from $Mtor^{+/+}$ and $Mtor^{A/A}$ mice. As expected, poly (I:C) increased TBK1 P-S172 and mTOR-dependent Akt P-S473 in wild type BMDMs (Fig 2.36). As in RAW264.7 macrophages, amlexanox suppressed Akt P-S473 to a basal level (Fig 2.36). Importantly, BMDMs from $Mtor^{A/A}$ mice displayed reduced poly (I:C) induced Akt P-S473 (Fig 2.36), thus demonstrating a required role for mTOR S2159 phosphorylation in TLR-3-mediated activation of mTORC2 signaling. Consistent with our prior work (44), amlexanox or *Mtor S2159A* knock-in reduced mTORC1 signaling in RAW264.7 macrophages and BMDMs (Fig 2.34, 2.36). These results demonstrate that TBK1 activity, mTOR activity, and mTOR S2159 phosphorylation are required for mTORC2 signaling to Akt upon activation of TLR-3 in macrophages. Finally, to demonstrate a role for mTOR S2159 phosphorylation in activation of mTORC2 signaling by TLR-3 *in vivo*, we injected $Mtor^{+/+}$ and $Mtor^{A/A}$ mice with poly (I:C) and harvested macrophage-rich spleen tissue. We

found that spleen tissue from *Mtor*$^{A/A}$ mice displayed reduced Akt S473 phosphorylation in response to poly (I:C) (Fig 2.37). Taken together, these results demonstrate that TBK1 phosphorylates mTOR S2159 to activate mTORC2 directly and increase downstream signaling to Akt in cultured cells and *in vivo* (Fig 2.38) (model cartoon). Moreover, they reveal that mTORC2 represents an essential link between TBK1 and Akt phosphorylation at physiological levels of protein expression. We find that in MEFs, basal TBK1 kinase activity signals in parallel to growth factors to augment mTORC2 (and mTORC1) activity, as EGF and other growth factors increased mTORC1/2 signaling in a TBK1 and mTOR P-S2159 dependent manner without increasing TBK1 S172 or mTOR S2159 phosphorylation. The relationship between growth factor signaling and TBK1 activity appears to be context dependent, however, as growth factors were shown recently to activate TBK1 (i.e. increase TBK1 P-S172) in lung cancer cells (296) (see Discussion). In macrophages, TLR-3 signaling increases TBK1 and mTORC1/2 activity in a linear pathway (Fig 2.38). It is important to note that other groups have reported that the PI3K/Akt pathway signals in parallel to, but independently of, TBK1/IKKε to promote full activation of IRF-3 and the innate immune response. Specifically, dsRNA-induced phosphorylation of TLR-3 on tyrosine 759 resulted in the recruitment of PI3K to the cytoplasmic domain of the receptor, and this mechanism further increased phosphorylation of IRF-3, effectively rendering it transcriptionally competent to drive innate immune target gene expression (317). This group proposed a two-step model in which initial phosphorylation of IRF-3 by TBK1, together with subsequent phosphorylation on additional serine/threonine residues (presumably by Akt) cooperate to yield a transcriptionally active IRF-3 homodimer. In addition, Lu et al

demonstrated that influenza A virus induced IRF-3 binding to interferon-sensitive response elements (ISREs) within the promoter of C-X-C motif chemokine ligand 10 (CXCL-10) to induce its expression, and several other groups have demonstrated that the influenza A viral protein NS1 activates the PI3K/Akt pathway (318-320).

2.4 Discussion

Several studies have demonstrated a positive role for TBK1 in Akt phosphorylation in various contexts. Increasing or decreasing TBK1 activity by various approaches in many cell types (i.e. MEFs, HEK293 cells, U2OS cells, HeLa cells, MNT1 melanoma cells, or HCT116 colorectal cancer cells) led to correspondingly similar changes in Akt S473 phosphorylation (177,199,295,296). These observations, together with evidence that mTORC2 serves as a major Akt S473 kinase (30,57,149,298), and our prior work that TBK1 directly activates mTORC1 (44), prompted us to investigate whether mTORC2 represents a missing link between TBK1 and Akt phosphorylation.

While it is challenging to demonstrate definitely that a kinase (i.e. TBK1) phosphorylates a substrate (i.e. mTOR) directly in intact cells rather than indirectly in a complex, several lines of evidence considered together support our model that TBK1 phosphorylates mTOR S2159 directly to increase mTORC2 activity and signaling to Akt (Fig 2.38): TBK1 phosphorylates mTOR S2159 within mTORC2 *in vitro*; TBK1 and mTORC2 co-immunoprecipitate and TBK1$^{-/-}$ MEFs display reduced mTOR P-S2159 in intact cells; TBK1$^{-/-}$ MEFs and mTOR$^{A/A}$ MEFs (lacking mTOR P-S2159) display reduced mTOR dependent Akt P-S473 and Akt P-T308 in response to EGF; Rictor$^{-/-}$ MEFs stimulated with EGF bear extremely low Akt P-S473 that remains strongly Torin1

sensitive; TBK1 overexpression at low levels increases Akt P-S473 in a largely Torin1 sensitive manner; and finally, both TBK1$^{-/-}$ MEFs and mTOR$^{A/A}$ MEFs display reduced mTORC2 intrinsic catalytic activity in response to EGF, as measured by mTORC2 IVK assay and by Rictor-associated mTOR S2481 autophosphorylation. To support these results in immortalized cells, we also provide evidence that in primary macrophages in culture (i.e. BMDMs) and spleen tissue *in vivo,* mTOR S2159 phosphorylation is required for Akt S473 phosphorylation in response to TBK1 activation with poly (I:C). In addition, Zhao et al found that adipocyte-specific knockout of TBK1 in diet-induced obese mice reduced Akt P-S473 in response to insulin in white adipose tissue (285). Taken together, these results argue that TBK1-mediated mTOR S2159 phosphorylation promotes mTORC2 signaling to Akt.

Several groups identified TBK1 as a direct Akt S473 and T308 kinase (177,199,297). We found that at physiological levels of TBK1 and Akt expression, the ability of growth factors or poly (I:C) to increase Akt S473 phosphorylation in a detectable manner required mTOR activity. mTOR inhibition also reduced Akt P-T308, a finding consistent with the observation that Akt S473 phosphorylation promotes and/or stabilizes Akt T308 phosphorylation (30,43,304). We found that overexpression of TBK1 increased Akt P-S473, similar to earlier work (177,199,297). This effect was largely dependent on mTOR activity at low to mid doses of TBK1 but displayed modest mTOR independence at higher doses. When both TBK1 and Akt were overexpressed, however, TBK1 increased Akt P-S473 in an mTOR-independent manner. Taken together, these data indicate that at physiological levels of TBK1 and Akt expression, TBK1 increases Akt phosphorylation through mTORC2.

Pathological or unique physiological contexts may modify mechanisms governing Akt S473 and T308 phosphorylation. For example, tissue-specific knockout of Rictor or mTOR from cardiac or skeletal muscle failed to ablate Akt S473 phosphorylation (an unexpected finding) (177,321-323). Even more surprising, mTOR knockout cardiac muscle displayed elevated Akt P-S473 (177,323). This finding prompted Xie et al to search for alternate Akt S473 kinases. Upon discovering TBK1 as an Akt S473 and T308 kinase, they noted that mTOR knockout cardiac muscle displayed elevated TBK1 expression (177). Thus, in this context of elevated TBK1 expression, TBK1 may phosphorylate Akt directly. Pathological contexts may also render Akt S473 phosphorylation TBK1 independent. For example, TBK1 inactivation reduced S6K1 T389 but not Akt S473 phosphorylation in response to growth factors in A549 human lung adenocarcinoma cells or primary mouse lung cancer epithelial cells (296). Therefore, we propose that in stress-related contexts (i.e. knockout of the essential kinase, mTOR), oncogenic contexts, or other pathological contexts, cells and tissues may re-wire signaling and metabolism by upregulating expression of TBK1/IKKε (or other Akt S473 kinases). Such an adaptive response may in turn enable TBK1/KKε (or other kinases) to directly phosphorylate Akt S473 and/or T308, thus increasing Akt activity in order to mitigate the pathologic insult and improve metabolic homeostasis and/or provide a proliferative or survival advantage.

Mechanisms governing regulation of TBK1 kinase activity in non-immune cells remain poorly defined. Our results indicate that in MEFs, growth factors increase mTORC2 signaling in a TBK1 and mTOR P-S2159 dependent manner without increasing TBK1 S172 phosphorylation (i.e. TBK1 activity) or mTOR S2159

phosphorylation. We therefore conclude that in this context, the basal kinase activity of TBK1 promotes mTORC2 signaling in parallel to growth factors. It is important to note, however, that stimulation of human A549 lung cancer cells and primary mouse lung cancer cells with several different growth factors (i.e. EGF, FBS, and insulin) increased TBK1 S172 phosphorylation, which required TBKBP1 (TBK binding protein 1), a TBK1 adaptor protein (296). We speculate that differential expression of numerous TBK1 adaptors explains differences in cellular context that underlies regulation of TBK1 activity by growth factors. These adaptors may also differentially control TBK1 subcellular localization and/or TBK1 substrate preference (200,292,307,324).

Taken together, our results identify TBK1 as a direct activator of mTORC2 in physiological contexts, which expands our limited understanding of mTORC2 regulation. In addition, they establish the TBK1-mTORC2 pathway as a potential target for therapeutic intervention to treat cancer and obesity linked metabolic disorders.

2.5 Experimental Procedures

Materials

General chemicals were from Thermo Fisher or Sigma. Protein A- and G-Sepharose Fast Flow were from GE Healthcare; NP40, Brij35, and CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1- propanesulfonate) detergents were from Pierce; cOmplete Protease Inhibitor Cocktail (EDTA-free) tablets were from Millipore Sigma (#11836170001); Immobilon-P polyvinylidene difluoride (PVDF) membrane (0.45 μM) was from Millipore; Bradford Reagent for protein assays was from Bio Rad (#5000001); reagents for enhanced chemiluminescence (ECL) were from either Alkali Scientific

(Bright Star) or Advansta (Western Bright Sirius HRP substrate). Recombinant active GST-TBK1 was from Thermo Fisher/ Life Technologies (#PV3504); recombinant His-Akt1 was from EMD Millipore (#14-279); recombinant GST-mTORf (containing a 30 fragment of mTOR encoding amino acids 2144-2175) was generated as described (44).

Antibodies

The following antibodies from Cell Signaling Technology (CST) were used in this study (all rabbit polyclonal antibodies, unless otherwise noted): Akt (#9272); Akt P-S473 (#4060; rabbit mAb D9E XP); Akt P-T308 (#4056, rabbit mAb 244F9); TBK1 (#3013; or #3504, rabbit mAb D1B4); TBK1 P-S172 (#5483, rabbit mAb D52C2 XP); S6K1 (#9202); S6K1 P-T389 (#9205; #9234, rabbit mAb 108D2; #9206, mouse mAb IA5); mTOR (#2972); MAPK (#9102); MAPK P-T202/Y204 (#4370, rabbit mAb D13.14.4E XP); GST (#2624; mouse mAb 26H1); IgG-conjugated Sepharose beads (#3423); Rictor- conjugated Sepharose beads (#5379). The following antibodies were from other commercial vendors: mTOR P-S2481 (MilliporeSigma #09-343); Myc (Millipore Sigma #05-419, mouse mAb 9E10); HA.11 (BioLegend # 901513, mouse mAb 1612B). The following custom, anti-peptide polyclonal antibodies were generated by us in-house with assistance from a commercial vendor: Rictor (amino acids 6-20; Covance; as described (48,325)); mTOR (amino acids 221-237, Covance; as described (48,325)); mTOR P-S2159 (Millipore; as described (44)).

Plasmids

pcDNA3/Flag-TBK1 wild type (WT) and pcDNA3/Flag-TBK1 kinase dead (K38M) were

obtained from Dr. A. Saltiel (UCSD School of Medicine, Institute for Diabetes and Metabolic Health; San Diego, CA). pcDNA3/Flag-HA-Akt was obtained from Addgene (#9021). pRK5/Myc-mTOR WT was originally from Dr. D. Sabatini (MIT and the Whitehead Institute, Boston, MA) and obtained via Addgene (#1861). pRK5/Myc-mTOR S2159A was generated via site-directed mutagenesis as described (44))). pCI/HA-Rictor was from Dr. E. Jacinto (Rutgers University, New Brunswick, NJ).

Cell culture, transfection, and drug treatments

All cell lines used in this study (i.e. MEFs, HEK293, RAW264.7 murine macrophages, and primary mouse BMDMs) were cultured in DMEM that contained high glucose [4.5 g/L], glutamine [584 mg/L], and sodium pyruvate [110 mg/L] (Thermo Fisher/ Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Gibco/Invitrogen). Note that dialyzed FBS was used to culture the RAW264.7 macrophages and BMDMs. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. HEK293 cells (from ATCC) were transfected using Mirus Trans-It LT1 in accordance with manufacturer's instructions and lysed ~24 to 48 hr. post-transfection. To stimulate cells with growth factors, MEFs and HEK293 cells were first serum starved via incubation in DMEM containing 20 mM Hepes pH 7.2 overnight, ~20 hr. The cells were then stimulated with EGF [25 or 50 ng/mL] (Sigma Aldrich #E9644 and #E4127), FBS [10% final], PDGF [10 ng/mL] (EMD Millipore #GF149), or insulin [100 nM] (Thermo Fisher/ Life Technologies #12585) for various amounts of time (0-60 mins). To stimulate macrophages with innate immune agonists, RAW264.7 macrophages and BMDMs cultured in complete DMEM (i.e. DMEM containing dialyzed FBS [10%]) were treated

with poly(I:C) [30 ug/mL] (Sigma Aldrich #P1530) or ultrapure LPS [100 ng/ml] (InVivo Gen #tlrl-3pelps). Cells were treated with the following drugs: Torin1 [100 nM] (30 min) (shared by Dr. D. Sabatini), Ku-0063794 [100 nM] (30 min) (Tocris #3725); amlexanox [100 uM] (1-2 hr) (EMD Millipore #SML0517); BX-795 [10 uM] (30 min) (Millipore CalBiochem #204011) (30 min); BYL-719 [10 uM] (30 min) (Selleck; #1020). To amino acid deprive and stimulate cells, MEFs were first incubated for 50 min in RPMI media lacking all amino acids (Millipore Sigma; # R8758) but supplemented with dialyzed FBS [10%]. The cells were then acutely stimulated with amino acids for 10 min by adding a mixture of total amino acids (RPMI 1640 Amino Acid Solution) (Millipore Sigma; # R7131) to a final concentration of [1x] (the concentration of amino acids in RPMI medium). Note that the pH of this amino acid solution is quite basic, and thus the pH of this solution was first normalized to 7.4 prior to addition to cells in order to maintain physiological pH. As the RPMI 1640 Amino Acid Solution lacks glutamine, the amino acid mixture was supplemented with L-glutamine (Millipore Sigma; #59202C) prior to pH normalization and addition to cells.

