Novel Regulation of mTOR Complex 1 Signaling by Site-Specific mTOR Phosphorylation

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Cell and Developmental Biology)
in The University of Michigan
2012

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“Our true mentor in life is science.”

(“Hayatta en hakiki mürşit ilimdir.”)

Mustafa Kemal Atatürk, the founder of Turkish Republic

Acknowledgements

This thesis would not have been possible without the enormous support and encouragement of my Ph.D. advisor Diane C. Fingar. I am sincerely thankful for her research insight and guidance during my Ph.D. training. I would like to express my great appreciation to Billy Tsai, Anne B. Vojtek, Ken Inoki, and Patrick J. Hu for serving on my thesis committee, whose advice and help have been valuable. I would like to thank all members of the Fingar, Tsai, and Verhey labs for the discussion in our group meetings. I also would like to thank the CDB administrative staff, especillay Kristen Hug, for their help.

I thank Ed Feener for performing the liquid chromatography tandem mass spectrometry analysis to identify novel phosphorylation sites on mTOR and Steve Riddle for performing the *in vitro* kinome screen to identify candidate kinases for mTOR S2159 phosphorylation site.

I thank Brian Magnuson, Hugo A. Acosta-Jaquez, and Jennifer A. Keller for contributing to my first-author paper published in Molecular and Cellular Biology Journal in 2011. I also thank Diane C. Fingar for generating data presented in Figure 2.2 Part D.

I cannot finish without saying how grateful I am with my family; my father Halil İbrahim Ekim, my mother Meral Ekim, my sister Çiğdem Ekim, and most importantly my husband Eser Üstünel for their unconditional love and support.

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List of Abbreviations

4E-BP1	Eukaryotic translation initiation factor 4E binding protein
5'TOP	5'track of polypyrimidines
m^7GTP	7-Methylguanosine 5'-triphosphate
AGC	Protein kinase A, Protein kinase G and Protein kinase C
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate activated kinase
AP-1	Activator protein 1
ATG	Autophagy-related protein
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
CBP	CREB binding protein
CD	Cluster of differentiation
Cdc	Cell division control
CNS	Central nervous system
Deptor	DEP domain-containing mTOR-interacting protein
DNA-PK	DNA dependent protein kinase
dsRNA	Double stranded RNA
eEF2	Eukaryotic elongation factor 2
eEF2K	Eukaryotic elongation factor 2 kinase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF	Eukaryotic translation initiation factor
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase

FAK	Focal adhesion kinase
FDA	Food and Drug Administration
FIP200	FAK family-interacting protein of 200 kDa
FKBP12	FK506-binding protein 12-kDa
FRAP	The FKBP-12-rapamycin associated protein
FRB	FKBP12-rapamcyin binding
GAP	GTPase activating protein
GEF	GTP exchange factor
Glut4	Glucose transporter type 4
GSK3β	Glycogen synthase kinase 3 beta
GTP	Guanosine 5' triphosphate
HEAT	Huntingtin, elongation factor 3, protein phosphatase 2A (PP2A), and the yeast PI3-kinase TOR1
HIF-1α	Hypoxia-inducible factor 1-alpha
HM	Hydrophobic motif
hVPS34	Mammalian vacuolar protein sorting 34 homologue
IB	Immunoblot
IGF	Insulin-like growth factor
IκBα	I-kappa-B-alpha
IKK	Inhibitor of nuclear factor-kappa B- kinase
ILK	Integrin-linked kinase
IP	Immunoprecipitate
IRS	Insulin receptor substrate
IVK	*In vitro* kinase
JAK	Janus kinase
KOG1	Kontroller of growth protein 1
LKB1	Liver kinase B1
LPS	Lipopolysaccharide
LRL	Leucine rich repeat
MAP4K3	MAP kinase kinase kinase kinase 3
MAPK	Mitogen-activated protein (MAP) kinase

MAPKAPK-2	MAP kinase-activated protein kinase 2
MBH	Mediobasal hypothalamus
MEF	Mouse embryonic fibroblast
MEK	MAPK/ERK kinase
mLST8	mTOR associated lethal with Sec 13 8/G protein β subunit-like
MP1	MEK-binding partner 1
mRNA	Messenger RNA
mSin1	Mammalian *s*tress-activated protein kinase interacting protein
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
MyD88	Myeloid differentiation primary response protein
NDRG1	N-Myc downregulated gene 1
NEMO	NF-kappa-B essential modulator
NF-1	Neurofibromatosis-related protein
NF-kB	Nuclear factor-kappa B
NLS	Nuclear localization signal
PA	Phosphatidic acid
PAK1	p21-activated kinase 1
PAMP	Pathogen associated molecular patterns
pDC	Plasmacytoid dendritic cell
PDCD4	Programmed cell death protein 4 PDCD4
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide-dependent kinase-1
PGC1α	Peroxisome-proliferator-activated receptor gamma coactivator-1 alpha
PI3K	Phosphatidylinositol 3 kinase
PIKK	Phosphoinositide kinase-related kinase
PIP_3	Phosphatidylinositol-3,4,5-trisphosphate
PKCα	Protein kinase c alpha isoform
PLD	Phospholipase D

Pol	DNA polymerase
Poly(I:C)	Polyinosinic:polycytidylic acid
PPARγ	Peroxisome proliferator-activated receptor gamma
PRAS40	Proline rich Akt substrate of 40 KDa
P-Rex1	Phosphatidylinositol-3,4,5-trisphosphate -dependent Rac exchange factor
PRR5/Protor	Proline-rich protein 5
PTEN	Phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase
Rac 1	Ras-related C3 botulinum toxin substrate 1
RAFT1	Rapamycin and FKBP12 target
RAPT	Rapamycin target
Raptor	Regulatory associated protein of mTOR
Ras	Rat sarcoma
RCC	Renal cell carcinoma
rDNA	Ribosomal DNA
Redd1	Regulated in development and DNA damage response
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
RNAi	RNA interference
ROS	Reactive oxygen species
RR	Rapamycin resistant
rRNA	Ribosomal RNA
S6K	S6 kinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEP	Sirolimus effector protein
SGK1	Serum/Glucocorticoid Regulated Kinase 1
SH3	SRC Homology 3 Domain
SKAR	S6K1 Aly/REF-like target
SLC7A5/SLC3A2	Solute carrier family 7, member 5/solute carrier family 3, member 2

SREBP	Sterol response element binding protein
STAT3	Signal transducer and activator of transcription 3
Tssk3	Testis-specific serine/threonine-protein kinase 3
TBK1	TANK-binding kinase 1
TIF	Transcription initiation factor
TIR	Toll/interleukin-1 receptor domain
TM	Turn motif
TNFα	Tumor necrosis factor alpha
TOS	TOR signaling
TRAM	TRIF-related adaptor molecule
tRNA	Transfer RNA
TRIF	Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN-β
TSC	Tuberous sclerosis complex
TTRAP	Trafficking protein particle
UBF	Upstream-binding factor
ULK	Unc-51-like kinase
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
YY1	Yin-yang 1

Abstract

Aberrant regulation of the kinase mTOR (mammalian target of rapamycin) within mTOR complex 1 (mTORC1) contributes to several pathologic states including cancer, type II diabetes, and cardiovascular diseases. mTORC1 functions as an environmental sensor to promote protein and lipid synthesis, cell growth/size, and cell proliferation in response to growth factors and nutrients. While diverse stimuli regulate mTORC1 signaling, the direct molecular mechanisms by which mTORC1 responds to these signals remain poorly defined. By employing LC-MS/MS and phospho-specific antibodies, we identified S2159 and T2164 as novel sites of mTOR phosphorylation. Mutational analyses of these phosphorylation sites indicate that mTOR S2159 and T2164 phosphorylation cooperatively promotes mTORC1 signaling to its well-characterized substrates S6K1 and 4E-BP1. Mechanistically, mTOR S2159 and T2164 phosphorylation modulates the interactions of mTOR with its partner proteins within mTORC1 and increases intrinsic kinase activity, which promotes biochemical signaling, cell growth, and cell cycle progression.

To identify the upstream kinase(s) that directly mediate mTOR S2159 phosphorylation, we performed an *in vitro* kinome screen. This approach identified TBK1 and IKKε, kinases best known to mediate immune signaling in response to viral and bacterial infection, as mTOR S2159 kinases. We confirmed the kinome screen by showing that TBK1 and IKKε phosphorylate mTOR *in vitro* and upon overexpression in intact cells. Moreover, TBK1 and IKKε interact with mTOR, and TBK1 or IKKε null mouse embryonic fibroblasts (MEFs) display impaired mTOR S2159 phosphorylation. In addition, TBK1 null MEFs show reduced S6K1 phosphorylation, suggesting that TBK1 promotes mTORC1 signaling. Activation of toll-like receptor 3 (TLR3) and TLR4 signaling pathways

that promote TBK1 and IKKε activity also increases downstream mTORC1 signaling to S6K1 and 4E-BP1.

Oncogenic Ras signaling requires TBK1 and IKKε activity to support tumorigenesis. Emerging data suggest that IKKε may also contribute to metabolic dysfunction (i.e. insulin resistance) by sustaining chronic, low-grade inflammatory state during obesity. Aberrantly regulated mTOR signaling also plays a central role in the pathophysiology of cancer and metabolic dysfunction. Thus, the novel molecular link between TBK1/IKKε and mTOR S2159 phosphorylation we describe here may represent a mechanism for, at least in part, the progression of these pathologic states.

Chapter 1

Introduction

The mammalian or mechanistic target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine protein kinase that functions as an environmental sensor to maintain cellular and organismal homeostasis (81, 100, 114, 198, 324). Aberrant regulation of mTOR contributes to several disease states including cancer, type II diabetes, and cardiovascular diseases, indicating its important role in physiology (70, 198, 341). Rapamycin (a.k.a sirolimus) is a macrolide antibiotic produced by the bacterium *Streptomyces hygroscopicus* (272). Rapamycin forms a complex with the cellular protein FKBP12 (FK506-binding protein 12 kDa) and binds to mTOR to inhibit its function in an allosteric manner (1, 19, 47, 79, 80, 114, 223, 307). mTOR is a member of the phosphoinositide kinase-related kinase (PIKK) superfamily, which also includes ATM (ataxia telangiectasia mutated), ATR (ataxia telangiectasia and Rad3-related protein), DNA-PK (DNA dependent protein kinase), and TTRAP (trafficking protein particle). mTOR is a large, atypical protein kinase (289kDa) with several functional domains. The HEAT (huntingtin, elongation factor 3 [EF3], protein phosphatase 2A [PP2A], and the yeast PI3K [phosphatidylinositol 3 kinase] TOR1) repeats that extend from the N-terminus of mTOR to its kinase domain are thought to mediate protein-protein interactions and allow mTOR to form distinct multi-protein complexes (10, 235). The FKBP12-rapamycin complex binds to the FKBP12–rapamycin-binding (FRB) domain, which lies immediately N-terminal to the mTOR kinase domain (168 2005, 289). Two evolutionarily conserved domains, the FAT (FRAP-ATM-TTRAP) and FAT-C (C-terminal FAT)

domains, flank the catalytic domain and play important roles in controlling mTOR kinase activity via unclear mechanisms (Fig. 1.1) (23, 237, 292).

mTOR acts in at least two functionally distinct multi-protein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Acute rapamycin treatment inhibits mTORC1, which contains mTOR, raptor (regulatory associated protein of mTOR), mLST8/GβL (mammalian lethal with SEC13/G protein β subunit-like), PRAS40 (proline rich Akt substrate of 40 KDa), and deptor (DEP [dishevelled, egl-10, pleckstrin] domain-containing mTOR-interacting protein) (119, 168, 169, 188, 238, 263, 308). Acute rapamycin treatment fails to inhibit mTORC2, which contains mTOR, mLST8/GβL, rictor (rapamycin-insensitive companion of mTOR), mSin1 (mammalian *s*tress-activated protein kinase [SAPK]-interacting protein), PRR5/protor (proline-rich protein 5), and deptor (101, 152, 169, 188, 228, 238, 264)**.** Two classes of mTORC1 substrates have been characterized extensively, the ribosomal S6 kinases (S6K1 and S6K2) and the 4E-BPs (eukaryotic translation initiation factor 4E [eIF4E] binding proteins; 4E-BP1, 4E-BP2, and 4E-BP3). mTORC1-mediated phosphorylation of S6K1 and 4E-BP1 increases cap-dependent translation, an event necessary for cell growth, cell cycle progression, and cell proliferation (93-95, 100, 198, 324). mTORC1-mediated S6K1 phosphorylation also promotes lipid synthesis (82, 181, 241). While nutrients (e.g. amino acids, glucose) and growth factors (e.g. insulin) upregulate mTORC1 signaling, energy depletion and diverse cellular stresses downregulate mTORC1 signaling. In comparison to mTORC1, mTORC2 remains less well characterized. Growth factor stimulation augments mTORC2 function in PI3K-dependent manner yet the signaling intermediates that link PI3K to mTORC2 remain unknown. Activated mTORC2 phosphorylates several AGC kinase family members (named after its founding members protein kinase A [PKA], PKG, and PKC) including Akt, SGK1 (serum/glucocorticoid regulated kinase 1), and PKCα (protein kinase C alpha) on their hydrophobic motif (HM), an event critical for catalytic activity (87, 104, 115, 143, 151, 185, 264, 327)**.**

Since rapamycin-FKBP12 complex cannot bind assembled mTORC2, mTORC2 displays insensitivity to acute rapamycin treatment (188). During long-

term treatment, however, rapamycin may bind to free mTOR (present during synthesis or during mTOR complex turnover) and inhibits mTORC2 assembly, thus suppressing mTORC2 function (152, 264, 265, 336). Recent studies indicate that while rapamycin completely blunts mTORC1-dependent S6K1 phosphorylation, it fails to inhibit mTORC1-dependent 4E-BP1 phosphorylation completely (57, 88, 302, 335). The newly developed mTOR catalytic site inhibitors (i.e. Torin1, KU-0063794, PP242), on the other hand, fully block phosphorylation of both mTORC1 targets S6K1 and 4E-BP1 as well as mTORC2 targets Akt and SGK1 (57, 88, 301, 302, 335). Interestingly, treatment of cells with mTOR catalytic site inhibitors impairs cell growth and proliferation to a far greater degree than rapamycin in an mTORC2-independent manner. These data reveal that some cell biological functions of mTORC1 are quite rapamycin-resistant, which are at least partially mediated by mTORC1-dependent and rapamycin-resistant 4E-BP1 phosphorylation (57, 88, 114, 301, 302, 335).

A. Discovery of rapamycin and TOR

Rapamycin is a macrolide antibiotic produced by a strain of *Streptomyces hygroscopicus*, isolated from a soil sample collected on the South Pacific island Rapa Nui (known better as Easter Island) (310). This anti-proliferative activity was discovered during antifungal screening performed by Ayerst Research Laboratories in 1975. Initial studies suggested that rapamycin functions as an antifungal agent by suppressing the G1-S phase transition during the cell cycle (1, 128, 178, 310). Subsequent studies revealed that rapamycin inhibits proliferation not only in yeast but also in mammalian cells and exhibits antitumor activity (85, 137). Rapamycin inhibits IL-2 (interleukin-2)-induced propagation of T cells, as well (1, 9, 79, 80, 201). Thus, in 1999, the FDA approved the use of rapamycin as an immunosuppressive agent to blunt kidney transplant rejection. In 2007, the FDA also approved the use of rapamycin analogs, temsirolimus (a.k.a CCI-779; Torisel) and everolimus (a.k.a RAD-001; Afinitor) to treat renal cell carcinoma (15, 249).

FKBP12, a peptidyl-prolyl cis/trans isomerase, is the intracellular receptor for the immunosuppressive drugs FK506 and rapamycin, which share structural similarity (1, 121, 128, 173, 178, 282). Deletion of FPR1 in yeast, the gene that encodes FKBP12, is not toxic, indicating that rapamycin does not function as an antifungal by inhibiting FKBP12 function (75, 128, 173, 322). Thus, in order to identify the molecular mechanisms underlying the antiproliferative effects of rapamycin, a genetic screen was performed in *Saccharomyces cerevisiae*. Mutations in three different genes conferred resistance to rapamycin treatment: the targets of rapamycin TOR1 and TOR2; and FPR1 (32, 128). The point mutations (S1972R in Tor1p and S1975I in Tor2p) that rendered TOR1 and TOR2 rapamycin-resistant mapped to a domain named the FRB (FKBP12-rapamycin binding) domain. These mutations prevented binding of the rapamycin- FKBP12 complex to Tor1p or Tor2p and thus allowed proliferation in the presence of drug (289). Tor1p and Tor2p are 67% identical and possess shared and distinct functions (32, 129, 178). When growth conditions permit, rapamycin-sensitive Tor signaling promotes anabolic processes and inhibits catabolic processes. Tor2p, but not Tor1p, functions further to regulate spatial aspects of yeast cell growth. Similar to mTORC2, the ability of yeast Tor2 to regulate these spatial aspects of yeast cell growth is insensitive to rapamycin (129, 178, 271).

Subsequent studies by five different groups led to the identification and cloning of the mammalian TOR ortholog (1, 25, 50, 55, 258, 259). These groups employed a similar approach to identify the mammalian target of the FKBP12-rapamycin complex, using recombinant FKBP12 protein in complex with rapamycin as bait to purify mTOR from several mammalian sources, including bovine brain (FKBP-rapamcyin-associated protein, FRAP) (25), rat brain (Rapamycin and FKBP12 target, RAFT; mammalian target of rapamycin,mTOR) (258, 259), and human lymphocytes (Sirolimus effector protein, SEP; rapamycin target, RAPT) (50, 55). Unlike *S. cerevisiae* and *S. pombe*, which possess two *TOR* genes, higher eukaryotes (i.e. worms, flies, mammals) possess only one *TOR* gene (1, 25, 50, 55, 191, 221, 258, 259, 337). mTOR S2035, which lies

within the FRB domain, is homologous to S1972/1975 in yeast Tor1p/Tor2p. Mutation of mTOR S2035 to residues other than alanine abrogates binding of FKBP12-rapamcyin complex to mTOR, rendering it rapamycin-resistant (26, 47).

B. Identification of mTORC1 partner proteins

Before the identification of mTORC1 or mTORC2 components, several lines of evidence suggested that mTOR indeed functions as a multi-protein complex: mTOR contains HEAT repeats, which are known to mediate protein-protein interactions, and it migrates at around 1.5–2.0 mDa on gel filtration chromatography (168). In addition, when washed with NP40-Brij containing buffer, immunoprecipitated mTOR loses its *in vitro* kinase activity towards 4E-BP1; when mTOR is purified by $(NH_4)_2SO_4$ salt precipitation, however, its kinase activity remains intact. These data suggest that the mTOR-dependent phosphorylation of 4E-BP1 and S6K1 requires additional molecules that copurify with mTOR in a detergent-sensitive manner (119, 168). Conventional purification conditions had been insufficient to identify mTOR-associated proteins, indicating a relatively unstable interaction between mTOR and putative partner proteins. Nevertheless, in 2002, two groups identified a novel mTOR interacting protein of 150 kDa, now known as raptor (119, 168). In order to copurify mTOR with raptor, Hara et al. employed the $(NH_4)_2SO_4$ salt precipitation approach, whereas Kim et al. used a reversible crosslinking reagent to stabilize the mTOR-raptor interaction. Raptor contains a highly conserved N-terminal domain followed by several HEAT repeats and seven C-terminal WD40 repeats (119, 168). Raptor interacts with mTOR HEAT repeats and acts as a scaffolding protein to recruit mTORC1 targets S6K1 and 4E-BP1 through their TOR signaling (TOS) motif (119, 268, 269). Downstream mTORC1 signaling and function requires the mTOR-raptor interaction (119, 168, 268, 269). In the absence of nutrients or growth factors, however, the mTOR-raptor interaction becomes more stable and this stronger mTOR-raptor interaction correlates with reduced mTORC1 signaling (84, 168).

Not long after the identification of raptor, studies by two different groups identified GβL (a.k.a mLST8) as another mTOR interacting protein (169, 188). GβL/mLST8 copurified with mTOR and appeared as a 36 kDa protein on a coomassie-blue stained gel, which was further analyzed by mass spectrometry and identified as GβL/mLST8 (169, 188). GβL/mLST8 contains seven WD40 repeats and binds to the mTOR kinase domain within both mTORC1 and mTORC2 (169). Although initial knockdown experiments suggested that GβL/mLST8 promotes mTORC1 signaling, studies in GβL/mlST8 knockout mice indicated that GβL/mLST8 is essential for mTORC2 but not mTORC1 function (115, 169).

Insulin stimulation of serum-deprived cells dramatically increases mTORC1-dependent S6K1 phosphorylation in intact cells. By *in vitro* kinase assay, however, mTORC1 purified from serum-deprived cells historically phosphorylated S6K1 to an extent similar to that of mTORC1 purified from insulin-stimulated cells, a curious result as insulin stimulates mTORC1-mediated phosphorylation of S6K1 robustly in intact cells. Thus, for a period of time in the mTOR field, researchers considered the possibility that growth factor signaling does not increase mTOR intrinsic catalytic activity but rather promotes mTORC1 signaling via an alternative mechanism. Washing mTORC1 immunorecipitates with buffers that contain lower salt concentrations, however, enabled the measurement of insulin-induced stimulation of mTORC1 *in vitro* kinase activity towards S6K1 and 4E-BP1 (263). These observations indicated that a salt-sensitive factor renders mTORC1 insulin responsive in *in vitro* kinase reactions. Search for the proteins that copurify with mTOR when immunoprecipitates are washed with buffers containing low but not high salt concentrations revealed PRAS40 as the salt-sensitive factor (263). Similar to S6K1 and 4E-BP1, PRAS40 contains a putative TOS motif and interacts with raptor (97, 222, 263, 308, 314). When bound to raptor, PRAS40 suppresses mTORC1 signaling. Insulin and serum stimulation promotes Akt- and mTORC1-mediated phosphorylation of PRAS40 on S246 and S183, respectively (222, 263, 308). These phosphorylation events release PRAS40 from raptor, promoting mTORC1 signaling. Due to the

presence of a putative TOS motif, PRAS40 may also suppress mTORC1 signaling by competing with other mTORC1 substrates such as S6K1 and 4E-BP1. A PRAS40 mutant bearing an inactivated TOS motif, however, still downregulates mTORC1 signaling; thus indicating that substrate competition likely does not represent a major mechanism for PRAS40-mediated mTORC1 inhibition (11).

In 2009, Peterson et al**.** reported deptor as a 48 kDa novel mTOR interacting protein. Similar to PRAS40, deptor copurifies with mTOR only under low-salt purification conditions (238, 263). Deptor contains tandem N-terminal DEP domains and a C-terminal PDZ (postsynaptic density 95, discs large, zonula occludens-1) domain through which it interacts with the mTOR FAT domain within both mTORC1 and mTORC2. Under serum-deprived conditions, deptor binds to mTOR and downregulates mTORC1 signaling. During growth factor sufficiency, however, mTOR-mediated deptor phosphorylation induces deptor release from mTOR and leads to its subsequent proteasomal degradation (238). Except for PRAS40 and deptor, all mTORC1 components have yeast orthologues, indicating their essential role in mTORC1 function (188).

C. Regulation of mTORC1 Signaling

mTORC1 acts an environmental sensor to promote anabolic cellular processes such as cap-dependent translation, cell growth, cell cycle progression, cell proliferation, lipid synthesis. Conversely, mTORC1 suppresses catabolic cellular processes such as autophagy (93, 198, 324). Both extracellular and intracellular signals regulate mTORC1 function through distinct mechanisms, as explained in detail below.

C.i. Canonical insulin signaling

The insulin/PI3K/Akt pathway represents the best-characterized pathway that leads to mTORC1 activation (Fig. 1.2). Stimulation of cells with insulin or IGF sequentially activates receptor tyrosine kinases, insulin receptor substrate (IRS), and PI3K, leading to the generation of PIP_3 (Phosphatidylinositol (3, 4, 5)-

triphosphate) lipids and recruitment of PDK1 to the plasma membrane. PDK1 and mTORC2 cooperate to phosphorylate Akt on two critical sites, the T-loop site (T308) and the hydrophobic motif (HM) site (S473) to increase Akt catalytic activity (5, 115, 152, 264). Activated Akt, in turn, phosphorylates and inactivates at least two inhibitors of mTORC1: Tsc2 and PRAS40 (33, 146, 200, 242).

The TSC1 and TSC2 (tuberous sclerosis complex 1 and 2) represent tumor suppressors, the loss of which causes an autosomal dominant disease called tuberous sclerosis complex (36, 44, 111, 139). This genetic disorder causes the development of benign tumors in various organs including brain, kidney, and heart (36, 44, 111, 139). The protein products of TSC1 and TSC2 (also called hamartin and tuberin, respectively; hereafter referred to as Tsc1 and Tsc2) act in a dimeric complex. Tsc2 possesses GAP (GTPase activating protein) activity and Tsc1 stabilizes Tsc2 by preventing its ubiquitin-mediated degradation (145, 297). Tsc1/2 plays a crucial role in the regulation of mTORC1 signaling as diverse signals from several pathways converge on Tsc1/2, which negatively regulates mTORC1 function by promoting the hydrolysis of Rheb-GTP to Rheb-GDP (145, 297, 298).

Rheb (Ras homologue enriched in brain) is a small GTP binding protein and represents the most proximal positive regulator of mTORC1 known to date (145, 189, 190, 267, 295). A GEF (GTP exchange factor) for Rheb remains unidentified, but Tsc1/Tsc2 complex acts as the GAP for Rheb (145, 298). Rheb weakly interacts with the mTOR kinase domain, yet the exact molecular mechanism by which Rheb-GTP binding to mTOR mediates mTORC1 activation remains ill-defined (189, 190, 267). Although earlier studies suggested that Rheb-GTP increases mTORC1 kinase activity (189, 190, 263), this notion was challenged when Sato et al. reported that Rheb-GTP does not promote mTORC1 kinase activity but instead enhances the recruitment of substrates such as 4E-BP1 to mTORC1 (267).

Activated Akt phosphorylates Tsc2 on several sites including S939 and T1462 to suppress Tsc2 function (200). Although, the exact mechanisms remain unclear, several models have been proposed: Akt-mediated Tsc2

phosphorylation may induce Tsc2 destabilization and degradation, altered Tsc2 subcellular localization, or sequestration of Tsc2 via 14-3-3 protein binding (33, 139, 141, 146, 200, 242).

C.ii. Amino acids

mTORC1 signaling absolutely requires sufficient levels of amino acids, particularly the branched chain amino acids leucine and isoleucine. Thus, growth factors fail to activate mTORC1 signaling during amino acid deprivation (214, 295, 298). Withdrawal of amino acids suppresses mTORC1 function in Tsc-deficient cells, indicating that the control of mTORC1 signaling by amino acids occurs downstream or independently of Tsc1/Tsc2 (283). The bidirectional amino acid permease SLC7A5-SLC3A2 (solute carrier family 7 member 5 - solute carrier family 3 member 2), which regulates the simultaneous efflux of glutamine out of cells and transport of leucine into cells, is essential for mTORC1 activation (213). Although, it remains unclear how cells sense amino acids levels, several molecules have been reported to link amino acid sensing to mTORC1, including hVPS34 (mammalian [vacuolar protein sorting] 34 homologue), MAP kinase kinase kinase kinase 3 (MAP4K3), the RalA GTPase, and the Rag GTPases (31, 92, 197, 214, 261, 262).