Cell lysis, immunoprecipitation, and immunoblotting

Cells were washed twice with ice-cold PBS pH 7.4 and scraped into ice-cold lysis buffer A (10 mMKPO4 pH 7.2; 1 mM EDTA; 5 mM EGTA; 10 mM MgCl2; 50 mM β-glycerophosphate; 1 mM sodium orthovanadate [Na3VO4]; a cocktail of protease inhibitors) containing NP-40 [0.5%] and Brij35 [0.1%], as described (325).
To maintain detergent sensitive mTOR-partner protein interactions during Rictor or mTOR immunoprecipitation, cells were lysed in ice-cold buffer A containing mild

CHAPS [0.3%] detergent. Lysates were spun at 13,200 rpm for 5 min at 4°C, and the post-nuclear supernatants were collected and incubated on ice (15 min). Bradford assay was used to normalize protein levels for immunoblot or immunoprecipitation analysis. For immunoprecipitation, whole cell lysates were incubated with antibodies for 2 hr. at 4°C, followed by incubation with Protein G- or A- Sepharose beads for 1 hr. Sepharose beads were washed three times in lysis buffer and resuspended in 1x sample buffer. Samples were resolved on SDS-PAGE and transferred to PVDF membranes in Towbin transfer buffer containing 0.02% SDS, as described (325). Immunoblotting was performed by blocking PVDF membranes in Tris-buffered saline (TBS) pH 7.5 with 0.1% Tween-20 (TBST) containing 3% non-fat dry milk, as described (325), and incubating the membranes in TBST with 2% bovine serum albumin (BSA) containing primary antibodies or secondary HRP-conjugated antibodies. Blots were developed by ECL and detected digitally with a Chemi-Doc-It System (UVP).

Lentiviral transduction

TBK1$^{-/-}$ MEFs stably expressing Flag-TBK1 (wild type or kinase dead K38M) were generated by lentiviral transduction. Flag-TBK1 was subcloned into a modified lentiviral vector, pHAGE-Puro-MCS (pPPM) (326) (modified by Amy Hudson (Medical College of Wisconsin) to include an expanded multiple cloning site, MCS). Lentivirus particles were packaged in HEK293T cells by co-transfection with empty pPPm vector or pPPM/Flag-TBK1 together with pRC/Tat, pRC/Rev, pRC/gag-pol, and pMD/VSV-G using Mirus TransIT-LT1 transfection reagent. Supernatants containing viral particles were collected 48 hr. post transfection and filtered through a 0.45 μm filter. TBK1$^{-/-}$ MEFs were infected

with fresh viral supernatants containing 8 μg/ml polybrene. 24 hr. post infection, cells were re-fed with DMEM/10% FBS supplemented with 10 μg/ml puromycin for 2-3 days to select for stably transduced cells, trypsinized, and plated at limiting dilution in order to isolate clones originating from single cells. TBK1$^{-/-}$ MEF clones transduced with wild type Flag-TBK1 lentivirus were analyzed for expression of exogenous Flag-TBK1 relative to expression of endogenous TBK1 found in wild type MEFs. TBK1$^{-/-}$ MEF clones expressing Flag-TBK1 at a level similar to endogenous TBK1 were chosen for analysis. Alternately, TBK1$^{-/-}$ MEFs transduced with wild type or kinase dead (K38M) Flag-TBK1 were selected in puromycin and pooled for analysis. Rictor $^{-/-}$ MEFs (from Dr. E. Jacinto; Rutgers University, New Brunswick, NJ) stably expressing vector control or HA-Rictor were generated by lentiviral transduction and stable selection, as described (43). These rescued lines were maintained in DMEM/FBS [10%] containing puromycin [8 μg/mL].To knockdown TBK1, Rictor$^{-/-}$ MEFs were infected with lentiviral particles encoding an shRNA targetingTBK1 (Sigma) (mouse TBK1 # TRCN0000323444; non-targeting # SHC016V) and then selected in puromycin [8 μg/mL] for 4 days.

In vitro kinase assays

Phosphorylation of recombinant His-Akt1 or GST-mTOR$_f$ by recombinant active TBK1. *In vitro* kinase (IVK) assays were performed by incubating recombinant His-Akt1 [50 ng] or GST-mTORf [50 ng] substrates with recombinant active GST-TBK1 [50 ng] in kinase buffer containing 25 mM Tris-HCl pH 7.5,10 mM $MgCl_2$, 1 mM DTT, and 200 μM ATP. Reactions were incubated at 30°C for 30 min and stopped by addition of sample buffer followed by incubation at 95°C for 5 min. Samples were resolved on SDS-PAGE,

transferred to PVDF membrane, and immunoblotted. The phosphorylation of His-Akt1 was measured using anti-Akt P-S473 antibodies, and the phosphorylation of GST-$mTOR_f$ was monitored using anti-mTOR P-S2159 antibodies. For certain IVK reactions, recombinant kinases were pre-incubated with BX-795 [10 μM] in kinase buffer on ice for 30 min.

Phosphorylation of Myc-mTOR or Rictor-associated mTOR isolated from cells by recombinant active TBK1. HEK293 cells were transfected with Myc-mTOR wild type or mutant S2159A, and lysates were immunoprecipitated with anti-Myc antibodies. Myc-mTOR S2159A was generated by QuikChange mutagenesis, as described previously (104). Alternately, Rictor from non-transfected HEK293 cells was immunoprecipitated with anti-Rictor antibodies. After washing in lysis buffer and kinase buffer, the immune complexes (containing substrate) were incubated with recombinant active GST-TBK1 [50 ng] in kinase buffer and ATP, as described above. The phosphorylation of Myc-mTOR or Rictor-associated mTOR was monitored using anti-mTOR P-S2159 antibodies.

Phosphorylation of His-Akt1 by cellular mTORC2 (i.e., mTORC2 IVKs). mTORC2 *in vitro* kinase (IVK) assays was performed as described (43,70). Briefly, Rictor was immunoprecipitated from serum starved MEFs pre-treated without or with Torin1 [100 nM] (30 min) and then stimulated without or with EGF [50ng/mL] (10 min) (1-2 10 cm plates for each immunoprecipitate). After washing in lysis buffer and kinase buffer, the immune complexes (containing mTOR kinase) were incubated with ATP [500 μM] and

recombinant His-Akt1 [100 ng/reaction] in kinase buffer containing 25 mM HEPES, 100 mM potassium acetate, and 1 mM $Mgcl_2$ at 30°C for 30 mins. Certain reactions were also pre-treated with Torin1 [10 μM] in kinase buffer on ice for 30 min prior to initiating the IVK reaction. Immunoprecipitates were pre-incubated with Torin1 [10 μM] on ice (30 min) and then were incubated with ATP [500 uM] and recombinant His-Akt1 [100 ng/reaction] in kinase buffer containing 25 mM HEPES, 100 mM postassium acetate, and 1 mM $Mgcl_2$, at 30°C for 30 min.

Co-immunoprecipitation of TBK1 and mTORC2

$TBK1^{+/+}$ or $TBK1^{-/-}$ MEFs were lysed in buffer A containing CHAPS [0.3%] detergent. Protein levels in each cell type were normalized after performing protein assays with the Bradford assay. IgG control Sepharose beads (CST) or Rictor-conjugated Sepharose beads (CST) were washed 2x with PBS, blocked ~1 hr in PBS containing 2% bovine serum albumin (BSA) to reduce non-specific binding, and then washed in PBS 2x more. Whole cell lysates were then added to the washed beads, rotated at 4°C overnight, washed 3x in lysis buffer, and resuspended in 1x SDS-PAGE sample buffer.

Mtor S2159A knock-in mice

mTOR knock-in S2159A mice (*$Mtor^{A/A}$*) were generated using CRISPR-Cas9 genome editing technology and genotyped, as described (44). Mice were housed in a specific pathogen-free facility with a 12-hour light/12-hour dark cycle and given free access to food and water. All animal use was in compliance with Institutional Animal Care and Use Committee (IACUC) at the University of Michigan.

Isolation and immortalization of MEFs

Male and female heterozygous *Mtor*$^{+/A}$ mice were mated, and MEFs from plugged females were isolated on day 13.5 of pregnancy, generally as described (327,328). Dissected embryos were washed with 3x in PBS pH 7.4 and minced with fine scissors in the presence of trypsin-EDTA. The minced tissue was titrated with 5 mL serological and fine-tip Pasteur pipettes to prepare a homogenate and transferred into a 15 mL conical tube containing 8 mL DMEM. The homogenate was centrifuged 4 min at 300g, and the supernatant was discarded. The pellet was washed 1x in PBS pH 7.4 and resuspended in complete medium (DMEM/FBS [10%] containing 50 U/mL penicillin and 50 μg/mL streptomycin). The resuspended cells were transferred to a 10 cm culture dish with fresh complete medium and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The MEFs were washed 1x with PBS pH 7.4, detached with 0.05% trypsin-EDTA, centrifuged 4 min at 300g, and transferred to a 15 cm culture dish. At confluency, the MEFs were trypsinized and aliquoted to multiple cryovials for long-term storage in liquid N_2. MEFs were immortalized spontaneously through serial passaging. As they reached confluency in 15 cm culture dishes following isolation, primary MEFs were plated into 10 cm culture dishes at a 1:9 split ratio into 10 ml complete medium (passage #2). Upon reaching confluency, the MEFs, were again, passaged into 10 cm culture dishes with a split ratio of 1:3. This process was repeated every 3-4 days until reaching senescence (approximately passage 5-7). Culture media was replaced every 3-4 days, and the cells were trypsinized and transferred into a new culture dish once a week. Once the MEFs began proliferating, they were passaged with a 1:3 split ratio

every 3-4 days until cell number doubled approximately every 24-30 hrs. MEFs were genotyped as described (44) using dissected head tissue.

Isolation of primary bone marrow derived macrophages (BMDMs)

Bone marrow from 8-14 week old *Mtor*$^{+/+}$ and *Mtor*$^{A/A}$ mice was harvested by flushing femora and tibiae with ice-cold PBS pH 7.4 using a 30G needle under sterile conditions. Bone marrow cells were suspended in MEM with L-glutamine supplemented with 10% HI-FBS, 50 U/ml penicillin, 50 µg/ml streptomycin, and 20 ng/ml M-CSF (R&D Systems; #416-ML) and plated into 6-well tissue culture plates. Cells were incubated at 37°C in 5% CO_2, and medium was replaced every other day until day 5, at which point the monocytes had differentiated into macrophages. Macrophages were studied at approximately 80% confluency.

***In vivo* poly (I:C) treatment of mice**

Mtor$^{+/+}$ and *Mtor*$^{A/A}$ mice (mostly C57BL6) (12 weeks old) fed a normal chow diet were fasted (5 hr) and injected with saline or poly (I:C) [10 mg/kg-BW] (2 hr). Spleen tissue was isolated, homogenized, and analyzed by western blotting. A motorized tissue homogenizer (Tissue Ruptor, Qiagen) was used to homogenize whole spleen in 1 ml ice-cold RIPA buffer that contained protease and phosphatase inhibitors. Lysate was incubated on ice (10 min), centrifuged at 13,200 rpm (15 min, 4°C), and supernatant was collected. A DC (Detergent Compatible) protein assay (Bio-Rad; #5000111) was used to standardize protein amount for immunoblot analysis.

Western blot editing, quantification, and statistical analysis

Western blot images were prepared for publication using Adobe Photoshop. Only the parameters levels, brightness, and contrast were employed to fine-tune exposure time and band sharpness. Importantly, these parameters were adjusted equivalently across the entire blot, and the final image shown reflects the raw image. In certain panels, thin dotted lines indicate excision of an irrelevant lane(s) from a western blot. Western blot signals were quantitated using FIJI to measure integrated densities of protein bands. The ratios of phosphorylated proteins over cognate total protein were calculated and normalized as indicated in the figure legends. Statistical significance was tested using paired Student's t-test assuming equal variances. Error bars represent either standard deviation (SD) or standard error of the mean (SEM), as indicated in the figure legend.

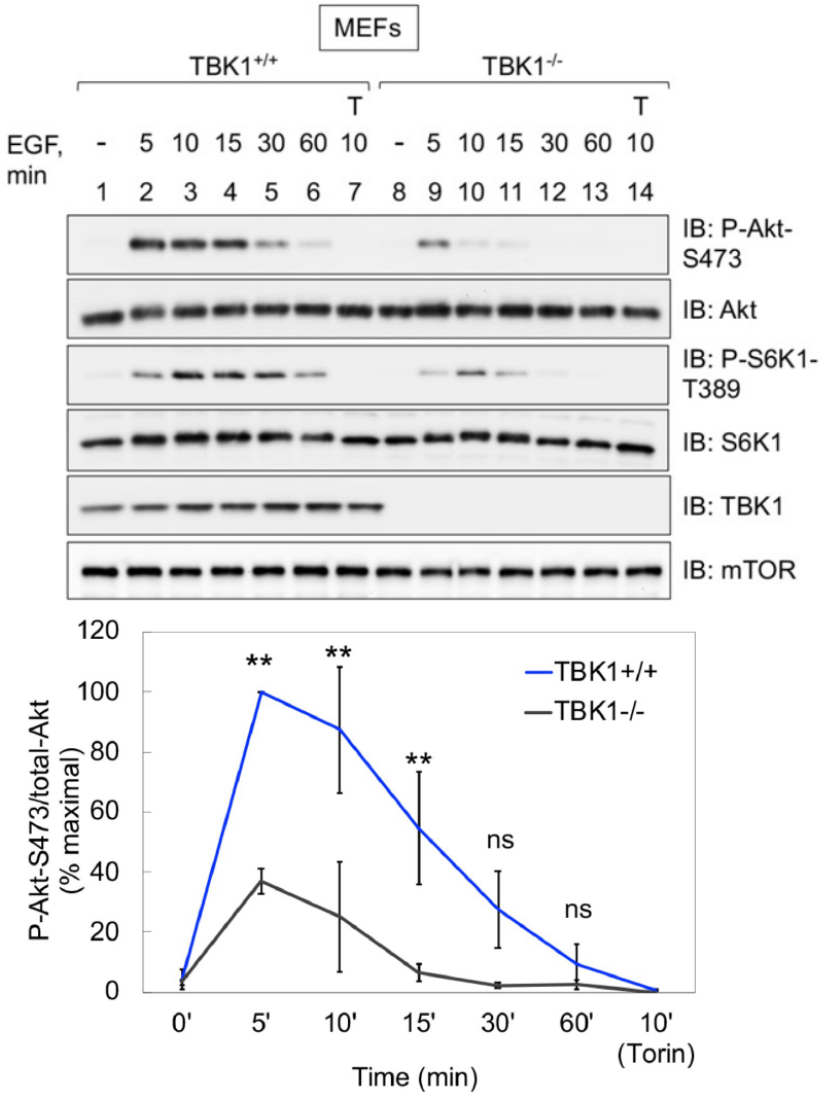

Figure 2.1: TBK1 null MEFs exhibit significantly impaired EGF-stimulated Akt S473 phosphorylation. TBK1$^{+/+}$ and TBK1$^{-/-}$ MEFs were serum starved overnight (20 hr), pre-treated with Torin1 (T) [100 nM] (30 min), and stimulated without (-) or with (+) EGF [50 ng/mL] for the indicated times (in minutes, min). Whole cell lysates (WCLs) were immunoblotted with the indicated antibodies. Graph: Quantification of results**.** Mean ratio +/- SD of Akt P-S473 over total-Akt from three independent experiments, normalized as percent of maximal (+EGF 5 min in TBK1$^{+/+}$ MEFs set to 100%). Statistical significance was measured using paired Student's t-test (assuming equal variances). **p < .01; "ns", not significant.

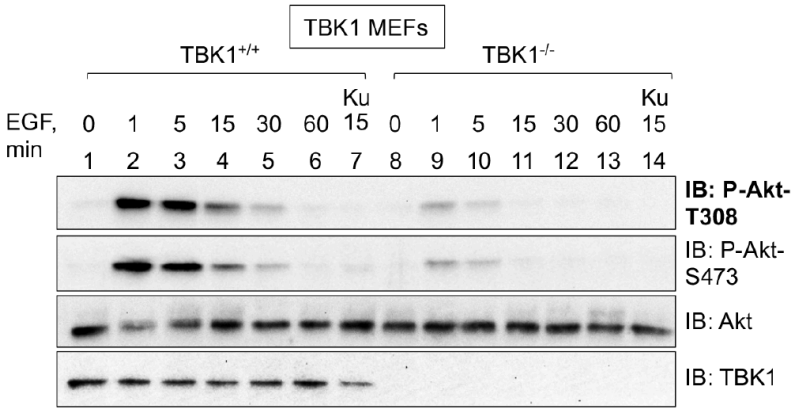

Figure 2.2: TBK1 null MEFs exhibit reduced EGF stimulated Akt T308 phosphorylation. TBK1$^{+/+}$ and TBK1$^{-/-}$ MEFs were serum starved overnight (20 hr), pre-treated with Torin1 (T) [100nM] (30 min), and stimulated without (-) or with (+) EGF [50 ng/mL] for the indicated times. Whole cell lysates were immunoblotted with the indicated antibodies.

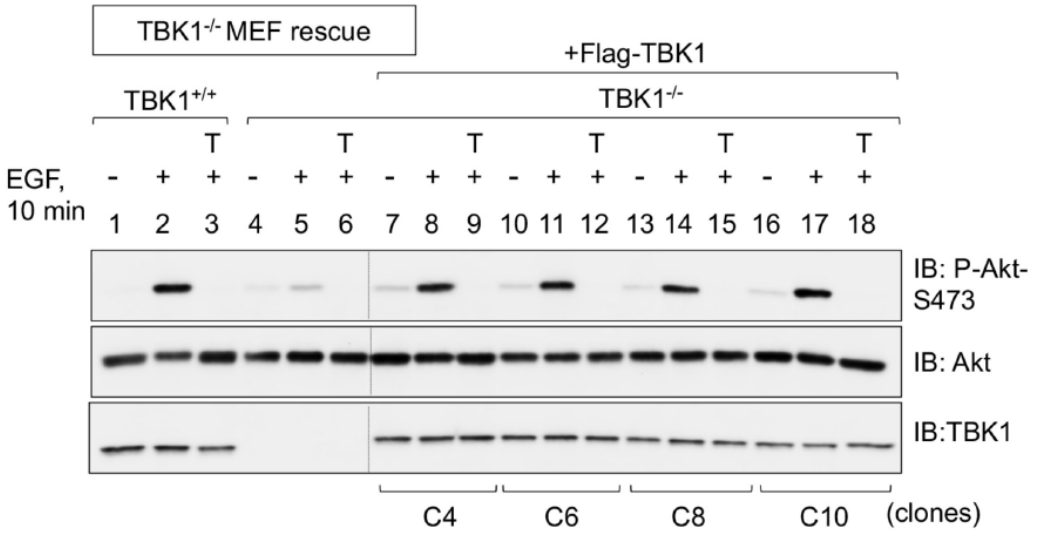

Figure 2.3: Stable expression of Flag-TBK1 WT rescues impaired EGF-stimulated Akt S473 phosphorylation in TBK1 null MEFs. TBK1$^{+/+}$ MEFs, TBK1$^{-/-}$ MEFs, and clones of TBK1$^{-/-}$ MEFs stably expressing Flag-TBK1 were serum starved, pre-treated with Torin1 [100 nM] (30 min), and stimulated without (-) or with (+) EGF [50 ng/mL] for 10 min. WCLs were immunoblotted with the indicated antibodies.

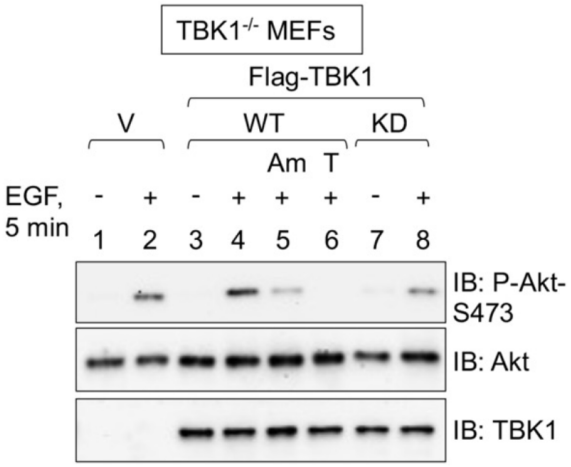

Figure 2.4: Stable expression of wild type (WT), but not kinase dead (KD), Flag-TBK1 rescues impaired EGF-stimulated Akt S473 phosphorylation in TBK1 null MEFs. Pools of drug resistant TBK1$^{-/-}$ MEFs stably expressing wild type or kinase dead (K38M) Flag-TBK1 were analyzed as in Figs 2.1 and 2.3.

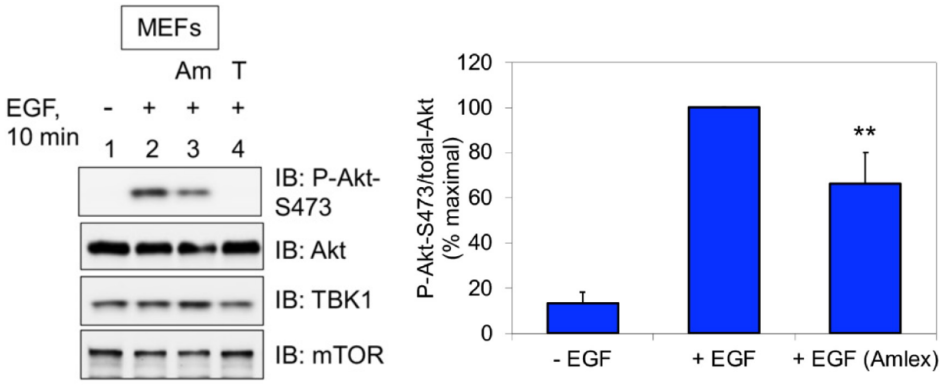

Figure 2.5: Amlexanox significantly blunts EGF-stimulated mTORC2 signaling in MEFs. TBK1$^{+/+}$ MEFs were serum starved overnight (20 hr), pre-treated with amlexanox (Am) [100 mM] (2 hr) or Torin1 (T) [100 nM] (30 min), and stimulated with EGF as in Fig 2.1. Whole cell lysates were immunoblotted with the indicated antibodies. Graph: Quantification of results. Mean ratio +/- SD of Akt P-S473 over total-Akt from five independent experiments, normalized as percent of maximal (+EGF 10 min set to 100%). Statistical significance was measured using Student's paired t-test (assuming equal variances). **p < .01 relative to TBK1$^{+/+}$ MEFs stimulated +EGF in the absence of amlexanox.

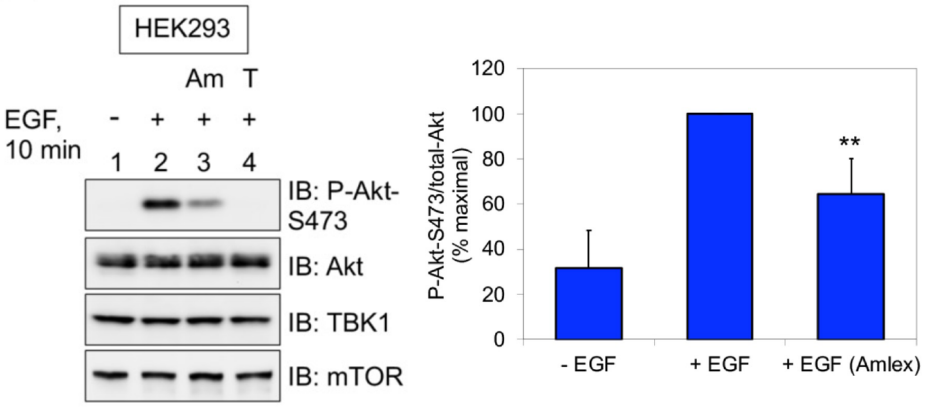

Figure 2.6: Amlexanox significantly blunts EGF stimulated mTORC2 signaling in HEK293 cells. HEK293 cells were analyzed as in Fig 2.5. Graph: Quantification of results. Mean ratio +/- SEM of Akt P-S473 over total- Akt were calculated from five independent experiments as in Fig 2.5.

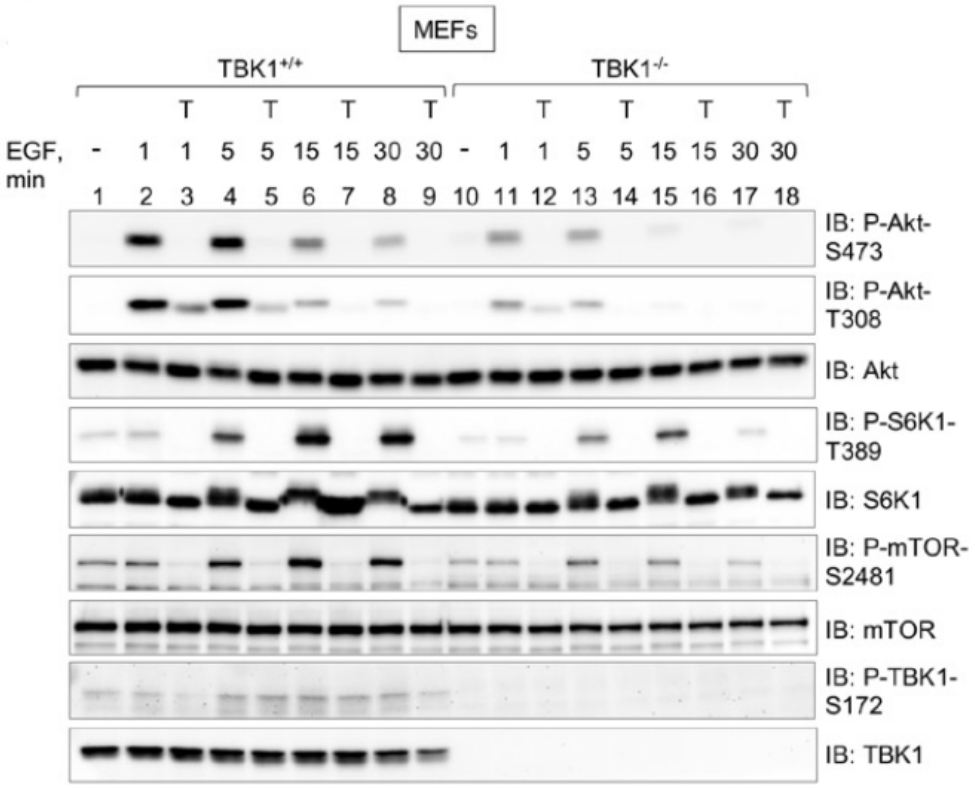

Figure 2.7: Torin1 profoundly reduces EGF stimulated Akt S473 phosphorylation over an EGF time course. TBK1$^{+/+}$ and TBK1$^{-/-}$ MEFs were serum starved overnight (20 hr), pre-treated with Torin1 (T) [100 nM] (30 min), and stimulated without (-) or with (+) EGF [50 ng/mL] for the times indicated (in minutes, min). Whole cell lysates (WCLs) were immunoblotted with the indicated antibodies.

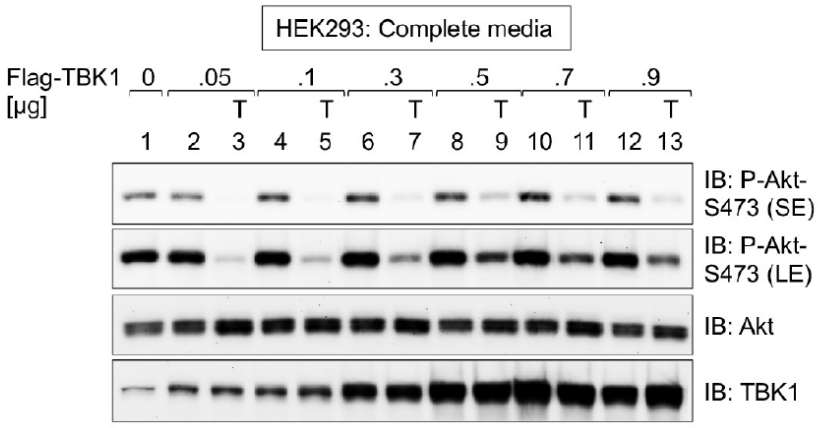

Figure 2.8: Overexpression of Flag-TBK1 WT increases Akt P-S473 in a largely Torin1-sensitive manner. HEK293 cells were transfected with increasing amounts of Flag-TBK1 ([0-0.9 mg] per 60 mm plate) in duplicate. ~24 hr post-transfection, cells in

complete media were treated with Torin1 (T) [100 nM] (30 min). WCLs were immunoblotted with the indicated antibodies.

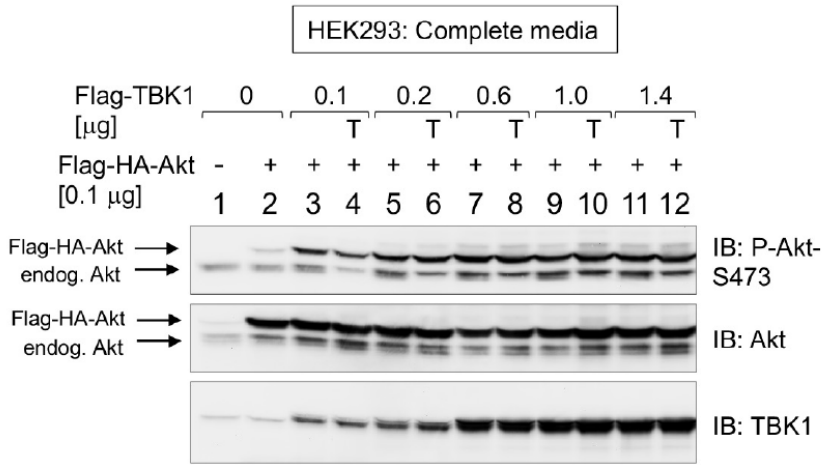

Figure 2.9: Co-expression of Flag-TBK1 WT and Flag-HA-Akt increases Akt S473 phosphorylation in a Torin1-insensitive manner. HEK293 cells were co-transfected with increasing amounts of Flag-TBK1 ([0-1.4 mg] per 60 mm plate) together with a constant amount of Flag-HA-Akt [0.1 mg] in duplicate. Cells were treated with Torin1 and analyzed as in Fig 2.8.