RagGTPases are the best-characterized regulators of mTORC1 in response to amino acid stimulation (170, 261, 262). There are four RagGTPases, RagA, RagB, RagC, and RagD. RagA and RagB represent mammalian orthologues of the yeast Gtr1p, while RagC and RagD represent mammalian orthologues of the yeast Gtr2p. In yeast, both Gtr1p and Gtr2p control the intracellular sorting of the amino acid permease Gap1p and microautophagy. RagA or RagB heterodimerize with RagC or RagD, with each Rag class bearing opposite nucleotide bound states. Amino acid stimulation increases the formation of active Rag heterodimers (e.g. $RagB^{GTP}$-$RagD^{GDP}$) which interact with mTORC1 via raptor (170, 262). This interaction allows mTORC1 to translocate from the cytoplasm to a Rab7-positive, late endosomal/lysosomal membrane compartment that also contains exogenously expressed mTORC1 activator Rheb

(262). The multi-protein "*ragulator*" complex, which contains three conserved proteins called MP1 (MEK [MAPK/ERK kinase] -binding partner 1), p14, and p18, resides on lysosomal membranes and binds to activated Rag heterodimers to recruit mTORC1 for Rheb mediated activation (261, 262). In addition, forced localization of mTORC1 to the lysosomal surface eliminates the requirement of amino acids, Rag GTPases, and the *ragulator* for mTORC1 activation (261).

C.iii. Ras/MAPK Signaling

Independent of the PI3K/Akt pathway, mitogen activated Ras/MEK/MAPK signaling also activates mTORC1. Activated ERK (extracellular signal-regulated kinase) and its substrate RSK (ribosomal S6 kinase) phosphorylate Tsc2 (S540; S644 and S1798, respectively) to inhibits its function and promote mTORC1 signaling (193, 255). Similar to its Akt-mediated regulation, phosphorylation of Tsc2 on S540 and/or S644 by ERK disrupts the Tsc1-Tsc2 interaction and prevents its inhibitory function on mTORC1 (193). In addition, upon mitogenic stimuli, ERK and RSK activate mTORC1 via direct phosphorylation of raptor on S8, S696/S863 and S719/S721/S722, respectively (39).

C.iv. PLD signaling

The canonical PLD (phospholipase D) signaling controls vesicle trafficking and cytoskeleton organization (208). Recent findings, however, suggest an important role for PLD in mediating signals of nutrient and growth factor sufficiency to mTORC1. Both amino acids and growth factors activate PLD and activated PLD cleaves phosphatidylcholine to produce phosphatidic acid (PA), which in turn stimulates mTORC1 signaling (291). Interestingly, PA binds to the mTOR FRB domain, and this PA-mTOR interaction upregulates mTORC1 signaling in a rapamycin sensitive manner (291, 304).

C.v. Cytokine signaling

TNFα (tumor necrosis factor α) signaling also regulates the mTORC1 pathway. Stimulation of cells with TNFα activates IKKβ (inhibitor of nuclear

factor-kappa B [NF-κB] kinase subunit beta), which in turn binds to and phosphorylates Tsc1 on S487 and S511 (184). These phosphorylation events destabilize the Tsc1-Tsc2 interaction, promoting biochemical mTORC1 signaling, tumor angiogenesis, and insulin resistance (184). In addition, insulin and TNFα promote mTORC1 signaling via a mechanism that involves direct interaction of IKKα (inhibitor of NF-κB kinase subunit alpha) with mTORC1 in an Akt-dependent manner, which in turn promotes NF-κB transcriptional activity (67-69). Furthermore, emerging data suggest that IKK-related kinases TBK1 (TANK-binding kinase 1) and IKKε (inhibitor of NF-κB kinase epsilon) phosphorylate Akt on its T-loop and HM-sites, T308 and S473, which activates Akt and promotes mTORC1 signaling (155, 224, 325).

C.vi. Energy Availability and Cellular Stress

Energy depletion or cellular stress (e.g. hypoxia, DNA damage, endoplasmic reticulum stress, etc.) downregulates mTORC1 signaling (28, 73, 148). The increase in cellular AMP/ATP ratio during energy deprivation activates AMPK (AMP activated kinase), which in turn phosphorylates Tsc2 (S1227; S1345) and promotes Tsc1/Tsc2 function to downregulate mTORC1(148). Phosphorylated S1345 acts as a priming site for further Tsc2 phosphorylation and activation by GSK3β (S1337; S1341), establishing crosstalk between mTORC1 and Wnt signaling (147).

Hypoxia-induced Redd1 (regulated in development and DNA damage response) also promotes Tsc1/Tsc2 function to downregulate mTORC1 signaling (28, 73). Increased levels of Redd1 during endoplasmic reticulum stress and unfolded protein response (UPR) downregulate mTORC1 signaling, as well (321). Upon DNA damage, activated p53 transcriptionally induces the target genes sestrin 1 and 2, which bind to and activate AMPK via an unknown mechanism to downregulate mTORC1 signaling (29). Overall, diverse forms of cellular stress employ a variety of molecular mechanisms to downregulate mTORC1 signaling under unfavorable conditions.

D. Bona Fide mTORC1 Substrates

4E-BP1 and S6K1 are the best-characterized mTORC1 substrates that mediate the role of mTORC1 in many of its cell biological functions including cap-dependent translation, cell growth, and cell cycle progression (93-95, 198). Thus, this section will focus on mTORC1 substrates, S6K1 and 4E-BP1. Other mTORC1 substrates such as ULK1/2, Atg13, and HIF1α will be discussed under "Cell biological functions of mTORC1" section.

D.i. S6 Kinases (S6K)

S6K1 and S6K2 belong to the AGC family of serine/threonine protein kinases, which contains 59 other members (198, 229). The *S6K1* gene encodes for two alternatively spliced isoforms: p70-S6K1 and p85-S6K1. The p70 isoform is 502 amino acids long and the p85 form has an additional 23 N-terminal amino acids, which contains a nuclear localization signal (NLS) (Fig. 1.3). Despite the presence of an NLS within p85-S6K1, both isoforms of S6K1 localize to the cytoplasm (167). The *S6K2* gene also encodes for two alternatively spliced isoforms: p54-S6K2 and p56-S6K2. The p54 isoform is 482 amino acids long, and the p56 form has an additional 13 N-terminal amino acids, which again contains an NLS (183, 198). Among these four different S6Ks, p70-S6K1 is the best-characterized.

In addition to its catalytic domain, which contains the T-loop phosphorylation site (T229), S6K1 has several functional domains and motifs (Fig. 1.3). The acidic N-terminus contains a TOS motif (FDIDL [the single-letter amino acid code for its sequence]; amino acids 5-9), enabling the S6K1-raptor interaction and S6K1 phosphorylation by mTORC1 (198, 268, 269). The linker region contains two other critical phosphorylation sites, the turn motif (TM) site and the hydrophobic motif (HM) site and the basic C-terminus contains an autoinhibitory pseudosubstrate domain. The C-terminal RSPRR motif (from the single-letter amino acid code for its sequence) (amino acids 410-414) suppresses S6K1 activity possibly by interacting with a negative regulator such as a phosphatase.

Upon growth factor or mitogenic stimuli, phosphorylation of several sites on S6K1 increases catalytic activity. Insulin/IGF (insulin-like growth factor) stimulation, for instance, signals through the PI3K/Akt/mTORC1 pathway to promote S6K1 phosphorylation and function (200). Ras/MAPK (mitogen-activated protein kinases) pathway, on the other hand, upregulates mTORC1 in a PI3K-independent manner to activate S6K1 (39, 40, 193, 255).

Activation of p70-S6K1 absolutely requires three critical phosphorylation events: T-loop (a.k.a activation loop) phosphorylation on T229 by PDK1 (phosphatidylinositol dependent kinase 1), HM site phosphorylation on T389 by mTORC1, and TM site phosphorylation on T371 by GSK3β (Glycogen synthase kinase 3 beta) (6, 30, 149, 244, 279). In addition to these phosphorylation events, the C-terminal autoinhibitory pseudosubstrate domain also bears five phosphorylation sites: S404, S411, S418, S421, and S424, which contribute to activation of S6K1 but are not absolutely required (90, 198).

In its unphosphorylated form, the basic C-terminal pseudosubstrate domain interacts with the acidic N-terminus and occludes the kinase domain (13, 243). Upon mitogenic stimuli, phosphorylation of these C-terminal sites in the psuedosubstrate domain exposes the HM and T-loop sites (13, 243). The physiological kinase(s) for these C-terminal phopsphorylation sites in intact cells, however, remains elusive (209).

The S6K1 TOS motif is also critical for S6K1 activation, as its deletion or mutation abolishes S6K1 activity due to the loss of S6K1-raptor interaction and thus mTORC1-mediated S6K1 phosphorylation (268, 269).

D.ii. 4E-BP

The 4E-BP family consists of three members: 4E-BP1 (118 amino acids [aa]), 4E-BP2 (120 aa), and 4E-BP3 (100 aa), which exhibit distinct patterns of tissue-specific expression. 4E-BP1 is ubiquitously expressed in skeletal muscle, adipose tissue, pancreas, liver, heart, and kidney (275). 4E-BP2 expression, on the other hand, is higher in liver and kidney. 4E-BP3 displays a tissue distribution very similar to 4E-BP1 (i.e. skeletal muscle, pancreas, heart, and kidney) but

unlike 4E-BP1, its expression is very low in liver. Although 4E-BP3 shares the basic structural and functional features of 4E-BP1, its regulation appears to be different (172).

4E-BPs and eIF4G (eukaryotic translation initiation factor 4G) share a motif for eIF4E recognition, thus compete with each other for eIF4E binding (196). By interacting with eIF4E, 4E-BPs blunt the initiation of cap-dependent translation. Among the three 4E-BPs, 4E-BP1 is the best characterized and is considered the prototype of the family (Fig. 1.4). In response to growth factor stimulation, mTORC1 mediates phosphorylation of 4E-BP1 at four well-characterized serine/threonine (S/T) sites: T36; T47; S65; and T70 to activate mRNA translation by releasing 4E-BP1 from eIF4E (106-108). Phosphorylation of T36/47 acts as priming site for T70 phosphorylation, which in turn is required for S65 phosphorylation (106-108). Both 4E-BP2 and 4E-BP3 share homologous S/T sites that correspond to these 4E-BP1 phosphorylation sites. Although the kinase(s) are unknown, S101 and S112 are other phosphorylation sites on 4E-BP1 required for its dissociation from eIF4E. Interestingly, the corresponding phosphorylation sites for 4E-BP1 S101 and S112 do not exist in 4E-BP2 or 4E-BP3 (316).

4E-BP1 and 4E-BP2 are regulated in a similar manner, and they share an N-terminal regulatory motif called the RAIP motif (from the single-letter amino acid code for its sequence), which is absent in 4E-BP3 (187, 299). The RAIP motif facilitates the insulin-mediated phosphorylation of 4E-BP1 and 4E-BP2 and their subsequent release from eIF4E, suggesting that this motif may be a kinase docking site. Due to lack of the RAIP motif in 4E-BP3, its insulin-mediated phosphorylation is not sufficient to induce its dissociation from eIF4E, suggesting that 4E-BP3 has functions distinct from 4E-BP1 and 4E-BP2 (187, 299). 4E-BP1 also contains a TOR signaling (TOS) motif (FEMDI [the single-letter amino acid code for its sequence]) at its extreme C-terminus (aa 114-118), which mediates its interaction with the mTORC1 component raptor and facilitates its multi-site phosphorylation on T37/T46, S65, and T70. When overexpressed, the TOS motif

mutant 4E-BP1-F114A shows impaired phosphorylation on T37/T46, S65, and T70 and reduces cell size (268, 269).

E. Cell Biological Functions of mTORC1

mTORC1 is an important positive regulator of the rate-limiting initiation step during cap-dependent translation. Increased protein synthesis promotes cell growth (increase in cell mass/size), which in turn promotes cell cycle progression and cell proliferation. mTORC1 also promotes *de novo* lipid synthesis and inhibits autophagy.

E.i. Cap-dependent translation

The rate-limiting step in cap-dependent translation is initiation, which is tightly regulated by various mechanisms. For cap-dependent translation initiation, the eIF4F complex has to form at the m^7GTP (7-methly-guanosine 5' triphosphate) cap structure at the 5' end of mRNAs. The eIF4F complex contains eIF4E, eIF4G, and eIF4A. Once assembled, this complex recruits other factors including eIF3, the small ribosomal subunit, the ternary complex containing eIF2, ^{met}tRNA (methionyl initiator transfer RNA), and GTP in order to form the 48S translation preinitiation complex (132, 194). mTORC1-mediated phosphorylation of 4E-BP1 and S6K1 enhances protein synthesis by regulating some of these initiation factors.

In the absence of mTORC1 activating stimuli, hypophosphorylated 4E-BP1 binds to eIF4E at the 5'end of the mRNAs and inhibits the binding of eIF4G, preventing the formation of the eIF4F complex and translation initiation. When activated, mTORC1 mediates 4E-BP1 phosphorylation on several sites (T37; T46; S65; T70), causing its dissociation from eIF4E and enables the recruitment of eIF4G and other complexes to the cap structure to initiate cap-dependent translation (2, 17). mTORC1 also promotes eIF4G function by mediating its phosphorylation on S1148, S1188, and S1232 upon growth factor stimulation (126, 194).

eIF3 is a dynamic scaffolding protein that plays role in protein synthesis. Inactive S6K1 binds to eIF3 (134, 194, 198). Once activated, mTORC1 binds to eIF3 and phosphorylates S6K1, upon which S6K1 dissociates and phosphorylates its substrates, including eIF4B (eukaryotic translation initiation factor 4B), PDCD4 (programmed cell death protein 4), SKAR (S6K1 Aly/REF-like target), eEF2K (eukaryotic elongation factor 2 kinase), and rpS6 (ribosomal protein S6 – a component of the 40S ribosome; a.k.a S6) (77, 91, 134, 194, 195, 198, 245, 248, 317, 328). The binding of mTORC1 to eIF3 also promotes the interaction between eIF3 and eIF4G, promoting translation initiation (123).