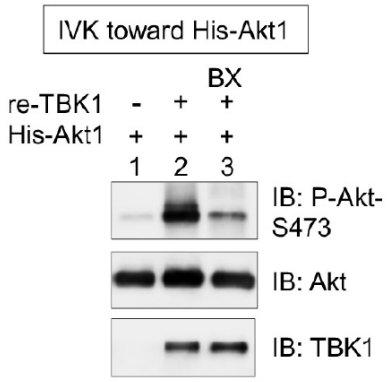

Figure 2.10: Recombinant TBK1 phosphorylates His-Akt1 *in vitro*. Recombinant, active TBK1 (re-TBK1) [50 ng] and His-Akt1 [50 ng] were incubated together in an *in-vitro* kinase (IVK) reaction with ATP at 30°C for 30 min, as indicated. The IVK reaction in lane 3 included pre-treatment with BX-795 (BX) [15 mM] for 30 min prior to initiation of the reaction with ATP. IVK reactions were immunoblotted with the indicated antibodies.

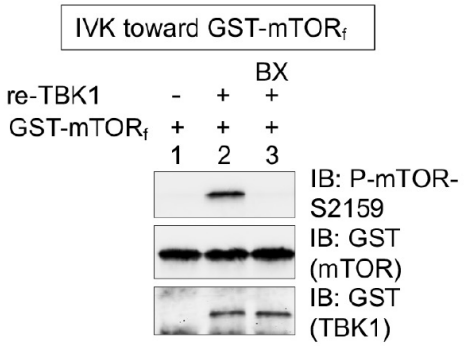

Figure 2.11: Recombinant TBK1 phosphorylates GST-mTOR$_f$ *in vitro*. Re-TBK1 [100 ng] was incubated with GST-mTORf [50 ng] at 30°C for 30 min, as indicated. As in Fig 2.10, the IVK reaction in lane 3 included pre-treatment with BX-795 (BX) [15 mM]. IVK reactions were immunoblotted with the indicated antibodies.

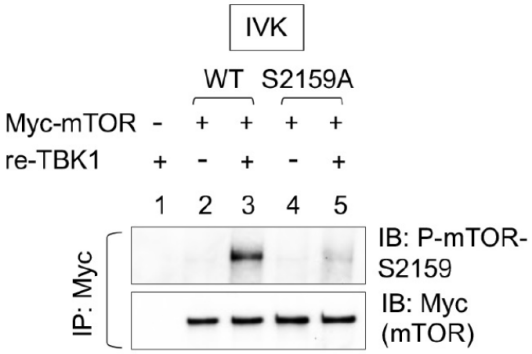

Figure 2.12: Recombinant TBK1 phosphorylates Myc-mTOR wild type but not S2159A *in vitro*. HEK293 cells were transfected with vector control (-), Myc-mTOR wild type (WT), or Myc-mTOR S2159A. mTOR was immunoprecipitated (IP) with Myc-9E10 antibody and subjected to IVK reactions with re-TBK1 [50 ng] per reaction, as in Figs 2.10 and 2.11. IVK reactions were immunoblotted with the indicated antibodies.

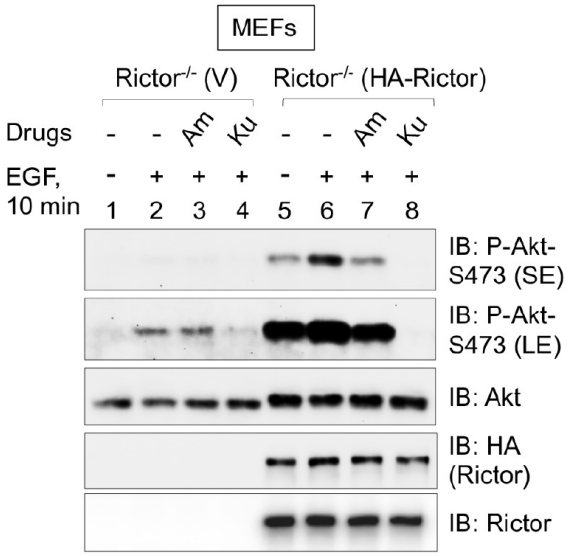

Figure 2.13: Ku-0063794 reduces, while amlexanox does not affect, EGF-stimulated Akt S473 phosphorylation in Rictor null MEFs stably expressing vector control. Rictor$^{-/-}$ MEFs rescued with vector control (V) or HA-Rictor were serum starved overnight (20 hr), pre-treated with amlexanox (Am) [100 mM] (2 hr) or Ku-0063794 (Ku) [100 nM] (30 min), and stimulated without (-) or with (+) EGF [50 ng/mL] for 10 min. Whole cell lysates (WCLs) were immunoblotted with the indicated antibodies. SE, short exposure; LE, long exposure.

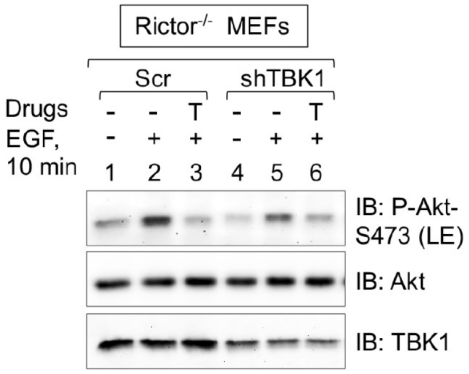

Figure 2.14: shRNA-mediated knockdown of TBK1 reduces EGF-stimulated Akt S473 phosphorylation in Rictor null MEFs. Rictor$^{-/-}$ MEFs were transduced with lentiviral particles encoding scrambled (Scr) shRNA or an shRNA targeting TBK1 and selected in puromycin. The MEFs were then serum starved overnight (20 hr), pre-treated with Torin1 (T) [100 nM] (30 min), and stimulated without (-) or with (+) EGF [50 ng/mL] for 10 min. WCLs were immunoblotted with the indicated antibodies

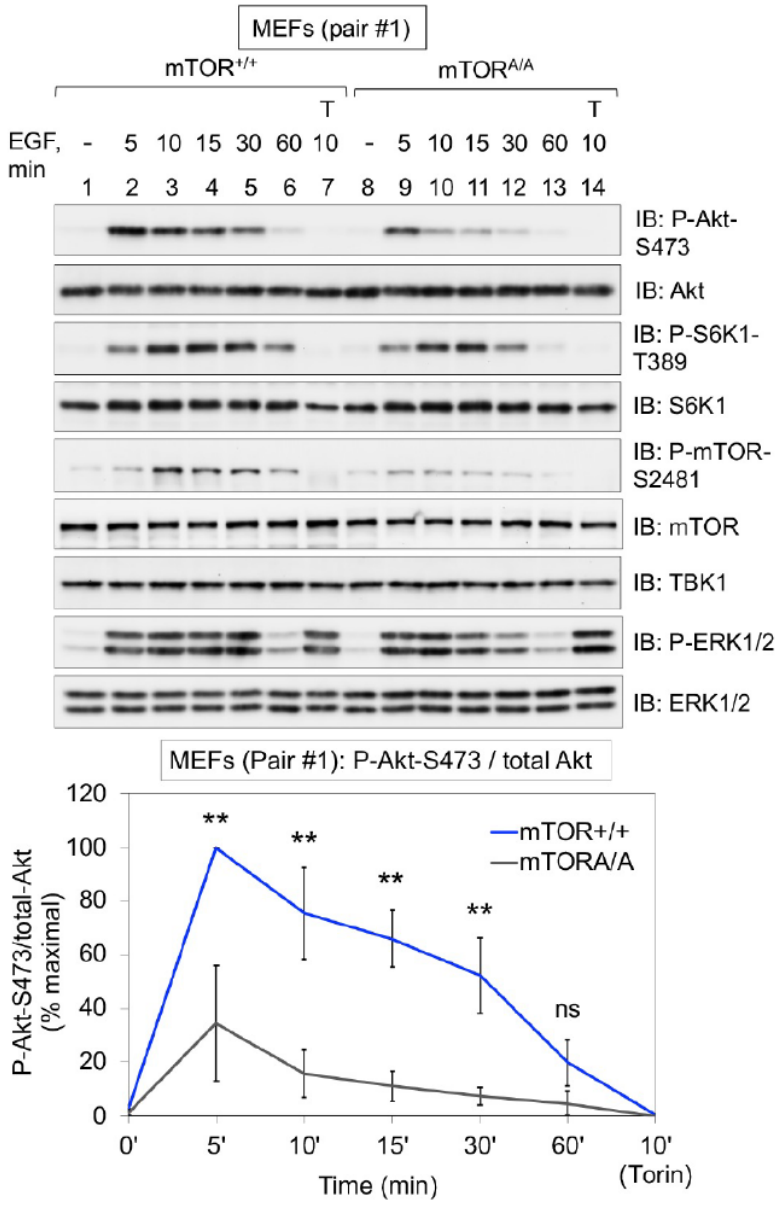

Figure 2.15: mTOR S2159A knock-in MEFs exhibit significantly impaired EGF-stimulated Akt S473 phosphorylation. Immortalized mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs (pair #1) were serum starved overnight (20 hr.), pre-treated with Torin1 (T) [100 nM] (30 min.), and stimulated without (-) or with (+) EGF [50 ng/mL] for the indicated times (in minutes, min). Whole cell lysates (WCLs) were immunoblotted with the indicated antibodies. Graph: Quantification of results. Mean ratio +/- SD of Akt P-S473 over total-Akt from four independent experiments, normalized as percent of maximal (+EGF 5 min. in mTOR$^{+/+}$ MEFs set to 100%). Statistical significance of differences was measured using Student's paired t-test (assuming equal variances). **p < .01; "ns", not significant.

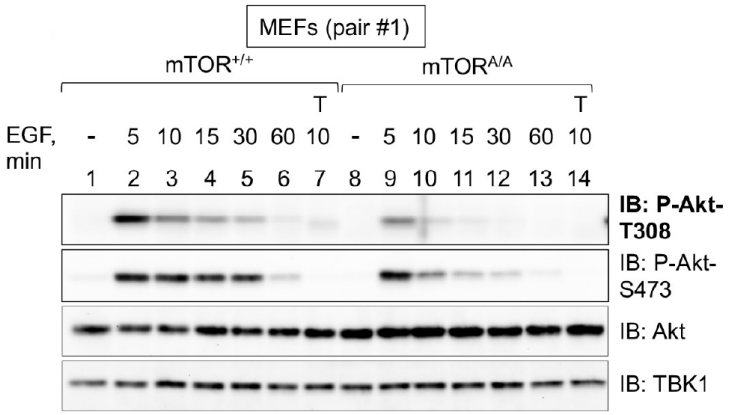

Figure 2.16: mTOR S2159A knock-in MEFs exhibit reduced EGF-stimulated Akt T308 phosphorylation. Immortalized mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs (Pair #1) were serum starved overnight (20 hr), pre-treated with Torin1 (T) [100nM] (30 min), and stimulated without (-) or with (+) EGF [50 ng/mL] for the indicated times. Whole cell lysates (WCLs) were immunoblotted with the indicated antibodies.

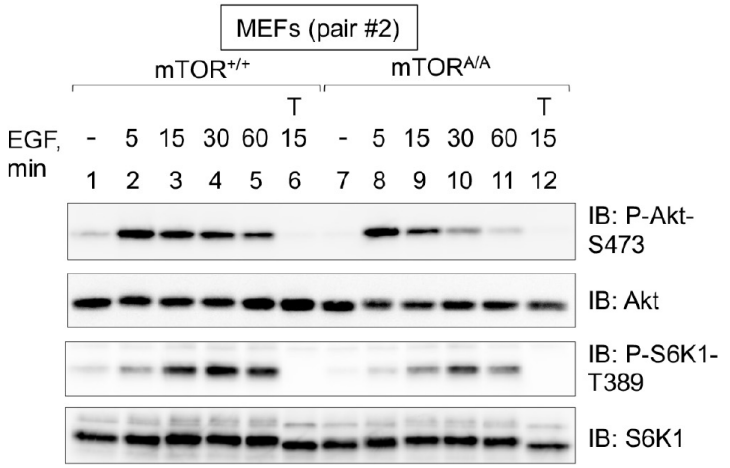

Figure 2.17: A second immortalized pair of wild mTOR S2159A knock-in MEFs exhibit impaired EGF-stimulated Akt P-S473. Immortalized mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs (pair #2) were treated as in Fig 2.15.

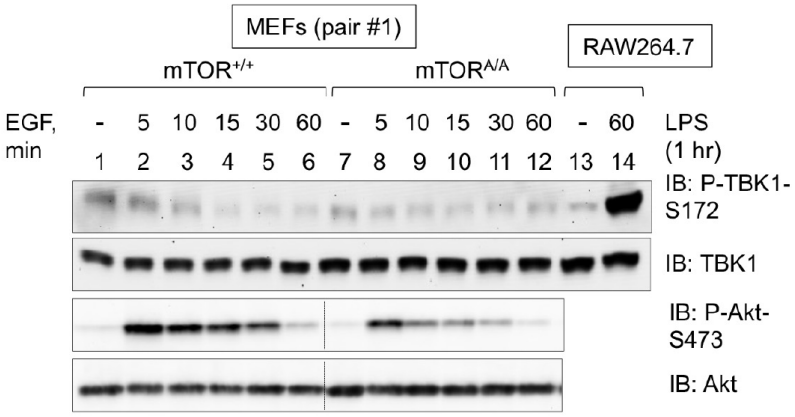

Figure 2.18: EGF does not increase TBK1 activation loop site (S172) phosphorylation in mTOR MEFs. mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs (pair #1) were serum starved and stimulated with EGF for the times indicated, as in Fig 2.15. RAW264.7 macrophages in complete media were stimulated without (-) or with (+) LPS [100 ng/mL] (60 min.) to serve as a positive control for TBK1 P-S172 western blotting. WCLs from mTOR MEFs and RAW264.7 macrophages were resolved on the same gel and immunoblotted with the indicated antibodies.

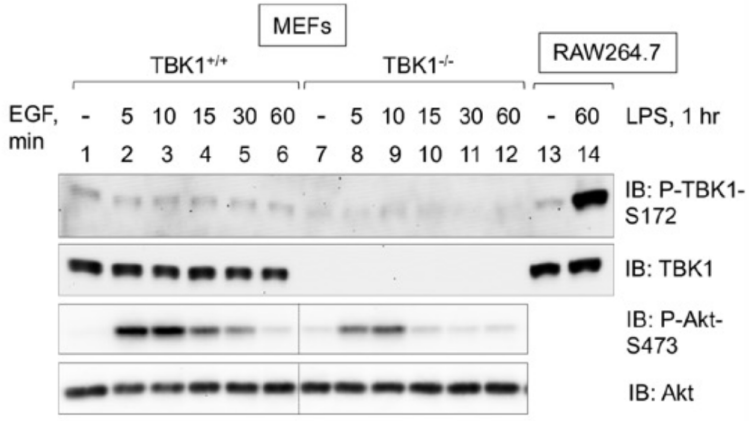

Figure 2.19: EGF does not increase TBK1 S172 phosphorylation in TBK1 wild type MEFs. TBK1$^{+/+}$ and TBK1$^{-/-}$ MEFs were serum starved overnight and stimulated with EGF for the indicated times, as in S2A. RAW264.7 macrophages in complete media (DMEM/FBS [10%]) were stimulated without (-) or with LPS [100 ng/mL] (1 hr) to serve as a positive control for TBK1 P-S172 western blotting. WCLs from MEFs and RAW264.7 macrophages were resolved on the same gel and immunoblotted with the indicated antibodies. Note that total protein amounts were not normalized within the two cell types.

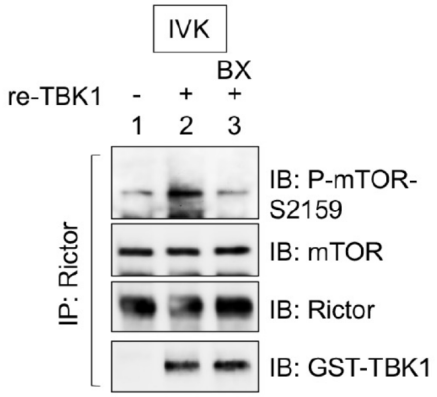

Figure 2.20: Recombinant TBK1 phosphorylates mTOR S2159 within mTORC2 *in vitro*. Rictor was immunoprecipitated from HEK293 cells and incubated with recombinant, active TBK1 (re-TBK1) [100 ng] for 30 min at 30°C. The IVK reaction in lane 3 was pre-treated with BX-795 (BX) [15 mM] for 30 min, as indicated.

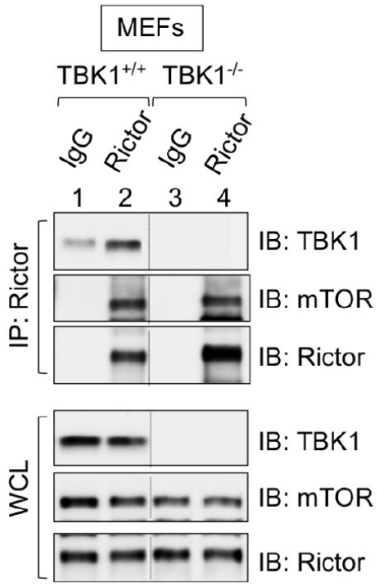

Figure 2.21: TBK1 co-immunoprecipitates with mTORC2 (Rictor/mTOR). Whole cell lysates from TBK1$^{+/+}$ and TBK1$^{-/-}$ MEFs cultured in complete media (DMEM/FBS) were incubated with Sepharose beads conjugated to either control IgG or anti-Rictor antibodies overnight at 4°C. The immunoprecipitates (IPs) and whole cell lysates (WCLs) were immunoblotted with the indicated antibodies.

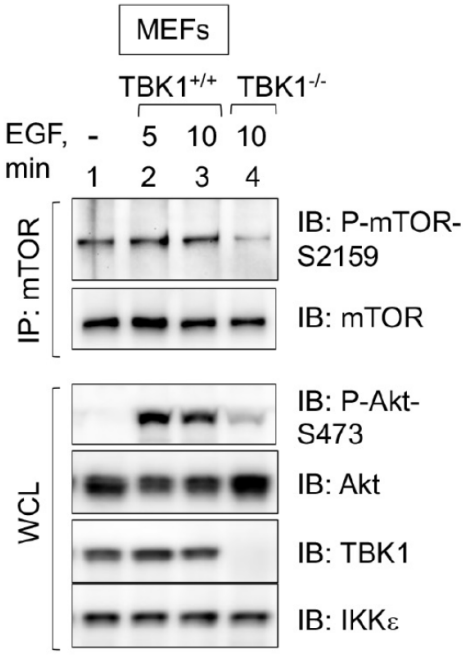

Figure 2.22: EGF does not increase mTOR S2159 phosphorylation in TBK1 wild type MEFs and mTOR S2159 phosphorylation is reduced in TBK1 null MEFs. mTOR was immunoprecipitated from TBK1$^{+/+}$ and TBK1$^{-/-}$ MEFs that had been serum starved overnight and stimulated with EGF [25 ng/mL] (10 min). IPs and WCLs were immunoblotted with the indicated antibodies.

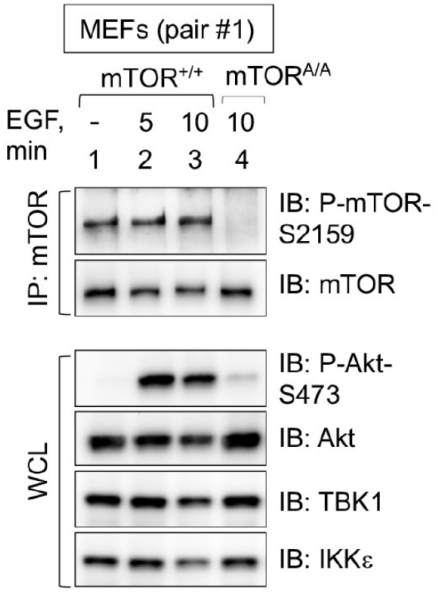

Figure 2.23: EGF does not increase mTOR S2159 phosphorylation in mTOR wild type MEFs and mTOR S2159A knock-in MEFs completely lack mTOR S2159 phosphorylation. mTOR was immunoprecipitated from mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs as in Fig 2.20. IPs and WCLs were immunoblotted with the indicated antibodies.

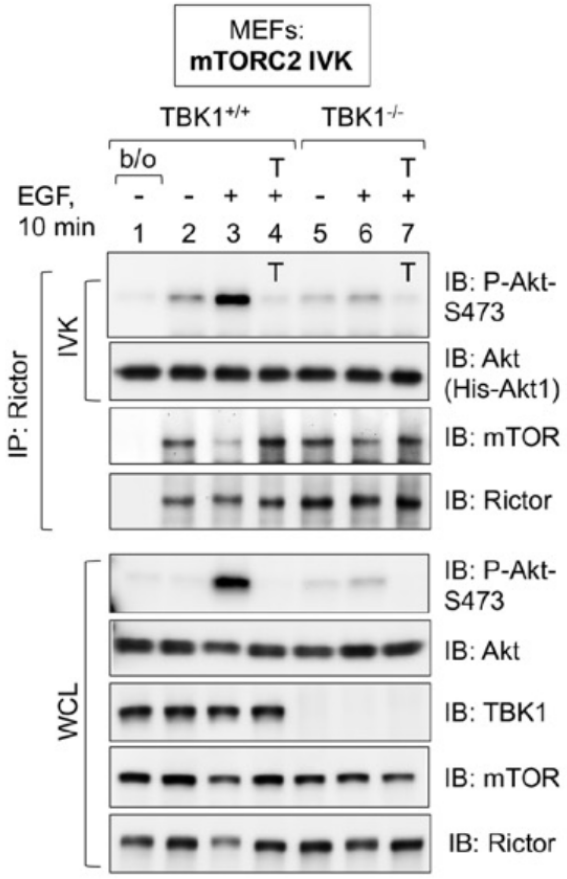

Figure 2.24: TBK1 is required for EGF to increase mTORC2 intrinsic catalytic activity toward His-Akt *in vitro*. Rictor was immunoprecipitated (IP) from TBK1$^{+/+}$ and TBK1$^{-/-}$ MEFs that had been serum starved overnight, pre- treated with Torin1 [100 nM] (30 min), and stimulated with EGF [50 ng/mL] (10 min). The immune complexes were washed and subjected to *in vitro* kinase (IVK) reactions with His-Akt1 [100 ng] substrate and ATP [500 mM] at 30°C for 30 min. Torin1 was included in the IVK reactions from cells pre-treated with Torin1, as indicated (T on the blot). IVKs and whole cell lysates (WCLs) were immunoblotted with the indicated antibodies.

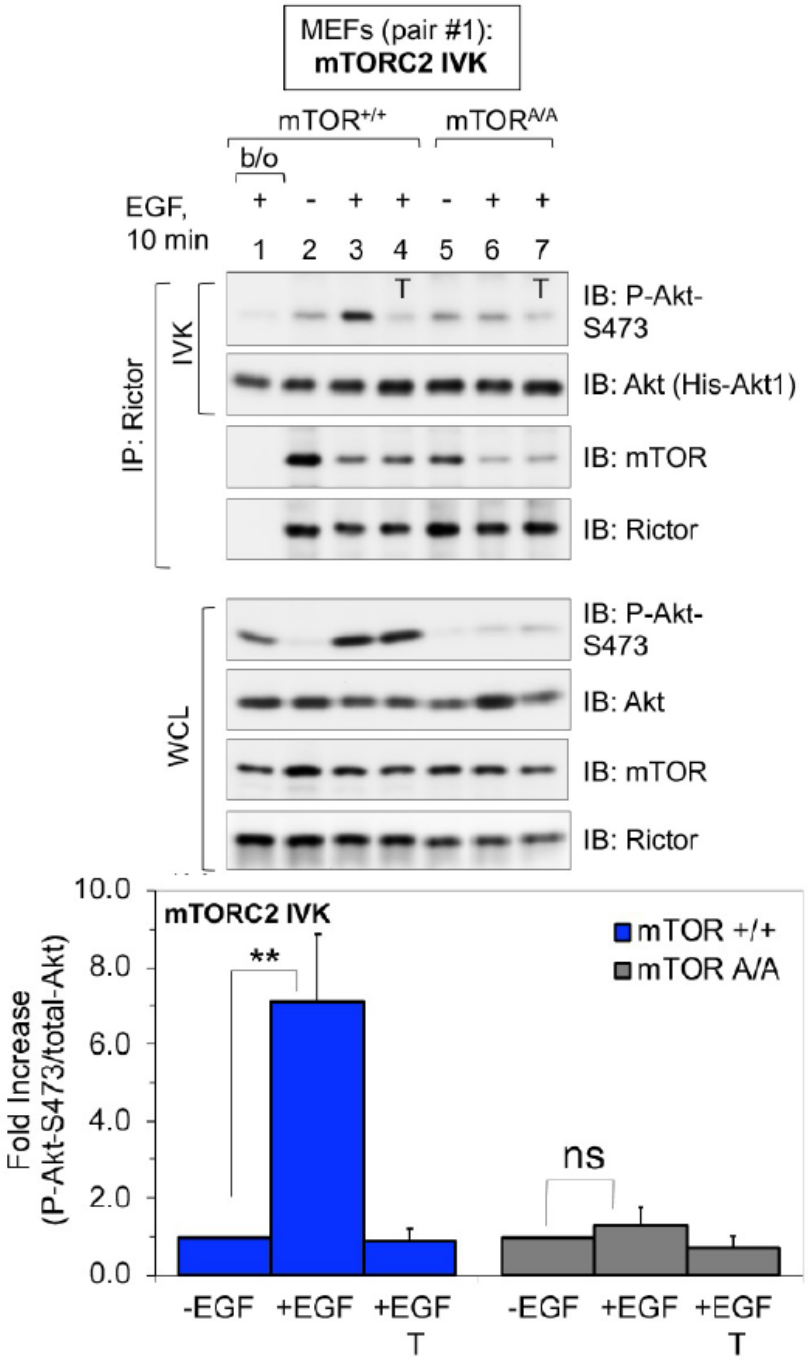

Figure 2.25: mTOR S2159 phosphorylation is required for EGF to increase mTORC2 intrinsic catalytic activity toward His-Akt *in vitro*. Rictor was immunoprecipitated from mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs (pair #1) that had been treated with EGF. IVK reactions were performed on the immune complexes and analyzed as in Fig 2.24, except that only certain IVK reactions, but not the cells, were treated with Torin1, as indicated (T on the blot). Graph: Quantification of results. Mean ratio +/- SEM of the fold increase in Akt P-S473 over total-Akt1 from four independent experiments, normalized within each genotype, setting the - EGF condition to 1.0. Statistical significance was measured using paired Student's t-test (assuming equal variances).

**p < .01, comparing -/+ EGF in $mTOR^{+/+}$ MEFs; “ns”, not significant, comparing -/+ EGF $mTOR^{A/A}$ MEFs.

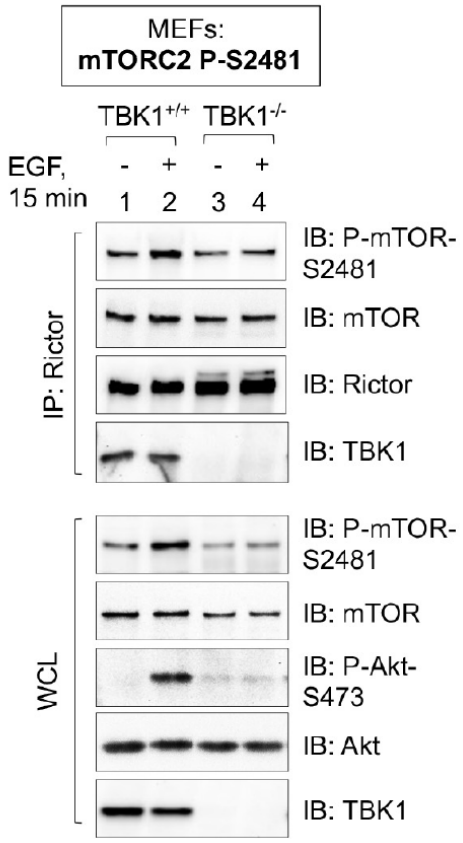

Figure 2.26: TBK1 is required for EGF to increase mTOR S2481 autophosphorylation within mTORC2. Rictor was immunoprecipitated from $TBK1^{+/+}$ and $TBK1^{-/-}$ MEFs that had been serum starved overnight and stimulated with EGF [50 ng/mL] (15 min), as in Fig 2.24. IPs and WCLs were immunoblotted with the indicated antibodies.