Small ribosome subunit binding and further scanning towards the initiation codon requires the linearization of untranslated regions of mRNAs with complex secondary structures. eIF4A is an RNA helicase that unwinds these secondary structures. Upon growth factor stimulation, S6K1 phosphorylates and activates eIF4B (S422). Activated eIF4B promotes eIF4A helicase activity to augment unwinding and translation initiation (245). In addition, activated S6K1 phosphorylates the tumor suppressor and eIF4A inhibitor PDCD4 (on S67), and promotes its degradation (77, 328). SKAR, another downstream target of S6K1, couples transcription with mRNA splicing and nuclear export by interacting with the exon junction complex of the spliced mRNAs. S6K1 binds to and phosphorylates SKAR (S383/S385) to enhance the translational efficiency of newly spliced mRNAs (195, 248). In order to enhance translation elongation, S6K1 also phosphorylates and inhibits eEF2K (S366), which phosphorylates and inactivates eEF2 (eukaryotic elongation factor 2) (317). S6K1 phosphorylates S6 on several sites (S235; S236; S240; S244), as well. The functional significance of S6 phosphorylation sites, however, remains to be elucidated (91, 204, 205, 232). Overall, these findings indicate that both 4E-BP1 and S6K1 control diverse steps during cap-dependent protein translation.

mTORC1 also has a role in translation of ribosomal proteins, the mRNAs of which share a 5'track of polypyrimidines (5'TOP mRNAs). Although earlier studies suggested that phosphorylation of S6 by S6K1 mediates the role of mTORC1 in translation of 5'TOP mRNAs, this idea was dismissed by additional

work, indicating that mTOR enhances the translation of these 5'TOP mRNAs in S6K1 and S6 independent manner (227, 257, 290, 294).

E.ii. Cell growth

Studies in *Drosophila melanogaster* first identified the role of TORC1 in regulation of cell growth (206, 220, 231). Inactivation of dTOR or dS6K1 causes developmental delay and lethality with reduced cell, organ, and body size in surviving flies (206, 220, 339). This reduced organ and body size phenotype is due to decrease in cell size without an effect on cell number (206, 220). Consistently, inhibition of mTORC1 and S6K1 via rapamycin treatment decreases the relative size of the cultured mammalian cells, whereas expression of rapamycin-resistant S6K1 alleles ($E_{389}D_3E$ and $E_{389}\Delta CT$) rescues this phenotype (95). Moreover, acute knockdown of S6K1 with RNAi (RNA interference) reduces cell size, whereas overexpression of S6K1 increases cell size (72, 95, 248). As in flies, S6K1 null mice show reduced body and cell size especially in pancreatic β cells and myoblasts (219, 231). Due to the possible functional redundancy between S6K1 and S6K2, in 2004 Pende et al compared the single $S6K1^{-/-}$ and single $S6K2^{-/-}$ knockout mice to the double $S6K1^{-/-}$;$S6K2^{-/-}$ knockout mice (DKO), which was essential to understand the specific role(s) of each S6K. In contrast to the singles, the $S6K1^{-/-}$; $S6K2^{-/-}$ double knockout mice were perinatal lethal. Interestingly the $S6K2^{-/-}$single knockout mice were larger in size compared to wild type mice and the surviving double $S6K1^{-/-}$;$S6K2^{-/-}$ knockout mice were similar in size to single $S6K1^{-/-}$ animals (232). In addition, overexpression of constitutively active S6K1 allele rescues the reduced cell size phenotype of mouse embryonic fibroblasts (MEFs) isolated from $S6K1^{-/-}$; $S6K2^{-/-}$ double knockout mice (78). Overall, these findings indicate that S6K1 but not S6K2 promotes cellular and organismal growth (78, 232). Studies in $S6K1^{-/-}$; $S6K2^{-/-}$ double knockout mice led to the discovery of another S6 kinase, RSK (ribosomal protein S6 kinase), as S6 phosphorylation was not completely abolished in these animals. RSK-mediated S6 phosphorylation (S235; S236) is upregulated by high serum levels and is insensitive to rapamycin treatment (232).

These findings set a nice example for how different pathways (in this case mTORC1 and MAPK pathways) can feed into the same substrate upon different stimuli. In addition, MEFs isolated from knockin mice with a phospho-site defective S6 allele ($S6^{P-/-}$) show reduced cell size compared to wild type (WT) controls, indicating a role for S6 phosphorylation in cell growth (257). SKAR may be another substrate of S6K1 that controls cell size, as its knockdown reduces cell size, as well (248).

Overexpression of eIF4E partially rescues the reduced cell size phenotype induced by rapamycin treatment (95). In addition, expression of the TOS motif mutant of 4E-BP1 (F114A) reduces cell size (269). These data indicate that not only S6K1 but also 4E-BP1 mediates the the role of mTORC1 in cell size.

E.iii. Cell cycle progression and cell proliferation

TOR is an important regulator of cell cycle progression and cell proliferation in yeast, flies, and mammals (1, 93, 94, 311). Overexpression of rapamycin resistant (RR)-S6K1 partially rescues the delay in G1/S phase progression and cell proliferation in rapamycin treated cells. Acute knockdown of S6K1 with RNAi also slows down G1/S-phase progression (94). Consistently, overexpression of S6K1 and S6K2 confers a proliferative advantage to cells in culture (63, 94, 109). These data clearly indicate a role for mTORC1-S6K1 axis in cell cycle progression and cell proliferation. Nevertheless, the $S6K1^{-/-}$; $S6K2^{-/-}$ double knockout mouse embryonic fibroblasts (MEFs) and myoblasts do not show any defect in cell cycle progression and proliferation compared to wild type controls (219, 232). It is, however, possible that under chronic inactivation of S6K (as during $S6K1^{-/-}$; $S6K2^{-/-}$ knockout), there are compensatory mechanisms that prevent any cell cycle progression and cell proliferation defects.

The mTORC1-4E-BP1 axis controls cell cycle progression independently of mTORC1-S6K1 axis (78). Overexpression of eIF4E accelerates G1/S-phase progression and confers partial rescue from rapamycin treatment. In addition, the dominant inhibitory TOS-motif mutant 4E-BP1-F114A or the phospho-site defective mutant 4E-BP1-T37A/T46A slows down the G1/S-phase progression

(94). Furthermore, inhibition of 4E-BP1 by mTORC1 increases the translation of cyclin D1 and c-myc, thus promoting cell cycle progression (11, 286). More recently, Dowling et al. reported that MEFs lacking all three 4E-BPs were resistant to inhibition of cell cycle progression and cell proliferation upon inhibition of mTORC1 signaling. These data suggest that mTORC1-4E-BP1 axis acts as the major controller of cell cycle progression and cell proliferation (78).

Additional studies in mice support the role of mTOR in cell growth and cell proliferation. In 2009, Nardella reported that prostate specific knockout of mTOR led to 7.5% decrease in cell size, but there was no other morphological or functional phenotypes (211). Nevertheless, when conditional mTOR knockout was induced on PTEN$^{-/-}$ (phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase) mice, which are predisposed to generate tumors, there was a reduction in prostate enlargement due to G1-phase arrest and decrease in overall proliferation rate (211). These data suggest that although mTOR is dispensable in post-mitotic tissues, it is crucial for cells that undergo mitosis during tumorigenesis or embryonic development, both of which require mitotically active cells (102, 210).

E.iv. Autophagy

Both in yeast and in metazoans TOR functions as a negative regulator of autophagy. In mammals, ULK1 and ULK2 [unc-51-like kinase 1/2; mammalian homologues of yeast Atg1 (autophagy-related protein 1)], and Atg13 (autophagy-related protein 13) are direct targets of mTORC1 for regulating autophagy. Atg13 is required for ULK1 and ULK2 to interact with and phosphorylate FIP200 (focal adhesion kinase [FAK] family-interacting protein of 200 kDa), which is essential for the formation of the autophagosome and induction of autophagy (120, 136, 157). Similarly FIP200 itself is required for ULK1 to interact with Atg13 (136). mTOR interacts with the ULK1/2-Atg13-FIP200 complex in a nutrient dependent manner (136). Under conditions of nutrient sufficiency, active mTORC1 interacts with the ULK1/2-Atg13-FIP200 complex and phosphorylates ULK1, ULK2, and Atg13 to inhibit their function. During nutrient deprivation, mTORC1 no longer

interacts with ULK1/2-Atg13-FIP200 complex and thus cannot phosphorylate ULK1, ULK2, and Atg13 to inhibit autophagy, resulting in increased autophagy (136). Interestingly knockdown of ULK1, ULK2 or Atg13 leads to increased S6K1 phosphorylation, indicating the presence of a negative feedback loop from this complex to mTORC1 (157).

E.v. Transcription

mTOR contributes to ribosome biogenesis by controlling the transcription of ribosomal RNA (rRNA) and translation of ribosomal proteins (270). mTORC1 promotes Pol I (DNA Polymerase I)-mediated transcription of rRNAs, Pol II (DNA Polymerase II)-mediated transcription of ribosomal protein genes, and Pol III (DNA Polymerase III)-mediated transcription of 5S rRNA and tRNAs (transfer RNAs) (202, 320).

mTOR enables the association of Pol I with transcription factors TIF-IA (transcription initiation factor IA) and TIF-IB at the promoter regions of rRNA genes. mTOR mediates the phosphorylation of TIF-IA on S44 to enhance its activity. In addition, S6K1 phosphorylates and activates the rDNA (ribosomal DNA) transcription factor UBF (upstream-binding factor) to promote 45S ribosomal gene transcription and ribosome biogenesis (118, 320).

The role of mTOR in transcription is not only limited to ribosomal genes. In the presence of amino acids, for instance, mTOR increases IGF-II mRNA levels required for myogenesis in a rapamycin sensitive manner. Very interestingly, mTOR kinase activity is not required for this transcriptional function (86). Cunningham et al. (64) also reported a role of mTOR in transcriptional control of mitochondrial genes. In order to induce mitochondrial gene expression, mTOR and raptor interact with the transcription factor YY1 (yin-yang 1) to recruit its co-activator PGC1α (peroxisome-proliferator-activated receptor γ coactivator-1 alpha). Rapamycin treatment prevents the YY1-PGC1α interaction, inhibiting the transcription of mitochondrial genes, thus blunting mitochondrial oxidative function (64).

mTORC1 also controls the functions of transcription factors STAT3 (signal transducer and activator of transcription 3), HIF1α (hypoxia-inducible factor 1-alpha), and SREBP-1. mTOR mediates STAT3 activation in response to cytokine signaling in a rapamycin sensitive manner (82, 180, 334). Initial phosphorylation of STAT3 on Y705 by JAK (Janus kinase 2) signaling is followed by mTOR-mediated phosphorylation on S727, which leads to its full activation (334). The mTORC1-4E-BP1 axis also leads to increased translation of the transcription factor HIF1α, which induces the expression of glycolytic genes and angiogenic factors such as VEGF-1 (vascular endothelial growth factor) (180). HIF1α contains a TOS motif through which it interacts with raptor. HIF1α-raptor interaction is important for Hif1α transcriptional activity. Mutation of the HIF1α TOS motif prevents HIF1α from binding its co-activator CBP (CREB binding protein)/p300 (180), suggesting that mTORC1 may also phosphorylate HIF1α and this phosphorylation may enable the HIF1α-CBP/p300 interaction. The mTORC1-S6K1 axis also promotes the activity of transcription factor SREBP-1 (sterol response element binding protein 1), that plays a central role in *de novo* lipid and sterol biosynthesis (82, 241). Full-length SREBP-1 resides as inactive transmembrane precursor in the endoplasmic reticulum (ER). In response to insulin stimulation, SREBP-1 translocates from ER to Golgi, where it is processed via proteolytic cleavage. The active processed SREBP-1 form then translocates to the nucleus to turn on its target genes to induce lipid biosynthesis. The mTORC1-S6K1 axis promotes the processing of SREBP-1 from its inactive precursor to its active form through an as yet unknown molecular mechanism (82). Overall, these findings indicate that directly or indirectly mTOR employs several mechanisms to control gene expression at the transcriptional level.

F. mTORC1 signaling in animal physiology and disease

Aberrant regulation of mTORC1 signaling contributes to myriad of diseases including cancer, benign tumor syndromes, type II diabetes, cardiovascular diseases, organ hypertrophy, neurological disorders (e.g. autism spectrum disorders, Alzheimer's disease), and aging-related pathology,

underscoring the role of mTORC1 signaling in maintaining organismal homeostasis (70, 133, 341).

In 2004, two groups (102, 210) attempted to generate $mTOR^{-/-}$ mice to understand the role of mTOR signaling in animal physiology. $mTOR^{-/-}$ mice, however, die very early during embryonic development at e5.5, due to impaired cell proliferation of the inner cell mass and trophoblast as well as defects in the extraembryonic tissue (102, 210). In order to characterize the physiological functions of mTORC1 and mTORC2 separately, Guertin et al. generated raptor, rictor, and mLST8/GβL knockout mice (115). Their data demonstrated that mLST8/$G\beta L^{-/-}$ and $rictor^{-/-}$ single knockout mice are embryonic lethal at day e10.5 due to defects in fetal vasculature. $Raptor^{-/-}$ mice, however, die at day e5.5, similar to $mTOR^{-/-}$ mice (102, 115, 210). Thanks to the research with $S6K^{-/-}$ and 4E-$BP^{-/-}$ mice as well as studies that involve tissue specific $mTOR^{-/-}$ and $raptor^{-/-}$ knockout mice, the role of mTOR signaling in maintenance of animal physiology is now appreciated. Thus, this part of the chapter will focus on our current understanding of how mTOR contributes to organismal physiology and how its deregulation contributes to pathologic disease progression.