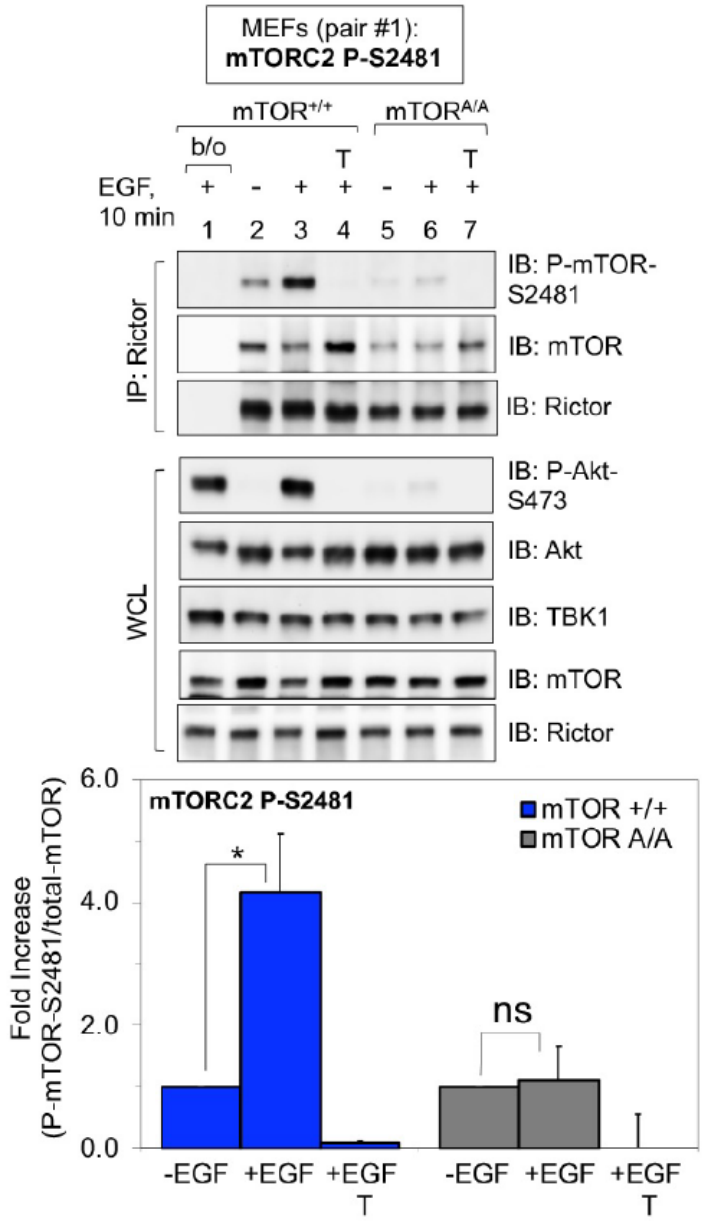

Figure 2.27: mTOR S2159 phosphorylation is required for EGF to increase mTOR S2481 autophosphorylation within mTORC2. Rictor was immunoprecipitated from mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs (pair #1) that had been treated with EGF and Torin1, as in Fig 2.24. IPs and WCLs were immunoblotted with the indicated antibodies. Graph: Quantification of results. Mean ratio +/- SEM of the fold increase in mTOR P-S2481 over total-mTOR from three independent experiments. *p < .05, comparing -/+ EGF in mTOR$^{+/+}$ MEFs; “ns”, not significant, comparing -/+ EGF in mTOR$^{A/A}$ MEFs

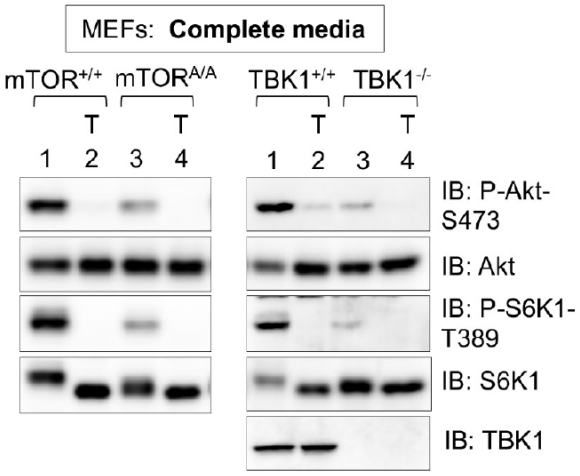

Figure 2.28: mTOR S2159 phosphorylation and TBK1 are required steady state mTORC2 and mTORC1 signaling in complete media. $mTOR^{+/+}$ vs. $mTOR^{A/A}$ MEFs (pair #1) and $TBK1^{+/+}$ vs. $TBK1^{-/-}$ MEFs were cultured in complete media (DMEM/FBS [10%]). At ~80% confluency, cells were re-fed with complete media for 1.5 hr, treated without or with Torin1 (T) [100 nM] (30 min), and lysed. Whole cell lysates (WCLs) were immunoblotted with the indicated antibodies.

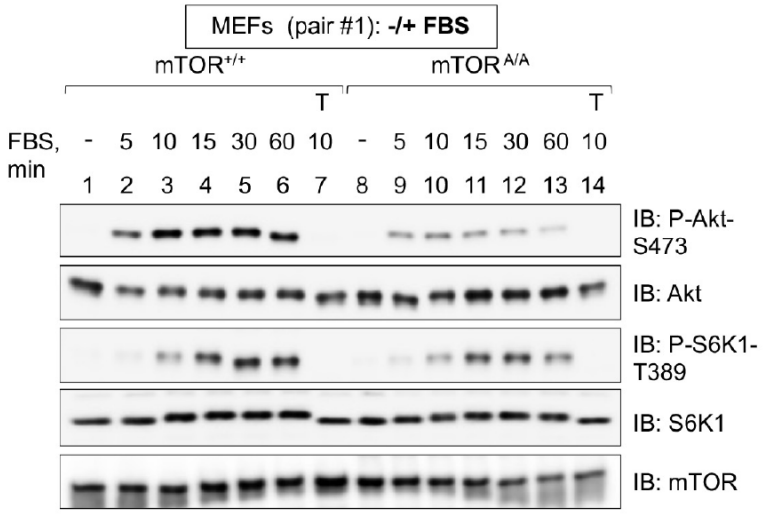

Figure 2.29: mTOR S2159 knock in MEFs exhibit profoundly reduced FBS-stimulated Akt S473 phosphorylation. $mTOR^{+/+}$ and $mTOR^{A/A}$ MEFs (pair #1) were serum starved overnight (20 hr), pre-treated with Torin1 (T) [100nM] (30 min.), and stimulated without (-) or with (+) FBS [10% final] for the indicated times. WCLs were immunoblotted with the indicated antibodies.

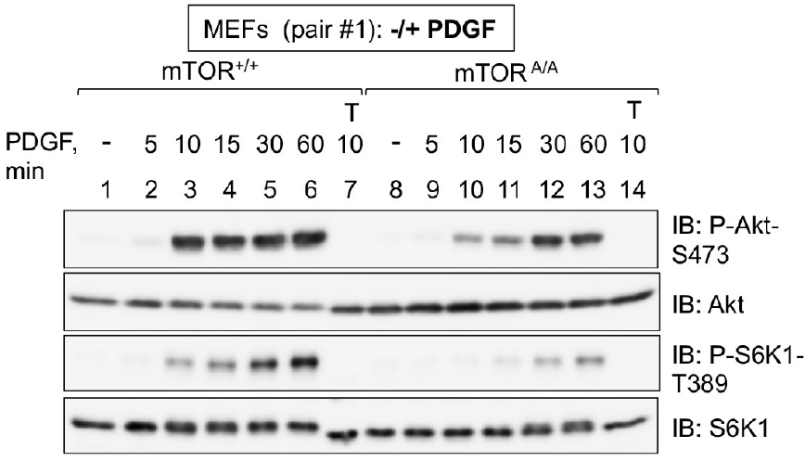

Figure 2.30: mTOR S2159 knock in MEFs exhibit profoundly reduced PDGF-stimulated Akt S473 phosphorylation. mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs (pair #1) were serum starved overnight and treated as in Fig 2.29, except they were stimulated with PDGF [10 ng/mL]. WCLs were immunoblotted with the indicated antibodies.

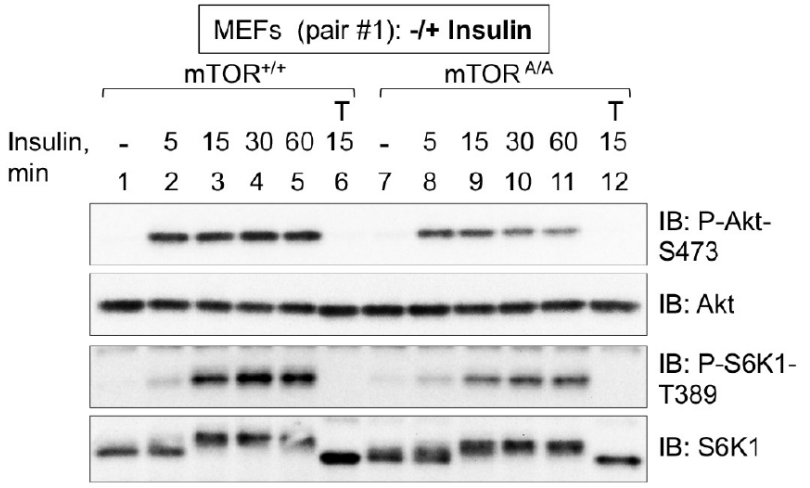

Figure 2.31: mTOR S2159 knock in MEFs exhibit profoundly reduced insulin-stimulated Akt S473 phosphorylation. mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs (pair #1) were serum starved overnight and treated as in Fig 2.29, except they were stimulated with insulin [100 nM]. WCLs were immunoblotted with the indicated antibodies.

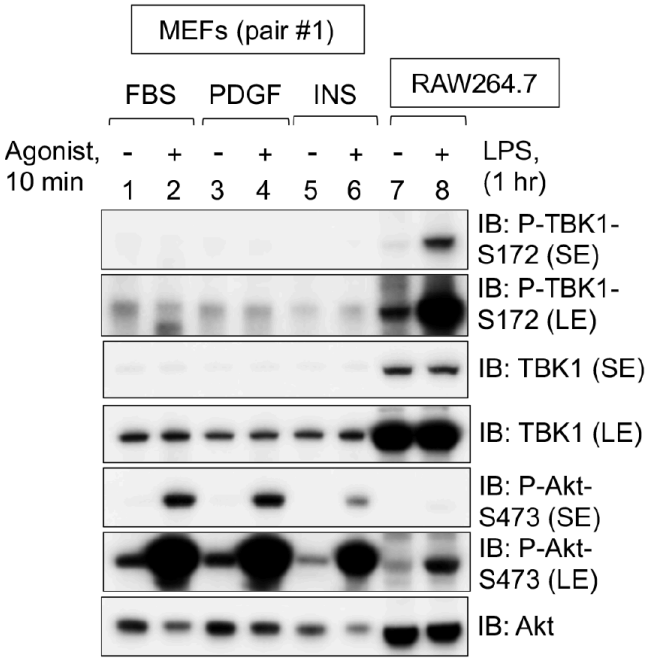

Figure 2.32: Diverse growth factors do not increase TBK1 P-S172 in mTOR MEFs. mTOR$^{+/+}$ and mTOR$^{A/A}$ MEFs (pair #1) were serum starved overnight and stimulated without (-) or with (+) FBS [10%], PDGF [10 ng/mL], or insulin [100 nM] (10 min). RAW264.7 macrophages in complete media (DMEM/FBS [10%]) were stimulated without (-) or with (+) LPS [100 ng/mL] (1 hr) to serve as a positive control for TBK1 P-S172 western blotting. Whole cell lysates from MEFs and RAW264.7 macrophages were resolved on the same gel and immunoblotted with the indicated antibodies. Note that total protein amounts were not normalized between the two cell types.

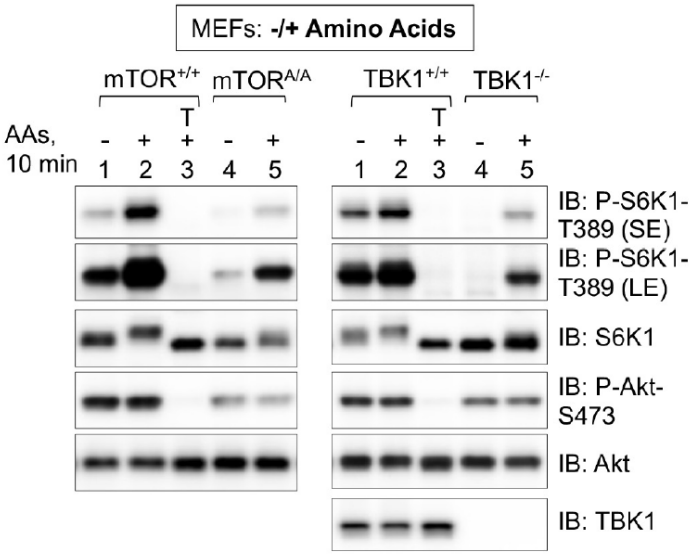

Figure 2.33: mTOR S2159 phosphorylation and TBK1 are required both for amino acid-induced mTORC1 signaling and overall mTORC2 signaling. $mTOR^{+/+}$ vs. $mTOR^{A/A}$ MEFs (pair #1) and $TBK1^{+/+}$ vs. $TBK1^{-/-}$ MEFs, cultured in complete media (DMEM/FBS [10%]), were amino acid deprived in DMEM lacking all amino acids but containing 10% dialyzed FBS (dFBS) (50 min). MEFs were then stimulated with a mixture of 1x total amino acids (pH 7.4) (10 min.). WCLs were immunoblotted with the indicated antibodies. SE, short exposure; LE, long exposure.

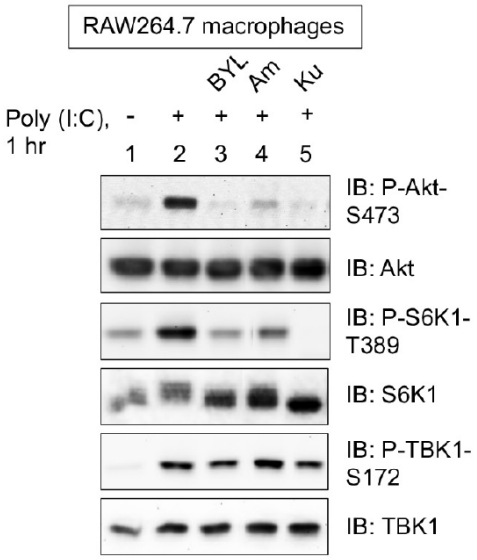

Figure 2.34: Inhibition of PI3K (with BYL-719), TBK1 (with amlexanox), or mTOR (with Ku- 0063794) reduces mTORC2 signaling in RAW264.7 macrophages in response to poly (I:C). RAW264.7 macrophages, cultured in complete media (DMEM/FBS [10%]), were pre-treated with BYL-719 [10 mM] (30 min), amlexanox [100 mM] (1 hr), or Ku-0063794 [100 nM] (30 min) and stimulated without (-) or with (+)

poly(I:C) [30 mg/mL] (60 min). Whole cell lysates (WCLs) were immunoblotted with the indicated antibodies.