F.i. Glucose Homeostasis, body mass, and energy balance

In addition to bearing smaller organ and body size, $S6K1^{-/-}$ mice are hypoinsulinemic and glucose intolerant due to reduced insulin secretion by pancreatic β-cells, which are smaller in size (232, 277). Consistently, $rpS6^{P-/-}$ knockin mice are hypoinsulinemic and glucose intolerant due to reduced insulin secretion by pancreatic β-cells of reduced size (257). Despite the lower levels of insulin production, $S6K1^{-/-}$ mice are more sensitive to insulin upon high-fat diet compared to the wild type controls (306). These observations led to the discovery of a negative feedback loop exerted by the mTORC1-S6K1 axis on IRS-1 to reduce PI3K/Akt signaling. Activated S6K1 directly phosphorylates IRS-1(S301; S636; S639; S1101) to inhibit its function by promoting proteasome dependent degradation (38, 122, 225, 306). S6K1 also represses IRS-1 gene expression at the transcriptional level. This negative feedback loop exerted by S6K1 on IRS-1

thus uncouples the insulin receptor from PI3K and downregulates PI3K/Akt signaling, rendering cells insulin resistant (38, 122, 225, 306). The activation of this negative feedback loop may explain, at least in part, why overnutrition during obesity correlates commonly with insulin resistance (306).

mTORC1 also promotes adipogenesis and modulates adipocyte metabolism. $S6K1^{-/-}$ mice have fewer adipocytes due to decreased differentiation of stem cells into early adipocyte progenitors. Independently of S6K1, mTORC1 also controls terminal adipocyte differentiation (37, 331, 338). Due to increased triglyceride lipolysis, fatty acid β-oxidation, mitochondrial biogenesis, and metabolic rate, $S6K1^{-/-}$ mice store less fat than wild type mice (4, 256). Consistent with these observations, inhibition of mTORC1 in cultured adipocytes via rapamycin treatment or raptor knockdown increases lipolysis and suppresses lipogenesis, reducing fat storage (43, 285). These data demonstrate the important role of mTORC1 signaling in controlling fat metabolism.

$S6K1^{-/-}$ mice are resistant to age- and diet-induced obesity, indicating a role for the mTORC1-S6K1 axis in control of body mass and energy balance (306). Modulation of S6K1 activity in the mediobasal hypothalamus (MBH) of rat brain indicates that S6K plays a critical role in the regulation of food intake and energy balance in rats (20). Adenovirus-mediated delivery of constitutively active S6K1 into rat MBH decreases food intake and body weight, whereas dominant negative S6K1 shows an opposite phenotype (20). These observations are consistent with leptin- or leucine-induced mTOR activation in rat hypothalamus, which also reduces food intake and body weight (62). Studies in whole-body $S6K1^{-/-}$ mice, which are resistant to diet- and age- induced obesity, however, suggested a positive role for mTORC1-S6K1 axis in promoting weight gain and obesity (306). This discrepancy may be due to distinct tissue-specific functions of the mTORC1-S6K1 axis. It is possible that the mTORC1-S6K1 axis has differential roles in controlling food intake and weight gain in response to nutrient availability or deficiency in the central nervous system (CNS) versus the peripheral organs (e.g. liver, muscle, adipose tissue) (20). In addition, mice with adipose specific raptor knockout ($raptor^{ad-/-}$) weigh less due to reduced white

adipose tissue mass. Similar to S6K1$^{-/-}$ mice, raptor$^{ad-/-}$ mice are resistant to diet-induced obesity and show improved insulin sensitivity (239). In addition to S6K1, 4E-BPs also play role in controlling whole body glucose homeostasis and body mass. The 4E-BP1 and 4E-BP2 double knockout mice develop severe high-fat diet induced obesity due to reduced energy expenditure, reduced lipolysis, and increased adipogenesis, demonstrating that 4E-BPs function as metabolic brake in the development of obesity (182).

F.ii. Muscle Function

Muscle-specific knockout of mTOR leads to contractile dysfunction and myopathy similar to muscular dystrophy, indicating an important role for mTOR in muscle function (250). Deletion of mTOR in muscle tissue reduces dystrophin mRNA and protein levels, impeding mitochondrial biogenesis and oxygen consumption (250). While deletion of mTOR in muscle tissue does not affect the whole body glucose homeostasis, basal glucose uptake is higher in these mice due to increased insulin sensitivity, similar to S6K1$^{-/-}$ mice (306).

Similar to mTOR deficient muscle tissue, muscle-specific knockout of raptor causes progressive muscle dystrophy with a reduction in muscle mass as well as S6 and 4E-BP1 phosphorylation (16). In raptor null muscle, the expression of mitochondrial regulators such as PPARγ (peroxisome-proliferator-activated receptor gamma) and PGCα1 (PPARγ coactivator 1 alpha) are impaired, causing mitochondrial loss and reduced oxidative capacity (16).

F.iii. Immune Function

The use of rapamycin as an immunosuppressive drug clearly implicates the role of mTOR in the immune system. Cytokines and activated T- and B-cell receptors activate mTOR signaling to boost adaptive immune responses (319), and rapamycin functions as an immunosuppressive agent by inhibiting the IL-2-induced proliferation of T and B cells (1, 9, 79, 80, 201, 329). In IL-2- and CD28-stimulated CD+4 helper T cells, Aurora B and survivin form a complex with mTOR and promote G1- to S-phase cell cycle progression (287).

In addition to its roles in adaptive immunity, mTOR also promotes innate immunity. Mouse embryonic fibroblasts or mice lacking both 4E-BP1 and 4E-BP2 are resistant to vesicular stomatitis virus infection due to increased production of of type-I interferons (IFN) (61). This enhanced type I IFN production relies on upregulation of IRF-7 (interferon regulatory factor 7) mRNA translation, since 4E-BPs repress IRF-7 expression at the translational initiation level (61). S6K1, on the other hand, promotes IRF-7 phosphorylation to increase type I IFN production for antiviral innate immune response in plasmacytoid dendritic cells (pDCs) (35).

F.iv. Cancer: Cell number control

Hyperactive mTOR signaling contributes to development of benign tumors and cancer. In the genetic disorder tuberous sclerosis complex (TSC), loss of Tsc1 or Tsc2 causes hyperactive mTORC1 signaling, which leads to overgrowth (increase in cell size/mass) and excess proliferation of cells, causing the formation of benign tumors known as hamartomas in various organs such as kidney, skin, eye, and brain (36, 111, 139). Various hamartomas caused by the loss of other tumor suppressors such as PTEN, LKB1 (liver kinase B1), and NF1 (neurofibromatosis-1) depend on mTORC1 signaling, as these tumors exhibit increased sensitivity to the anti-growth and anti-proliferative effects of rapamycin (296). One common feature of such hamartomas is their increased angiogenic potential. Hyperactive mTORC1 signaling contributes to angiogenesis in hamartomas since it increases the expression of angiogenic VEGF1 (28, 184, 296). Notably, tumors caused by aberrant mTORC1 signaling do not typically possess malignant potential due to the presence of a negative feedback loop from hyperactive mTORC1/S6K1 to IRS-1/PI3K/Akt signaling, which is an oncogenic pathway required for malignancy (125, 296). Due to this negative feedback loop, inhibiting mTORC1 signaling with rapamycin analogs (rapalogs) activates oncogenic Akt signaling, decreasing the rapalogs' efficacy in tumor treatments (114, 216). Emerging data also suggest that mTORC1 has rapamycin-resistant functions in cap-dependent translation and autophagy (57,

83, 88, 301, 302, 335). Rapamycin, for instance, partially inhibits mTORC1-mediated 4E-BP1 phosphorylation, which may be another reason for rapalogs' low efficacy in cancer treatment (57, 83, 88, 104, 301, 302, 335). Indeed, several breast and ovarian cancers display impaired 4E-BP function due to constitutively active mTORC1 signaling (41, 252). Nevertheless, the FDA-approved rapalogs CCI-779 (a.k.a temsirolimus) and RAD-001 (a.k.a everolimus) have been successful to treat renal cell carcinoma (RCC). Future studies will show whether complete inhibition of both mTORC1 and mTORC2 by mTOR catalytic site inhibitors (e.g. Torin, KU-0063794, WAY600, PP242) will prevent tumor growth (57, 83, 88, 105, 301, 302).

F.v. Hypertrophy: Organ growth

Besides its proliferative functions as mentioned above, aberrant mTORC1 signaling also causes diseases due to its role in promoting cell growth (increase in cell size/mass), especially in post-mitotic tissues. Hyperactive mTORC1 signaling, for instance, causes cardiac and renal hypertrophy (48, 280, 296), both of which involve abnormal organ overgrowth due to enlargement of cell size and mass rather than an increase in cell number. Chronic organ hypertrophy impairs organ function and may cause mortality if not treated. Inhibition of mTORC1 by rapamycin treatment reduces cardiac hypertrophy and compensatory renal hypertrophy that occurs upon removal of one kidney (known as uniephrectomy) (48, 280), Consistently, S6K1 knockout mice are resistant to diabetes-induced or compensatory renal hypertrophy (49).

F.vi. Aging

Various studies in model organisms have defined a role for TOR in regulating life span. Similar to genetic inactivation of the components of the insulin/IGF signaling pathway, inactivation of TOR increases longevity in worms, flies, and mice (124, 159-161, 274, 309). More importantly, deletion of S6K1 in mice not only increases longevity but also decelerates age-related pathologies such as bone, immune, and motor dysfunctions (274). Considering the role of

mTOR as an essential nutrient sensor, one might suggest that loss of mTOR signaling mimics calorie restriction, which increases longevity, as well (98, 124). Indeed, lifespan studies in mice showed that there is a significant overlap between the transcriptional profiles of S6K1$^{-/-}$ mice versus diet-restricted mice (274).

Along with DNA damage and telomerase dysfunction, increased oxidative stress is also considered as a cause of aging (323). As cells age, the damaging effects of reactive oxygen species (ROS) induces the expression of tumor suppressor genes and eventually causes cell senescence and death. Since mTORC1 upregulates mitochondrial biogenesis, increasing mTORC1 signaling may speed up the aging process by raising ROS levels in cells (98). In addition, inhibition of mTORC1 signaling increases the expression of anti-oxidizing proteins, slowing down the damaging effect of ROS accumulation in the cell, and preventing age related pathologies such as cardiac dysfunction and muscle degeneration (98). In addition to the anti-oxidizing processes, cells also employ autophagy to remove damaged proteins and organelles due to ROS accumulation. For instance, inhibition of cTOR in *C. elegans* extends life span due to increased autophagy (124). More interestingly, in mice models of age-related neurodegenerative diseases of aggregate-prone proteins such as Huntington, Parkinson's, and Alzheimer's, mTOR inhibition by rapamycin treatment is very effective in inducing autophagy-mediated aggregate cleaning, thus reducing cell death (186, 246, 318).

Maintanence of hemataopoietic stem cells requires quiescence. Hyperactivated mTORC1 signaling due to loss of Tsc1, PTEN, or Wnt1 in these cells causes excessive stem cell proliferation followed by their depletion due to cell senescence (42, 45, 323, 332, 333). These data suggest that inhibition of TOR signaling in various organisms may increase the efficiency of adult stem cell maintenance by promoting quiescence and this may contribute to increased lifespan.

G. mTOR Complex 2

Due to its insensitivity to acute rapamycin treatment, our understanding of mTORC2 function has lagged behind that of mTORC1. Nevertheless, recently developed mTOR catalytic site inhibitors (e.g. Torin1, KU-0063794, PP242, and WAY600) and studies in tissue specific knockouts of mTORC2 components has enabled us to gain further insight into mTORC2 regulation and function (217).

Rictor, mSin1, and mLST8/GβL are the core components of mTORC2, all of which are conserved from yeast to mammals and important for downstream mTORC2 signaling (101, 115, 152, 169, 188, 217, 264, 330). mTORC2 stability requires both rictor and mSin1. Rictor and mSin1 interact tightly and require each other for stability (330). mLST8/GβL binds to mTOR kinase domain and promotes the rictor-mTOR interaction but not the raptor-mTOR interaction (115, 169, 217). mTORC2 also contains PRR5/protor and deptor (228, 238). PRR5/protor interacts with rictor and deptor interacts with the mTOR FAT domain within both mTORC1 and mTORC2. Deptor and mTOR complexes (mTORC1 and mTORC2) negatively regulate each other (238). Deptor is overexpressed in multiple myelomas, which inhibits mTORC1 and relieves the negative feedback from S6K1 to PI3K signaling, thus activating oncogenic Akt signaling (238).

H. Regulation of mTORC2

Insulin/PI3K signaling upregulates mTORC2 signaling and inhibiton of PI3K reduces mTORC2 kinase activity (140, 198, 217, 340). Consistently, the insulin/PI3K pathway promotes mTORC2-associated mTOR S2481 autophosphorylation as well as S1261 phosphorylation in a PI3K-dependent manner (2, 284). The function of mTOR S1261 and S2481 phosphorylation in mTORC2 signaling remains elusive, however. Interestingly, recent findings indicate that insulin/PI3K signaling promotes mTORC2-ribosome association, and this interaction enhances mTORC2 activity. In cancer cells that have increased PI3K signaling, the mTORC2-ribosome interaction is enhanced (340). Opposite to its inhibitory effect on mTORC1, the Tsc1/Tsc2 complex promotes mTORC2 signaling independently of its GTPase activating protein (GAP) function

towards Rheb (139, 140). This model, however, is questioned by a more recent study (66).