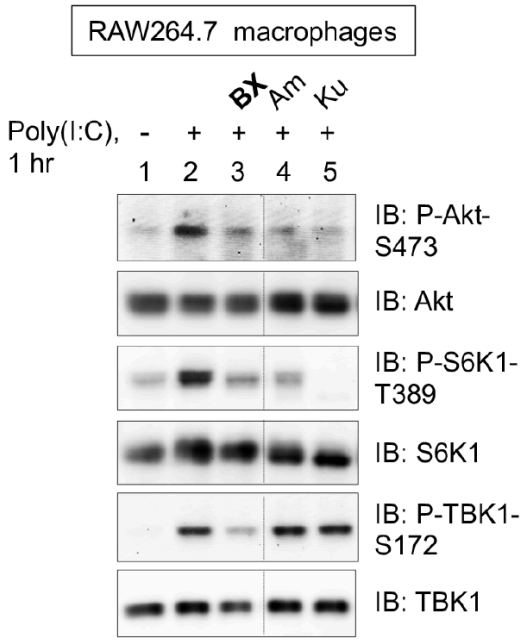

Figure 2.35: Inhibition of the TBK1/IKKε with BX-795 reduces mTORC2 signaling in RAW264.7 macrophages in response to poly(I:C). RAW264.7 macrophages, cultured in complete media (DMEM/FBS [10%]), were pre- treated with the TBK1/IKKε inhibitor BX-795 (BX) [10 mM] (30 min) as well as amlexanox (Am) [100 mM] (1 hr) and Ku-0063794 (Ku) [100 nM] (30 min) and stimulated without (-) or with (+) poly(I:C) [30 mg/mL] (1 hr.). Whole cell lysates were immunoblotted with the indicated antibodies.

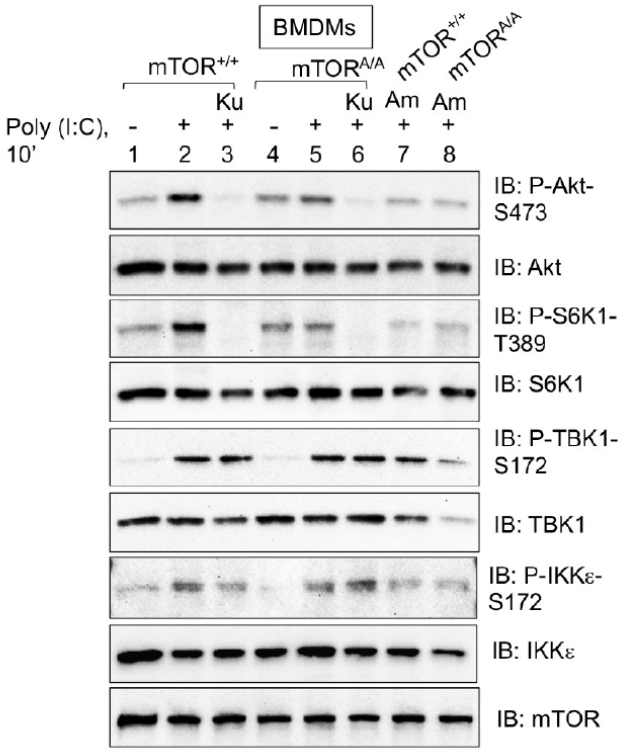

Figure 2.36: mTOR S2159 phosphorylation and TBK1 activity both promote mTORC2 signaling in primary BMDMs in response to poly(I:C). Primary bone marrow derived macrophages (BMDMs) derived from *Mtor*$^{+/+}$ and *Mtor*$^{A/A}$ mice were cultured in complete media (DMEM/FBS [10%]), pre-treated with Ku-0063794 [100 nM] (30 min) or amlexanox [100 mM] (1 hr), and stimulated without (-) or with (+) poly(I:C) [30 mg/mL] (10 min). WCLs were immunoblotted with the indicated antibodies.

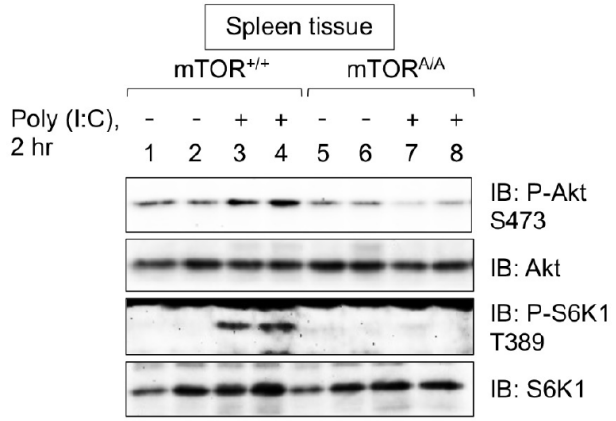

Figure 2.37: mTOR S2159 phosphorylation is required for mTORC2 signaling in mouse spleen tissue in response to poly(I:C) treatment *in vivo*. *Mtor*$^{+/+}$ and *Mtor*$^{A/A}$ mice were fasted 5 hr and injected intraperitoneally with poly(I:C) [10 mg/kg-BW] (2 hr). Spleen tissue was isolated, homogenized, and analyzed by western blotting with the indicated antibodies.

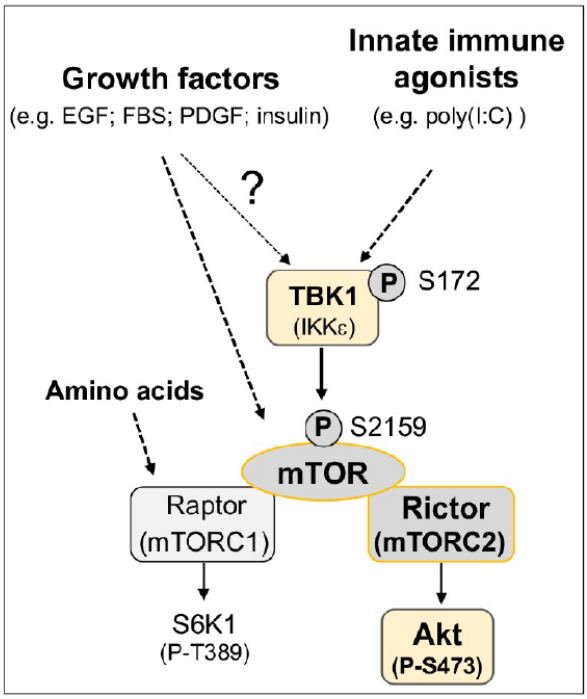

Figure 2.38: Model. See text for details.

Chapter 3

Conclusion

Collectively, these data demonstrate that TBK1 functions as a novel direct upstream activator of mTORC2. Through site specific phosphorylation of mTOR-S2159 within mTORC2, TBK1 provides an essential input in parallel to growth factor receptor signaling to promote mTORC2 intrinsic catalytic activity and downstream signaling to Akt. MEFs genetically lacking TBK1 display significantly reduced EGF-stimulated mTORC2 signaling, and inhibition of TBK1 with amlexanox significantly reduced EGF-stimulated mTORC2 signaling in MEFs and HEK293 cells. MEFs harboring an mTOR-S2159A mutation also displayed significantly impaired EGF-stimulated mTORC2 and mTORC1 signaling. Endogenous TBK1 and mTORC2 interact in intact cells, TBK1 phosphorylated mTOR-S2159 within mTORC2 *in vitro*, TBK1 knockout MEFs display reduced mTOR-S2159 phosphorylation, and $mTOR^{A/A}$ MEFs completely lack mTOR-S2159 phosphorylation. mTOR-S2159 phosphorylation not only promotes mTORC2 and mTORC1 signaling in response to EGF, but also FBS, PDGF, and insulin, indicating that TBK1-mediated mTOR phosphorylation likely represents a general mechanism mediating growth factor activation of mTOR signaling. In contrast, TLR-3 engagement by viral- or synthetic-derived dsRNA (i.e. poly (I:C)) activates TBK1 by increasing activation loop phosphorylation on TBK1 P-S172, which in turn promotes mTORC2 and mTORC1 signaling. The fact that growth factors do not activate TBK1 in MEFs, but

TBK1-activating innate immune agonists do in macrophages, implies that TBK1 assumes diverse roles/functions depending on the cell type, context, and stimulus.

This idea is supported by the fact that other groups have reported that growth factors activate TBK1. First, Ou et al concluded that EGF activates TBK1 in MEFs based on data that TBK1 immunoprecipitated from EGF-stimulated MEFs increased P-S473 on recombinant His-Akt1 *in vitro*. This data is weak, because it lacked essential controls, namely a stimulated sample pre-treated with a TBK1 inhibitor as well as a no antibody negative control (199). Interestingly, that was the only experiment in the entire paper reporting on a role for growth factors in increasing TBK1 kinase activity, and there was no assessment of TBK1 P-S172 in MEFs stimulated with EGF or other growth factors. This, coupled with our data demonstrating that EGF, FBS, PDGF, and insulin fail to increase TBK1 P-S172 in TBK1 and mTOR MEFs suggests that growth factors likely do not activate TBK1 in MEFs. However, this does not rule out the possibility that growth factors activate TBK1 in other cell types/contexts. Indeed, Zhu et al demonstrated that growth factors (EGF, FBS, and insulin) increase TBK1 P-S172 in human lung adenocarcinoma cell lines and that EGF increases TBK1 P-S172 through the adaptor molecule TBKBP1 (296). Mechanistically, TBK1 interacted with TBKBP1 to engage a signaling complex nucleated by the growth factor responsive kinase, protein kinase C theta (PKCθ), and the adaptor, CARD10, which leads to PKCθ-mediated activation of TBK1 in response to growth factors (296). Interestingly, Zhu did not report on TBK1 P-S172 status or whether or not TBKBP1 participates in TBK1 activation in MEFs stimulated with growth factors, likely because growth factors do not activate TBK1 in MEFs. However, these same MEFs lacking TBK1 exhibited impaired EGF-

stimulated mTORC1 and mTORC2 signaling, precisely consistent with our data indicating an important role for TBK1 in growth factor-stimulated mTORC1 and mTORC2 signaling. This suggests that either the basal kinase activity of TBK1 or a kinase-independent function of TBK1, such as a scaffold function, promotes growth factor-stimulated mTORC1 and 2 signaling in MEFs. Our data demonstrating that wild type, and not kinase dead TBK1, rescues EGF-stimulated mTORC1 and mTORC2 signaling in $TBK1^{-/-}$ MEFs suggests that the kinase activity of TBK1 profoundly contributes to growth factor-mediated activation of mTOR signaling. Moreover, our data demonstrating that mTOR-S2159 phosphorylation is detectable in MEFs, is reduced in MEFs lacking TBK1 or MEFs harboring an mTOR-S2159A mutation, and does not increase in response to EGF strengthens the plausible conclusion that, while EGF, FBS, PDGF, and insulin do not increase P-TBK1-S172, basal TBK1 kinase activity is necessary and sufficient for growth factors to robustly activate mTORC2.

Zhu et al also reported that, while shRNA-mediated knockdown of TBKBP1 in A549 lung cancer cells impairs TBK1 activation by EGF, TBKBP1 reduction has no effect on TBK1 activation by TBK1-activating innate immune agonists such as bacterial LPS and poly (I:C) (296). This suggests that, depending on the cell type, context and stimulus, TBK1 engages different adaptors to facilitate its activation in discrete cellular locations. Similar to the case concerning growth factors, no data were presented investigating a role for TBKBP1 in TBK1 activation in MEFs in response to TBK1-activating innate immune agonists, likely because TBKBP1 is also dispensable for these stimuli to activate TBK1. However, consistent with our data, TBK1 knockout MEFs exhibit impaired mTORC1 and mTORC2 signaling in response to innate immune

agonists. The ability of TBK1 to engage different adaptors in different cell types and contexts warrants further investigation, as it will provide additional clarity regarding potential cell type- and stimulus-specific roles for known and unknown TBK1 adaptors, as well as providing more concrete evidence to explain our data demonstrating that growth factors do no increase P-TBK1-S172. Based on the collective data, a viable hypothesis is that in MEFs TBKBP1 is dispensable for TBK1 activation by growth factors and innate immune agonists, likely attributed to differences in cell type (MEFs vs oncogenic Kras-driven lung cancer cells) or context (transformed vs oncogenic). Also, the fact that lung cancer is among the many cancer types strongly reliant on aberrant growth factor receptor signaling for proliferation and survival buttresses this hypothesis. Lastly, this likely provides a rational explanation for why TBK1 knockout in MEFs impairs growth factor-stimulated mTORC2 signaling, while growth factor-stimulated mTORC2 signaling in lung cancer cells appears to be TBK1 independent.

Numerous TBK1 interacting adaptors have been identified and characterized as critical participants in innate immune signal propagation following cellular detection of microbial stimuli. TBK1 engages several distinct adaptors via its C-terminal adaptor binding domain (located within the coiled-coil region of the scaffold/dimerization domain) to orchestrate the innate immune response, including TANK, NAP1, and the TRAF adaptors (200,329). The extent to which adaptors participate in TBK1-mediated signal transduction in other contexts such as growth factor receptor signaling remains elusive. In addition to Zhu, Uhm et al reported that TBK1 functions as a key kinase as part of an adaptor-like complex known as the exocyst complex, where, in response to insulin-stimulation, it phosphorylates Exo84 to enable trafficking of the essential glucose

transporter GLUT4 to the plasma membrane (282). These represent perhaps the only reports of TBK1 directly engaging other adaptors or adaptor-like complexes to transduce signals outside of the context of innate immune signaling. Thus, deciphering the mechanistic details of TBK1 involvement in crosstalk with pathways outside of innate immunity may require the identification of new adaptor molecules. Moreover, adaptor-mediated recruitment of TBK1 remains an important regulatory mechanism for achieving locally restricted activation of TBK1 to, as a way to circumvent aberrant TBK1 activity.

Cooper et al recently reported that TBK1, through association with a complex comprised of mTORC1 and mTORC1 regulatory proteins, promotes amino acid-stimulated mTORC1 signaling, in part by activating Akt. This is consistent with our data demonstrating that, while amino acids increase mTORC1 signaling in TBK1 knockout and $mTOR^{A/A}$ MEFs, both basal and amino acid-stimulated mTORC1 signaling are profoundly reduced in these cells. Interestingly, Cooper et al reports that in TBK1 knockout MEFs or wild type MEFs or cancer cell lines treated with TBK1 inhibitors, Akt P-S473 (mTORC2 signaling) is also reduced. Thus, they render the conclusion that amino acids increase both Akt T308 and S473 phosphorylation in a TBK1-dependent manner. This starkly contrasts with our data in TBK1 knockout MEFs, which demonstrate reduced basal Akt P-S473 relative to wild type MEFs that is not increased upon amino acid stimulation. While the reasons for these differences remain unclear, the unresponsiveness of Akt P-S473 to amino acids is entirely consistent with mTOR dogma that mTORC2 does not participate in amino acid sensing (58,300,312-314,316).