Several phosphorylation sites cluster at the conserved C-terminus of rictor (74, 156, 305). S6K1 phosphorylates rictor on T1135, which promotes binding to 14-3-3 proteins, suggesting that mTORC1 functions as an upstream regulator of mTORC2. Although controversial, some studies also suggest that rictor T1135 phosphorylation modestly downregulates mTORC2 signaling towards Akt S473 phosphorylation (74, 156, 305). Upon endoplasmic reticulum stress, GSK3β also phosphorylates rictor on S1235 and downregulates mTORC2 signaling towards Akt (46). mSin1 is another mTORC2 component that undergoes phosphorylation (253). In its hypophosphorylated form, mSin1 has low affinity for mTOR but not rictor. Short-term rapamycin treatment causes depohosphorylation of both mSin1 and rictor, indicating a role for mTORC1 in regulating mSin1 as well as rictor phosphorylation (253)

I. Substrates of mTORC2

mTORC2 phosphorylates the AGC family members Akt, PKCα/β, and SGK1 on their conserved hydrophobic motif (HM) sites (S473; S657/S660; and S422 respectively) and PDK1 phosphorylates them on their T-loop sites (T308; T638/T641; and T256, respectively), contributing to their full activation (5, 6, 84, 101, 112, 140, 147, 182, 260, 324).

I.i. Akt

Growth factors promote mTORC2-dependent Akt phosphorylation on S473 in a PI3K-dependent manner, which promotes Akt catalytic activity in cooperation with PDK1-mediated T-loop phosphorylation on T308 (5, 112, 260). Rictor$^{-/-}$, mSin1$^{-/-}$, and mLST8/GβL$^{-/-}$ MEFs display reduced Akt S473 phosphorylation (112, 147). Loss of S473 phosphorylation due to disruption of mTORC2 function does not reduce Akt signaling towards all substrates (e.g. TSC2; GSK3β), suggesting that HM site phosphorylation confers substrate specificity (87, 115, 138, 143, 151, 266). Eight other kinases other than mTORC2

have been reported to directly phosphorylate Akt S473. These include DNA-PK (DNA-dependent protein kinase), ILK (integrin-linked kinase), ATM, MAPKAPK-2 (MAP kinase-activated protein kinase 2), PKC, PAK1 (p21-activated kinase 1), and IKK-related kinases (IKKε and TBK1) (76, 89, 155, 224, 325). Currently, it is thought that mTORC2 represents the major Akt S473 kinase in the insulin signaling pathway. The other kinases may mediate Akt S473 phosphorylation under different conditions. Recent data suggest that mTORC2 associates with the actively translating ribosomes and also phosphorylates Akt on its TM site T450 co-translationally, in addition to phosphorylating Akt on its HM-site, S473 (340). TM phosphorylation of Akt facilitates its C-terminal folding, which stabilizes newly synthesized Akt by interacting with conserved basic residues in the kinase domain (87, 218).

I.ii. PKC

The lipid activated PKC subfamily of kinases play a role in spatial regulation of cellular signals (254). mTORC2 promotes maturation and stability of PKCα/PKCβ by mediating both TM (T638/T641) and HM site (S657/S660) phosphorylation events (87, 143, 185, 264). In rictor$^{-/-}$ or mSin1$^{-/-}$ MEFs, PKCα protein levels are impaired due to blunted HM and TM site phosphorylations. (143). Although mTORC2 fails to phosphorylate PKC in conventional *in vitro* kinase assays, it phosphorylates PKCα during a coupled *in vitro* kinase/translation assay (143, 218).

I.iii. SGK1

SGK1 represents another member of the AGC family kinases, whose best-studied function is control of sodium transport. Upon insulin or growth factor stimulation, mTORC2 phosphorylates SGK1 on its HM site S422 and promotes its activity (104, 327). mTORC2 signaling activates epithelial sodium channel dependent Na^+ transport in kidney epithelial cells (192), thus it is tempting to speculate that SGK1 mediates this role of mTORC2. PPR5/protor is an important component of mTORC2 for SGK1 phosphorylation on S422. Protor-1$^{-/-}$ mice

show markedly reduced SGK1 (S422) phosphorylation and reduced phosphorylation of SGK1 substrate NDRG1 (N-Myc downregulated gene 1) (230).

J. mTORC2 controls actin cytoskeleton reorganization and cell migration

mTORC2 controls actin cytoskeleton organization in both yeast and mammals in a PKCα-dependent manner (264). mTOR, rictor, and mLST8/GβL knockdown but not raptor knockdown interferes with actin cytoskeleton organization and induces stress fiber formation (152, 264). Recently, Saci et al. reported that the Rho family GTPase Rac1 interacts with mTORC1 and mTORC2, promoting their activities. Whether Rac1 mediates the role of mTORC2 in actin cytoskeleton organization remains elusive (260). In addition, an earlier study also suggested that the Rho GEF P-Rex1 (phosphatidylinositol (3, 4, 5)-trisphosphate-dependent Rac exchange factor 1) binds to mTORC2 and promotes cell migration in response to leucine and amino acid stimulation in a Rac-dependent manner (131). Elevated mTORC1 and mTORC2 activity may also regulate the epithelial-mesenchymal transition (EMT), motility, and metastasis of colorectal cancer cells via RhoA and Rac1 signaling (116). These observations provide rationale for including mTOR kinase inhibitors, as part of the therapeutic regimen for colorectal cancer patients.

K. Tissue-Specific Functions of mTORC2

Rictor, mSin1, and mLST8/GβL null mice are embryonic lethal at day e10.5 due to defects in the fetal vascular system, reflecting the essential role of mTORC2 during development (115). Tissue specific knockout studies of mTORC2 components have provided compelling evidence for significance of this signaling complex in animal physiology (217).

Conditional knockout of rictor in skeletal muscle (rictor$^{muscle-/-}$) impairs glucose transport due to decreased phosphorylation of Akt on S473 and its downstream target AS160 (Akt substrate of 160 kDa), which plays a role in Glut4 (glucose transporter type 4) transport from intracellular membranes to the plasma

membrane (174). mTORC2 also promotes terminal myoblast differentiation (281).

Conditional knockout of rictor in mouse adipose tissue (rictor$^{ad-/-}$) increases body size (65). Rictor$^{ad-/-}$ mice are also hyperinsulinemic and display impaired glucose uptake and Glut4 translocation to the plasma membrane, similar to the phenotypes observed with rictor$^{muscle-/-}$ mice (65, 174, 175)

Deletion of rictor in pancreatic β-cells reduces Akt S473 phosphorylation and causes mild hyperglycemia and glucose intolerance due to reduced β-cell mass, β-cell proliferation, pancreatic insulin content, and glucose-stimulated insulin secretion, reflecting a key role for mTORC2 in maintaining normal β-cell mass (112).

A relatively recent study also reported that mutations in *C. elegans* rictor elevated body fat content, and this metabolic role of mTORC2 in regulating fat mass is mediated by both SGK1 and Akt1/2 (288). Given the evolutionary conservation of TORC2 components, it is possible that mTORC2 also controls cellular and whole body metabolism in mice and humans.

L. Introduction to Dissertation

As described in earlier sections, mTOR as part of mTORC1 or mTORC2, functions as an important regulator of cellular and organismal homeostasis. Although the extra-and intra-cellular signals (e.g. nutrients; growth factors; cellular stress) that regulate mTORC1 function are well established, the molecular mechanisms that underlie direct regulation of mTOR remain poorly defined. To elucidate the molecular mechanisms underlying mTORC1 regulation, we investigated the role of site-specific mTOR phosphorylation in mTORC1 function.

At the outset of this work, four mTOR phosphorylation sites had been reported: S2481, T2446, S2448, and S1261 (2, 51, 52, 135, 212, 237, 273). S2481 is an mTOR autophosphorylation site (237). Stimulation of cells with amino acids or insulin promotes mTOR S2481 autophosphorylation, whereas inhibition of mTOR by drug inhibitors or amino acid depletion reduces S2481

autophosphorylation, suggesting that the phosphorylation status of S2481 is an indicator of mTORC1 intrinsic catalytic activity (285). Near S2481 lies the S2448 phosphorylation site. The mTORC1 substrate S6K1 phosphorylates mTOR S2448 in a feedback manner. Mitogenic signals, amino acids, and insulin stimulation increase while rapamycin and wortmannin treatment decreases mTOR S2448 phosphorylation (51, 52, 135, 273). mTOR phosphorylation on T2446, on the other hand, decreases upon insulin and nutrient stimulation, and increases when AMPK is activated (51, 273), suggesting that T2446 may function as a negative regulatory site on mTOR; little experimental evidence supports this notion, however. Although the regulation of mTOR S2481, S2448, and T2446 phosphorylation is well established, the functional significance of these phosphorylation sites remains poorly understood. S1261 is a more recently identified mTOR site, phosphorylation of which increases upon insulin stimulation via the PI3K/Akt pathway in 3T3-L1 adipocytes. mTOR S1261 phosphorylation promotes mTORC1 autokinase activity, downstream signaling to S6K1 and 4E-BP1, and cell growth, providing the first evidence that site-specific mTOR phosphorylation regulates mTORC1 function (2).

By employing liquid chromatography tandem mass spectrometry (LC-MS/MS) and phospho-specific antibodies, we identified S2159 and T2164 as novel sites of mTOR phosphorylation. S2159 and T2164 phosphorylation sites lie at the extreme N-terminus of mTOR kinase domain and are evolutionarily conserved (Fig. 1.1) (84). Mutational analysis of these phosphorylation sites revealed that mTOR S2159 and T2164 phosphorylation promotes biochemical mTORC1 signaling towards its well-characterized substrates S6K1 and 4E-BP1. mTOR S2159 and T2164 phosphorylation modulates the interaction of mTOR with its partner proteins raptor and PRAS40 and increases mTORC1 intrinsic catalytic activity. In addition, mTOR S2159 and T2164 phosphorylation promotes cell growth (increase in cell size/mass) and cell cycle progression, well-defined cell biological functions of mTORC1 (84).

Moving forward, we performed a kinome screen *in vitro* and identified TBK1 and IKKε as potential kinases that phosphorylate mTOR on S2159. TBK1

and IKKε (a.k.a. IKK-related kinases) share 64% sequence identity with each other and possess overlapping functions (22, 236, 278). We confirmed the kinome screen results and showed that both TBK1 and IKKε phosphorylate mTOR S2159 *in vitro* and in intact cells. Moreover, TBK1 and IKKε interact with mTOR and TBK1$^{-/-}$ or IKKε$^{-/-}$ single knockout MEFs display reduced mTOR S2159 phosphorylation relative to wild-type MEFs. In addition, TBK1$^{-/-}$ single knockout MEFs show impaired mTOR S2481 autophosphorylation and downstream mTORC1 signaling to S6K1, S6, and 4E-BP1, indicating that TBK1 promotes mTOR intrinsic catalytic activity and mTORC1 function.

Upon viral or bacterial infection, TLR3 (Toll-like receptor 3) and TLR4, respectively promote TBK1/IKKε function to increase type I interferon (IFN) synthesis and initiate innate immune responses (96, 130, 164, 166, 276). In this study, we show that stimulation of upstream activators of TBK1/IKKε (i.e. TLR3 and TLR4) promotes mTOR intrinsic catalytic activity as well as mTORC1 and mTORC2 downstream signaling. In addition, mTOR and TBK1/IKKε physically interact with each other, supporting our findings further.

Collectively, the work presented here elucidates the important role of two novel phosphorylation sites on mTOR S2159 and T2164 in mTORC1 function and provides insight into molecular mechanisms of mTORC1 regulation. Discovery of TBK1 and IKKε as mTOR S2159 kinases provides a novel link between TLR signaling pathways and mTORC1, which will improve our understanding of mTORC1 function in innate immunity. Both TBK1 and IKKε additionally play roles in oncogenesis. TBK1 and IKKε promote survival of oncogenic Ras- driven cancer cells (14, 21, 142). IKKε also plays role in chronic inflammation of adipose and liver tissue in obese mice (53). Thus, investigating the novel molecular link between TBK1/IKKε and mTOR S2159 phosphorylation we describe here may improve our understanding of cancer cell survival and chronic inflammation induced-tumorigenesis in obese patients, which may aid the development of novel therapeutics for mTORC1-associated diseases in the future.

Figure 1.1 mTOR domain structure and phosphorylation sites.
mTOR contains a tandem series of HEAT-repeats that mediate protein-protein interactions. Evolutionarily conserved FAT and FATC domains flank the kinase domain (KD). The rapamycin-FKBP12 complex binds the FKBP12-rapamycin binding (FRB) domain. Upon activation, mTOR autophosphorylates on S2481. Upon activation by mTORC1, S6K1 phosphorylates mTOR S2448 via a feedback loop. mTOR S1261, S2159, and T2164 phosphorylation promotes mTORC1 signaling.

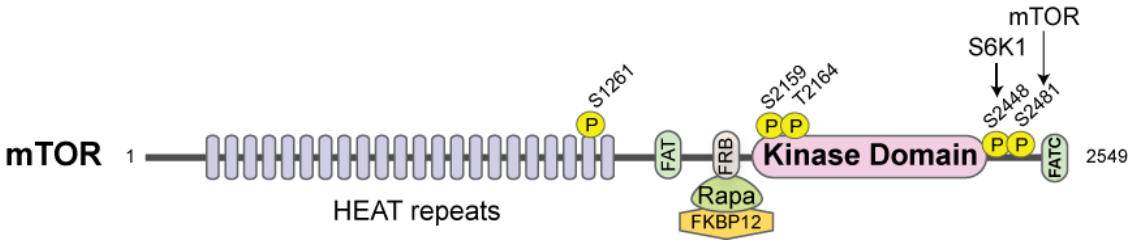


Figure 1.2 Intra- and extra-cellular signals regulate mTORC1 signaling.
Insulin stimulation of cells activates the insulin receptor at the plasma membrane, which in turn activates IRS and PI3K. Activated PI3K phosphorylates PIP_2 and generates PIP_3, which recruits PDK1 and Akt to the plasma membrane. PDK1, then phosphorylates and activates Akt, which in turn phosphorylates and inhibits Tsc2. Inhibition of Tsc1/Tsc2 by Akt promotes Rheb function, augmenting mTORC1 signaling. Upon stimulation with amino acids, Vps34, MAP4K3, and Rag GTPases mediate amino acid availability signals to mTORC1 to promote its function. During energy deprivation, activated AMPK phosphorylates Tsc2 and raptor, events that coordinately suppress mTORC1 signaling. Cellular stress activates Redd1, which in turn activates Tsc2 function to blunt mTORC1 signaling. mTORC1-dependent S6K1 and 4E-BP1 phosphorylation promotes cap-dependent translation, cell growth (increase in cell mass/size), and cell cycle progression.

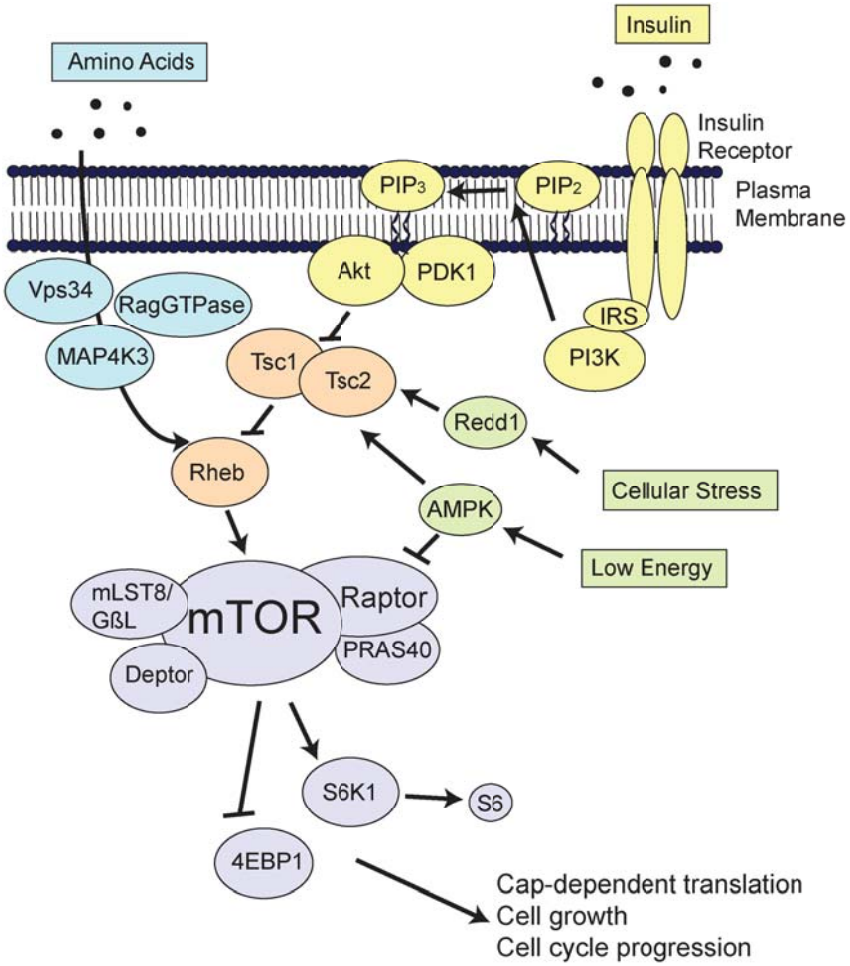


Figure 1.3 S6K1 isoforms, domain structure, and phosphorylation sites.
S6K1 isoforms include p70- and p85-S6K1. Alternative start site usage lengthens the p85-S6K1 N-termini by 23 amino acids. Nuclear localization sequences (NLS) lie within the N-terminal extensions of p85-S6K1. S6K1 contains an acidic N-terminal domain (NTD), kinase domain (KD), linker region, and acidic C-terminal domain (CTD). The NTD contains the TOS-motif while the CTD contains the autoinhibitory pseudosubstrate domain and RSPRR-motif. mTORC1 phosphorylates the hydrophobic motif (HM) site (T389) in the linker region and PDK1 phosphorylates the T-loop site (T229) within the kinase domain. Other regulatory phosphorylation sites including the turn motif (TM) site (S371) are shown.

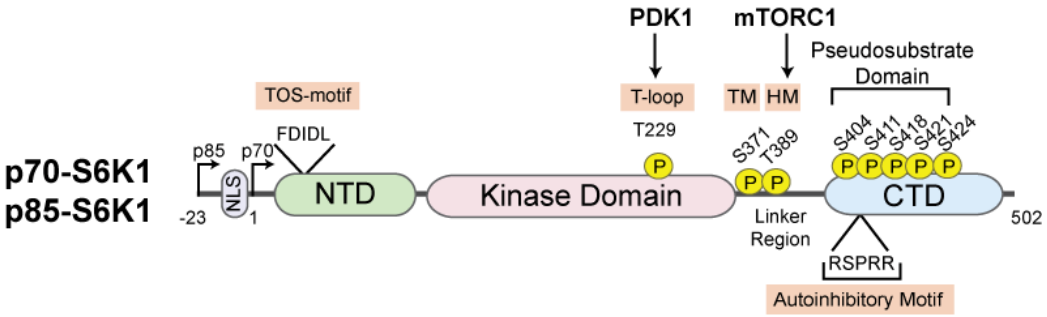
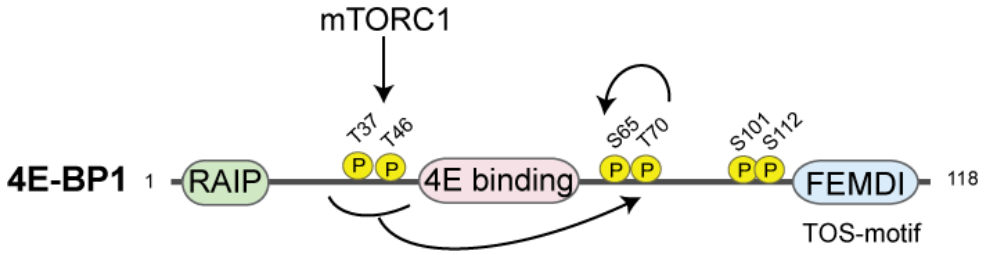


Figure 1.4 4E-BP1 domain structure and phosphorylation sites.
4E-BP1, a translational repressor, represents the best-characterized member of the 4E-BP family of proteins. The RAIP motif (named for the single-letter amino acid code for its sequence Arg13, Ala14, Ile15, Pro16) at the N-terminus facilitates insulin-mediated phosphorylation of 4E-BP1 by mTORC1 on T37/T46. Phosphorylation of T37/T46 acts as priming site for T70 phosphorylation, which in turn acts as a priming site for S65 phosphorylation. The kinases for S101 and S112 are unknown. The eIF4E binding motif (4E-binding motif) lies between amino acids 54-60, and the TOS-motif lies at the extreme C-terminus (amino acids 114-118).



Chapter 2

mTOR Kinase Domain Phosphorylation Promotes mTORC1 Signaling, Cell Growth, and Cell Cycle Progression

Introduction

Aberrant signaling by mTOR, the mammalian target of rapamycin, contributes to the pathogenesis of myriad human diseases (e.g. cancer; benign tumor syndromes; and type II diabetes) and pathophysiologic conditions (e.g. cardiac hypertrophy; coronary artery stent restenosis). Cellular mTOR regulation remains incompletely defined, however (70, 113, 144). mTOR senses and integrates signals from diverse environmental cues such as growth factors and hormones (i.e. insulin; IGF; EGF), nutrients (i.e. amino acids; glucose), and cellular stress (81, 100, 150, 247, 324). mTOR interacts with different partner proteins to form at least two functionally distinct signaling complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (7, 18). Acute rapamycin treatment inhibits the intrinsic catalytic activity and signaling capacity of mTORC1, which contains mTOR, mLST8/GβL, raptor, PRAS40, and deptor (114, 119, 168, 169, 188, 238, 263, 285, 308). Acute rapamycin treatment fails to inhibit mTORC2, which contains shared and distinct partners (7, 18, 100). At the cellular level, mTORC1 promotes cellular anabolic processes including ribosome biogenesis, protein and lipid synthesis, cell growth (increase in cell mass and size), and cell cycle progression, which drives cell proliferation (93, 100, 181, 194). During growth factor and nutrient sufficiency, mTORC1 phosphorylates the translational regulators S6K1 (p70 ribosomal S6 kinase 1) and 4E-BP1 (eukaryotic initiation factor 4E binding protein 1) to coordinately upregulate protein synthesis (93, 194). mTORC1-mediated phosphorylation of S6K1 aids the

assembly of the eIF3 translation initiation complex while phosphorylation of the translational repressor 4E-BP1 induces its release from eIF4E, allowing eIF4E to initiate cap-dependent translation (134, 194). Both S6K1 and 4E-BP1 contain a TOR signaling (TOS) motif that mediates an essential interaction with the scaffolding protein raptor to facilitate the recruitment of substrates to the mTOR kinase (56, 215, 268, 269). mTORC1 also inhibits autophagy, a catabolic process, by phosphorylating and inactivating the autophagic proteins ULK1/2 and ATG13 (158).

Intensive research effort has focused on identifying the biochemical pathways and molecular mechanisms that link environmental cues to mTORC1 regulation. The mTORC1-inhibitory tuberous sclerosis complex (TSC), a heterodimer composed of Tsc1 (hamartin) and Tsc2 (tuberin) proteins, functions as a nexus of convergent signals that regulate mTORC1 (139, 179). Inactivation of either Tsc1 or Tsc2 leads to strong and constitutive mTORC1 signaling, which causes benign tumors to develop in diverse organ systems (139, 179). Tsc2 contains a GTPase-activating protein (GAP) domain that acts on Rheb (Ras homologue enriched in brain), a small GTP-binding protein that activates mTORC1 through an incompletely defined mechanism, possibly involving enhanced substrate recruitment (12, 103, 267, 295, 298). The current model suggests that insulin/PI3K signaling promotes Akt-mediated phosphorylation of Tsc2, which suppresses the inhibitory effect of Tsc1/2 on mTORC1, thus activating Rheb (139, 146, 200, 297). Growth factor-mediated activation of mTORC1 absolutely requires sufficient levels of amino acids. A current model proposes that upon amino acid addition after factor deprivation, mTORC1 rapidly translocates from an ill-defined subcellular compartment to lysosomal membranes that contain Rheb in a manner dependent on the Rag GTPases (170, 261, 262).

Attention has focused more recently on the role of mTORC1 component phosphorylation in mTORC1 regulation. Insulin/PI3K signaling leads to Akt- and mTOR-mediated phosphorylation of PRAS40, which relieves the inhibitory effect of PRAS40 on mTORC1 (97, 222, 263, 308, 313). Insulin/PI3K signaling also

increases mTOR S1261 and mTOR-mediated raptor S863 phosphorylation, events that promote mTORC1 function (2, 100, 315). In addition to phosphorylating PRAS40 and raptor, activated mTOR also phosphorylates deptor, leading to its degradation and thus relieving its mTOR inhibitory action (238). Via a parallel pathway, Ras activation leads to MAPK- and RSK-mediated phosphorylation of Tsc2 (193, 255, 295) and raptor (39, 40) events that promote mTORC1 signaling. In response to energy deprivation, AMPK phosphorylates both Tsc2 and raptor to suppress mTORC1 function (117, 148). Thus, diverse upstream signals converge on Tsc2, PRAS40, and raptor to positively and negatively modulate mTORC1 function.

To elucidate the molecular mechanisms underlying mTORC1 regulation, we have investigated the phosphorylation of mTOR itself. Here we identify S2159 and T2164 as novel mTOR phosphorylation sites that lie at the beginning of the mTOR kinase domain. Collectively, our data demonstrate that mTOR kinase domain phosphorylation modulates the interactions of mTOR with raptor and PRAS40 and leads to increased intrinsic mTORC1 kinase activity, which promotes biochemical signaling, cell growth, and cell cycle progression.

Results

Identification of S2159 and T2164 as novel mTOR phosphorylation sites

To elucidate the biochemical mechanisms that directly control mTORC1 signaling, we have investigated a role for site-specific mTOR phosphorylation in regulation of mTORC1 function. We employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) and identified two novel *in vivo* phosphorylation sites (P sites) on AU1-tagged mTOR immunoprecipitated from transiently transfected HEK293 cells cultured under steady-state conditions (DMEM-FBS). LC-MS/MS analysis identified serine 1261 (S1261) as an unambiguous site of mTOR phosphorylation, as reported previously (1). Such analysis also revealed a doubly phosphorylated peptide with a potential match for mTOR phosphorylation on serine 2159 (S2159) and threonine 2164 (T2164). Unfortunately, we were

unable to unambiguously confirm this potential match, possibly due to low stoichiometry of phosphorylation on these sites (spectra not shown).