In addition, analysis of 94 non-small cell lung cancer (NSCLC) lines, through

whole exome sequencing and gene enrichment statistical analysis, identified genes involved in epithelial to mesenchymal transition (EMT) to be strongly correlated with oncogenic KRas-harboring NSCLC subtypes that are reliant on TBK1 for survival (295). EMT is characterized by a loss of epithelial cell features such as E-cadherin expression and apical-basal polarity and a gain in mesenchymal features such as increased vimentin expression, motility, and cytoskeletal reorganization. Motility is a key metastatic phenotype that tumor cells employ to invade and occupy organs distant from the organ of origin. In particular, these NSCLC subtypes exhibited increased expression of the pro-mesenchymal gene *ZEB1* (295). The identification of mutations in additional Ras family genes (*H-Ras, N-Ras*, *PIK3CA*, and *BRAF*) and Akt-mTORC1 pathway components suggests that cooperation among these mutant proteins, in the context of oncogenic Ras, is responsible for the dependence of oncogenic Ras-driven cancers on TBK1. The correlation between TBK1 and pro-mesenchymal genes like *ZEB1* in the context of oncogenic KRas makes it enticing to hypothesize that TBK1 promotes metastasis of these types of cancers through EMT. Several independent studies link TBK1 to metastasis and EMT in various cancers, including those driven by oncogenic Ras.

Platelet-induced conditioning of EMT characteristics in mouse and human breast cancer cell lines was found to depend on TBK1 activity, and genetic depletion of TBK1 inhibited breast cancer metastasis to the lungs of mice in a xenograft tumor model (330). Also, in breast cancer cells lacking sufficient levels of the microRNA, miR-200c, which constrains EMT through transcriptional suppression of EMT-promoting genes, TBK1 expression was increased and associated with decreased apoptosis and

resistance to irradiation therapy (331-334). Also, TBK1 was demonstrated to promote radiation induced EMT in A549 lung adenocarcinoma cells through ZEB1 (335). TBK1 activity was increased in NRas-mutant melanoma cells, genetic depletion of TBK1 impaired the migration and invasive properties of N-Ras mutant melanoma cells, and through cooperation with MEK, TBK1 was found to promote an antiapoptotic program (336). The integration of genetic and chemical screens and patient data focused on BRAF-mutant melanomas lead to the identification of a subtype that selectively depends on TBK1 for survival (337). Collectively, these studies strongly support the notion that TBK1 promotes an EMT phenotype and concomitant metastasis of various types of cancers, including those harboring oncogenic Ras mutations.

Several studies have also linked mTORC2 to EMT associated migration through reorganization of the cell cytoskeleton. Perhaps the earliest reports describing a role for mTORC2 in regulation of the cell cytoskeleton emanated in 2004. Indeed, Rictor depletion in NIH-3T3 cells perturbed F-actin polymerization and depolymerization, effectively stifling the formation and extension of actin-mediated cellular processes known as lamellipodia (299). In addition, mTORC2 was shown to promote GTP loading of Rac1, a Rho family G-protein and important regulator of F-actin dynamics associated with motility and migration in various cell types (299,338-342). mTORC2 was also reported to promote actin reorganization and attachment to the plasma membrane through modulation of protein kinase C alpha (PKCα) phosphorylation (26,162,343). A few studies have also investigated a role for mTORC2 in modulation of the actin cytoskeletal architecture *in vivo* (162,343,344). Genetic perturbation of mTORC2 in the mouse central nervous system and in purkinje cells suppressed Akt P-S473 and P-

T308, expression of protein kinase C isoforms (PKCα, β2, γ, and ε), and activation of the PKC substrates, GAP-43 and MARCKS, two key mediators of actin cytoskeletal dynamics (162,343). This same group also reported that ablation of Rictor reduced overall neuron size and disrupted morphology (162). Abrogation of mTORC2 function in the mouse hippocampus curtailed formation of filamentous actin, Rac1-GTPase activity, and structural integrity of neuronal synapses, ultimately affecting long term memory function (344).

mTORC2 has also been linked to metastasis in various cancer types through the Rac1 Rho-GTPase. mTORC2 promotes migration and invasion of breast cancer cells harboring *HER2* gene amplifications through bimodal mechanisms, one involving mTORC2 activation of PKCα, which suppresses the Rac1 inhibitor RhoGDI2, a guanine dissociation inhibitor that binds GDP-bound Rac1 and impedes the release of GDP, and another involving mTORC2 activation of Akt, which promotes Rac1 activity through the Rac1 guanine nucleotide exchange factor (GEF), Tiam1 (148). Genetic or pharmacological inhibition of mTORC2 (and mTORC1) impaired migration of colorectal carcinoma cells and promoted a mesenchymal to epithelial shift, concomitant with reduced Rac1 activity, and importantly, genetic perturbation of mTORC2 and mTORC1 profoundly inhibited the broad formation of metastases throughout the entire mouse (345). In cancer cells driven by isocitrate dehydrogenase (IDH) mutations, such as glioma tumors, inhibition of mTORC2 reduced Rac1 activity and filamentous actin-mediated lamellipodia (346). These studies implicate mTORC2-mediated activation of Rac1 in promotion of cancer cell migration, an initial step preceding tumor EMT and metastasis. Collectively, these studies support a functional role for mTORC2 in

promoting actin-mediated cell migration in both normal and tumorigenic contexts, through PKC and Rho family G proteins.

Lastly, proteomic interactome analysis recently identified mTORC2 as a direct effector of oncogenic Ras (145,146). mTORC2 interaction with the effector binding domain of oncogenic Ras, but not wild type Ras, through the mTOR kinase domain, increased mTORC2 catalytic activity and transcription of proliferation genes, and promoted *in vivo* growth of tumors addicted to oncogenic Ras (145,146). The fact that both TBK1 and mTORC2 have been independently linked to EMT and oncogenic Ras warrants future investigation into a role for the TBK1-mTORC2 axis in promoting oncogenic Ras-driven tumor cell migration/metastasis, proliferation, and survival. Importantly, this work may reveal additional downstream effectors of oncogenic Ras that are more amenable to targeting, in combination therapy approaches, for treating notoriously difficult to eradicate oncogenic Ras-driven cancers.

While our data demonstrate that mTORC2 represents an important signaling conduit through which TBK1 increases Akt P-S473, several independent groups reported that TBK1 functions as a direct Akt P-S473 kinase (177,199,297). Joung et al reported that endogenous TBK1 and Akt interact in macrophages stimulated with the TLR-3 and 4 agonists, poly (I:C) and bacterial LPS, respectively (297). Joung et al also demonstrated that genetic perturbation of Akt impaired poly (I:C)- and LPS-induced IRF-3 activation and interferon beta production, and genetic depletion of TBK1 impaired Akt P-S473 (297). It is perplexing that this group failed to entertain the likelihood that mTORC2 functioned as the critical intermediate linking TBK1 to Akt P-S473, especially considering that mTORC2 has been the prominent Akt P-S473 kinase since 2005

(11,30,347). Furthermore, our data demonstrating significant Torin-sensitivity of Akt P-S473 in poly (I:C)-stimulated macrophages strengthens the conclusion that mTORC2 profoundly contributes to TBK1-mediated activation of Akt.

Ou et al demonstrated that TBK1 directly phosphorylates Akt S473 and T308 in various transformed and cancer cell lines, and genetic depletion of TBK1 in pancreatic and breast cancer cell lines significantly reduced Akt-mediated cell viability and tumor progression in xenograft mouse tumor models (199). Also, the absence of PDK1 (an Akt T308 kinase and major effector of PI3K signaling) attenuated TBK1-mediated Akt P-T308, and in MEFs, TBK1 was essential for increasing Akt P-S473 and P-T308 in response to EGF and glucose, but not insulin (199). Also, Xie et al demonstrated that TBK1 (and IKKε) directly phosphorylate Akt on S473 and T308 *in vitro*, and growth factor activation of Akt is blunted in MEFs devoid of both TBK1 and IKKε, suggesting that the residual growth factor stimulated Akt P-S473 and P-T308 observed in our TBK1 knockout MEFs is likely attributed to the presence of IKKε (177). Also, inhibition of PI3K with pharmacological agents profoundly blunted insulin stimulated Akt activation in Rictor knockout MEFs overexpressing TBK1, and both TBK1 and IKKε were markedly increased in cellular membrane fractions (177).

The major proposition from both of these groups that TBK1 and IKKε represent Akt S473 and T308 kinases contrasts with our compelling data that instead, TBK1 (and likely IKKε) employ mTORC2 to increase Akt phosphorylation at these sites. Our observation that gross overexpression of TBK1 increases Akt P-S473 independently of mTOR suggests that, either direct phosphorylation of Akt by TBK1/ IKKε represents a parallel mechanism modestly contributing to Akt activation under normal physiologic

conditions where TBK1/ IKKε expression are appropriately regulated, or this represents a potentially non-physiological mechanism that dominates in pathologic contexts where TBK1/IKKε are overexpressed. Also, Xie and Ou's report that Akt P-S473 remains sensitive to PI3K inhibition in the presence of TBK1 overexpression supports the idea that TBK1 exerts its regulation on Akt through mTORC2. Lastly, Xie's observation that Rictor knockout MEFs display reduced profoundly blunted growth factor-stimulated Akt P-S473 and P-T308 that is only partially rescued with TBK1 overexpression, again, buttresses the conclusion that TBK1 largely requires mTORC2 to promote Akt activation. Numerous independent studies previously identified several candidate Akt P-S473 kinases, with the major caveat that a majority of these studies based their conclusions on overexpression of these proposed candidates (see chapter 1). Also, our data demonstrating that TBK1 phosphorylates two distinct substrates with dissimilar consensus motifs *in vitro* suggests that TBK1 functions as a promiscuous kinase, capable of phosphorylating any substrate in close proximity. Therefore, overexpression of TBK1 and IKKε likely dysregulates canonical regulatory mechanisms, such as subcellular localization, which enable TBK1 to directly phosphorylate Akt-S473 and T308 in a proximal-permissive manner. Indeed, lower levels of TBK1 overexpression promotes mTORC1 signaling to S6K1, while higher levels of TBK1 overexpression inhibit mTORC1, likely due to dysregulated TBK1 activity (44).

While we report that TBK1-mediated mTOR-S2159 phosphorylation also promotes growth factor-stimulated mTORC1 signaling, several independent groups have reported on contexts where TBK1 either activates or suppresses mTORC1. Cooper et al employed chemogenomic and interactome approaches to identify a

context-specific role for TBK1 in promotion of oncogenic Ras-driven lung cancer cell growth through activation of mTORC1 (295). Our group demonstrated that mTOR-S2159 promotes mTORC1 signaling in response to growth factors (EGF) and innate immune agonists (LPS and poly I:C), which promoted INFβ production (44). Conversely, a study demonstrated that TBK1-mediated phosphorylation of the mTORC1 partner protein, Raptor, on S877 suppressed mTORC1 signaling under various conditions, including in response to LPS-stimulated TLR-4 activation and in human lung adenocarcinoma cell line A549 and colon carcinoma cell line HCT116 (50). Notably, the latter contrasts with Zhu et al's report that TBK1 activates mTORC1 in response to growth factors in A549 cells. Another study reported that ablation of Trex and correlative chronic activation of TBK1 via the cGAS-STING pathway suppressed mTORC1 signaling (348). For unknown reasons, these collective discrepancies remain unclear, but highlight that the diverse roles TBK1 plays in regulating mTOR seems context dependent. Moreover, this warrants further elucidation of the elusive upstream activating mechanisms governing TBK1 in these contexts.

Activation of TBK1 requires initial K63-linked poly-ubiquitination of its ubiquitin-like domain (ULD) and subsequent phosphorylation of its activation loop on S172 (293). The mechanism(s) underlying this phosphorylation event remain unclear. However, current proposed models center on the ability of the C-terminal domain of TBK1 to exclusively bind different adaptors, depending on the cellular stimulus/context. Adaptor binding recruits TBK1 to discrete subcellular locations, leading to high local concentrations of the kinase and either one of two outcomes. The first proposed outcome involves transautophosphorylation where high local concentrations of TBK1

homodimers enables them to phosphorylate each other on S172 (207,227,349). The second outcome involves adaptor-mediated recruitment of TBK1 and direct phosphorylation by an unknown kinase (206). While the role of adaptor-mediated recruitment of TBK1 in the context of innate immunity is well-established, evidence for its role in growth factor-mediated signaling to TBK1 remains minimal. However, Zhu demonstrated a requirement for the adaptor, TBKBP1 (also known as SINTBAD), in growth factor-mediated activation of TBK1 in A549 lung adenocarcinoma cells via P-TBK1-S172 (296). Interestingly, shRNA-mediated knockdown or knockout of TBKBP1 or TBK1 reduced EGF-stimulated P-TBK1-S172 and mTORC1 signaling, but left mTORC2 signaling unperturbed (296). While the latter contradicts TBK1's role in EGF-stimulated mTORC2 signaling in lung cancer cells, it does emphasize the role cellular context might play in dictating the degree to which TBK1 functions and the downstream substrates it might engage and activate.

Elucidation of a role for the TBK1-mTOR axis in the growth, proliferation, and survival of oncogenic Ras-driven cancers will include the following future experiments. CRISPR/Cas9 will be employed to generate mTOR S2159A knock in mutations in relevant cancer cell lines known to be addicted to oncogenic Ras (i.e. NSCLC and pancreatic cancer cell lines) to determine if preventing mTOR-S2159 phosphorylation stifles growth, proliferation, and/or increases apoptosis in these cell lines. This would initially support the hypothesis that TBK1-mediated mTOR-S2159 phosphorylation promotes oncogenic Ras-driven tumorigenesis. Also, our CRISPR $mTOR^{+/+}$ and $mTOR^{A/A}$ mice will be crossed with appropriate genetic mouse models of oncogenic Ras-driven cancer, the hypothesis being that in mice homozygous for mTOR-S2159

and bearing oncogenic Ras and other contextually essential mutations necessary to recapitulate the cancer of interest, will exhibit reduced in vivo tumor growth and burden and metastasis (if relevant for the cancer type). In conjunction with Zhu et al 2019, it is likely that an oncogenic Ras-driven cancer type strongly reliant on growth factor receptor activation of TBK1 such as NSCLC will display reduced tumorigenicity in vivo. This would be a pivotal result that would lay the foundation for future experiments geared toward potential therapeutic targeting of TBK1-mTOR in oncogenic Ras-driven cancers. While the mTOR field is massive and continues to evolve, our mechanistic link between TBK1 and mTORC2 not only contributes critical knowledge to the upstream regulation of mTORC2, but it also highlights a potentially druggable pathway for diseases driven by aberrant TBK1 and mTORC2 activity such as oncogenic Ras-driven cancers and metabolic diseases.

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