To gain further evidence for mTOR phosphorylation on S2159 and T2164, we generated rabbit polyclonal antibodies against mTOR peptides phosphorylated on either S2159 or T2164. To test the site specificity of these antibodies and confirm phosphorylation in intact cells, we generated phosphorylation site-defective Ala substitution mutants with mutations at S2159 and T2164. We transiently transfected HEK293 cells with a vector control or wild-type (WT), S2159A, T2164A, or double S2159A/T2164A (AA) Myc-mTOR. Myc-mTOR was then immunoprecipitated with Myc antibodies and immunoblotted with P-S2159 and P-T2164 antibodies. P-S2159 antibodies detected WT-mTOR but not S2159A- or AA-mTOR. P-T2164 antibodies, on the other hand, detected WT-mTOR but not T2164A- or AA-mTOR, thus confirming the site specificity of each antibody (Fig. 2.1A). Additionally, these data show that mTOR S2159 and T2164 phosphorylation events do not modulate each other, as P-S2159 antibodies detect phosphorylation on the T2164A-mTOR mutant and vice versa. To facilitate our analysis of mTOR phosphorylation, we employed the Invitrogen Flp-In system to generate stable HEK293 cell lines that express vector control or various AU1-mTOR alleles (WT, AA, or kinase dead [KD]) at equivalent levels. As shown in Fig. 2.1B, both P-S2159 and P-T2164 antibodies detected WT but not S2159A/T2164A (AA)-AU1-mTOR, consistent with the transient-transfection analysis shown in Fig. 2.1A. To test whether S2159 and T2164 represents sites of autophosphorylation, similar to S2481, we analyzed phosphorylation on WT versus KD-mTOR as well as phosphorylation on WT-mTOR isolated from cells treated with the ATP-competitive mTOR catalytic inhibitor Torin1. As expected, S2481 phosphorylation on KD-mTOR was abrogated, and Torin1 strongly reduced P-S2481 on WT-mTOR; mTOR S2159 and T2164 phosphorylation was normal on KD-mTOR and unaffected by Torin1, however, thus indicating that neither S2159 nor T2164 represents a site of mTOR autophosphorylation (Fig. 2.1B). Phosphorylation on mTOR S2159 but not T2164 was reduced by the wide-spectrum kinase inhibitor staurosporine, indicating that two different kinases

mediate these phosphorylation events (Fig. 2.1B). Additionally, in a subset of experiments, stable expression of AA-mTOR reduced phosphorylation of S6K1-T389 and the ribosomal protein S6 (a.k.a S6), an S6K1 substrate, suggesting a dominant negative effect on mTORC1 function (Fig. 2.1B). Importantly, we confirmed S2159 and T2164 phosphorylation on endogenous mTOR immunoprecipitated from HEK293 cells (Fig. 2.1C). As seen with exogenously expressed mTOR, P-S2159 on endogenous mTOR was sensitive to staurosporine whereas P-T2164 was not (Fig. 2.1C). Lastly, in our HEK293 cell system, well-described mTORC1-regulating signals (e.g., serum, insulin, amino acids, glucose, and Rheb overexpression) did not modulate phosphorylation on mTOR S2159 or T2164 (data not shown), suggesting either that these phosphorylation events are constitutive or that we have uncovered a novel mTOR regulatory paradigm. S2159 and T2164 lie at the beginning of the C-terminal mTOR kinase domain and show conservation down to vertebrates, with T2164 showing conservation down to flies, worms, plants, and yeasts (Fig. 2.1D).

Phosphorylation of mTOR S2159/T2164 promotes cellular mTORC1 signaling to S6K1

To investigate a role for mTOR S2159/T2164 phosphorylation in regulation of mTORC1 signaling, we compared the abilities of wild-type (WT) and mutant mTOR alleles to mediate the phosphorylation of S6K1 and 4E-BP1, two well-characterized mTORC1 substrates. We introduced phospho-mimetic (S2159D/T2164E) and phosphorylation site-defective (S2159A/T2164A) substitutions at both S2159 and T2164 into the rapamycin-resistant (RR) mTOR backbone (S2035I) (47), which allows the signaling capacity of mTORC1 containing exogenously expressed mutant mTOR alleles to be studied in the absence of endogenous mTORC1 action upon pretreatment of cells with rapamycin to chemically knockout mTORC1 signaling. Point mutation of S2035 within the FKBP12/rapamycin binding (FRB) domain confers rapamycin resistance by abrogating the interaction of mTOR with the FKBP12/rapamycin complex (47). Upon transfection, we compared the abilities of AU1-mTOR-WT

and mutant alleles in the RR backbone to mediate the phosphorylation of cotransfected hemagglutinin (HA)-S6K1 on T389 in the presence of rapamycin.

Rapamycin completely inhibited the insulin-stimulated phosphorylation of HA-S6K1 in cells that coexpressed WT-mTOR, and expression of RR-mTOR, but not RR/kinase-dead (RR/KD)-mTOR, conferred rescue of HA-S6K1 phosphorylation (Fig. 2.2A), as expected. Expression of RR-S2159D/T2164E (RR/DE)-mTOR mediated stronger phosphorylation of HA-S6K1 than expression of RR- or RR-S2159A/T2164A (RR/AA)-mTOR in both the presence and absence of serum growth factors; expression of RR/AA-mTOR mediated weaker phosphorylation of HA-S6K1 than RR-mTOR. Similar results were observed under steady-state conditions in which HEK293 cells cycled asynchronously in DMEM-FBS (Fig. 2.2B). We also generated an alternate rapamycin-resistant allele of mTOR (S2035W; here designated RR-2) that signals to S6K1 in the presence of rapamycin more strongly than the originally described allele, S2035I (203). Similarly, phospho-mimetic DE substitutions in the RR-2 background promoted mTORC1 signaling to S6K1 more strongly than phospho-defective AA substitutions, and phospho-defective AA substitutions mediated weaker mTORC1 signaling relative to RR-mTOR (Fig. 2.2C). These data indicate that mTOR S2159/T2164 phosphorylation in the kinase domain promotes mTORC1-mediated signaling to S6K1.

As an alternative approach to transfecting RR-mTOR alleles in conjunction with rapamycin treatment, we performed an mTOR knockdown/rescue experiment whereby lentivirally delivered shRNA reduced the endogenous expression of human mTOR in HEK293 cells but did not target exogenously expressed rat Myc-mTOR. After knockdown, Myc-mTOR-WT and mutant alleles in a wild-type FRB domain backbone (not rapamycin resistant) were transfected, and mTORC1 signaling capacity was assayed. As expected, WT-mTOR rescued the phosphorylation of S6, whereas KD-mTOR did not (Fig. 2.2D). Consistent with our data obtained by utilizing RR-mTOR alleles (Fig. 2.2A, B, and C), the DE-mTOR mutant mediated stronger phosphorylation of S6 than WT-mTOR, while the AA-mTOR mutant mediated weaker phosphorylation of S6 than DE-

mTOR. These data further confirm that phosphorylation of mTOR S2159/T2164 enhances mTORC1 signaling toward S6K1 and S6.

mTORC1 signaling uniquely requires sufficient levels of amino acids, even during growth factor abundance. We thus tested whether mTOR S2159/T2164 phosphorylation modulates the mTORC1 response to acute amino acid stimulation. HEK293 cells were cotransfected with HA-S6K1 and various RR-AU1-mTOR alleles, amino acid deprived for 60 min in the presence of rapamycin, and then stimulated with amino acid containing medium containing rapamycin for 30 min. Similar to results under insulin-stimulated conditions, RR/DE-mTOR signaled to HA-S6K1 more strongly than RR- and RR/AA-mTOR in response to acute amino acid stimulation (Fig. 2.2E). Consistently, RR/AA-mTOR signaled to S6K1 in a defective manner relative to RR- and RR/DE-mTOR. Unlike under growth factor starvation conditions, however, the RR/DE mutant failed to increase basal HA-S6K1 phosphorylation in the absence of amino acids, even upon prolonged exposure of the Western blot signal (data not shown). These data indicate that mTOR S2159/T2164 phosphorylation enhances mTORC1 signaling only under conditions of amino acid abundance.

We next tested the abilities of transfected RR-, phosphodefective RR/AA-, and phospho-mimetic RR/DE-mTOR alleles to mediate the phosphorylation of HA-S6K1 in response to various concentrations of insulin. Consistent with a gain-of function phenotype, RR/DE-mTOR reached maximal signaling capacity at a submaximal insulin dose (5 nM) whereas RR-mTOR exhibited the expected submaximal signaling at this dose (Fig. 2.3A). Importantly, RR/AA-mTOR exhibited defective signaling at 100 nM insulin relative to RR-mTOR. We also tested the responses of the RR-, RR/AA-, and RR/DE-mTOR alleles to insulin treatment for 0 to 240 min. RR/DE-mTOR signaled to S6K1 more strongly than RR- and RR/AA-mTOR at early time points (Fig. 2.3B). Additionally, while mTORC1 signaling to S6K1 decreased at 240 min after insulin stimulation, the phosphorylation of S6K1 in cells expressing RR/DE-mTOR was maintained at higher levels at this time point. Consistently, RR/AA-mTOR signaled in a defective manner at 30 min of insulin stimulation relative to RR-mTOR. These

data indicate that constitutive phosphorylation on mTOR S2159/T2164 accelerates the time course for insulin-stimulated phosphorylation of S6K1 and additionally renders mTORC1 more resistant to downregulation at later time points.

We next examined the relative contributions of the individual S2159D and T2164E substitutions to the gain-of-function phenotype conferred by the double phospho-mimetic S2159D/T2164E mTOR mutant. As shown in Fig. 2.3C, neither RR/S2159D nor RR/T2164E enhanced the insulin-stimulated phosphorylation of HA-S6K1 as strongly as the RR/DE double mutant did (Fig. 2.3C). Additionally, we tested the contributions of the individual S2159A and T2164A substitutions to the defective signaling conferred by the double S2159A/T2164A mTOR allele. As shown in Fig. 2.3D, single RR/S2159A- and RR/T2164A-mTOR mutants mediated phosphorylation of HAS6K1 more strongly than the double RR/AA-mTOR mutant did (Fig. 2.3D). Overall, these data indicate that phosphorylation events on S2159 and T2164 cooperate to regulate mTORC1 signaling to S6K1.

mTOR S2159/T2164 phosphorylation promotes cellular mTORC1 signaling to 4E-BP1

Recent work indicates that while rapamycin partially inhibits mTORC1-mediated phosphorylation of 4E-BP1, novel ATP-competitive mTOR kinase inhibitors completely block 4E-BP1 phosphorylation, indicating that not all mTORC1 substrates are equally sensitive to rapamycin (56, 57, 88, 301, 302). Thus, rather than employing our RR-mTOR assay in conjunction with rapamycin treatment to study the role of mTOR S2159/T2164 phosphorylation in mTORC1 signaling to 4E-BP1, we utilized AU1-mTOR alleles in a wild-type backbone in the absence of rapamycin treatment. We reasoned that such an approach would be feasible if expression of DE-mTOR was sufficient to augment 4E-BP1 phosphorylation over that mediated by endogenous mTORC1 or if AA-mTOR was to dominantly inhibit mTORC1 function. We thus cotransfected HEK293 cells with various AU1-mTOR alleles together with the mTOR partner Myc-raptor and HA-4E-BP1, followed by serum deprivation and acute insulin stimulation. To

more sensitively measure 4E-BP1 phosphorylation, we utilized a cap pull-down assay. In this assay, cellular eIF4E binds m^7GTP, a chemical moiety that mimics the cap structure found at the 5'ends of mRNAs, which is coupled to Sepharose beads. In the absence of insulin, hypophosphorylated 4E-BP1 binds strongly to eIF4E and thus binds to beads; insulin stimulation, and thus 4E-BP1 phosphorylation, induces the dissociation of 4E-BP1 from eIF4E. As expected, insulin stimulation of WT-mTOR transfected cells reduced 4E-BP1-eIF4E association and increased 4E-BP1 S65 phosphorylation (Fig. 2.4). In cells transfected with the active DE-mTOR allele, less 4E-BP1 associated with eIF4E in the absence of serum growth factors than did so in WT- and AA-mTOR-transfected cells, indicating greater 4E-BP1 phosphorylation. In cells transfected with AA-mTOR, however, more 4E-BP1 associated with eIF4E in the absence or presence of insulin than did so in WT- and DE-mTOR transfected cells, indicating reduced 4E-BP1 phosphorylation. Additionally, AA-mTOR-expressing cells mediated weaker phosphorylation of 4E-BP1 S65 than RR- or RR/DE-mTOR expressing cells. Notably, in the absence of Myc-raptor cotransfection with AU1-mTOR, we do not observe the phenotypes described above (data not shown). Myc-raptor coexpression likely facilitates recruitment of 4E-BP1 to mTOR, thus increasing the efficiency of HA-4E-BP1 phosphorylation. These data suggest that mTOR S2159/T2164 phosphorylation mimics insulin stimulation to promote the phosphorylation of 4E-BP1 and that overexpression of AA-mTOR functions dominantly to suppress the phosphorylation of 4E-BP1 mediated by endogenous mTORC1.

mTOR S2159/T2164 phosphorylation modulates the interaction of mTOR with regulatory partner proteins and augments mTORC1 intrinsic kinase activity

Nutrients (e.g., amino acids and glucose) weaken the mTOR-raptor interaction, as measured by coimmunoprecipitation (168, 169), suggesting a conformational change in mTORC1 structure that correlates with active mTORC1. Similarly, we observe that insulin stimulation also weakens the mTOR-

raptor interaction (2) (Fig. 2.5A). Insulin stimulation weakens the inhibitory raptor-PRAS40 interaction via both Akt- and mTORC1-mediated phosphorylation of PRAS40 (97, 222, 263, 313, 314). We thus tested whether mTOR S2159/T2164 phosphorylation modulates the mTOR-raptor, mTOR-GβL (also known as mLST8), or raptor-PRAS40 interactions. To examine the mTOR-raptor and mTOR-GβL interactions, HEK293 cells were cotransfected with various wild-type backbone Myc-mTOR alleles together with HA-raptor and HA-GβL and lysed in a buffer that enables ready observation of the insulin-induced destabilization of mTOR and raptor. After immunoprecipitation of Myc-mTOR, we found that insulin destabilized the interaction of wild-type Myc-mTOR with HA-raptor, as expected (Fig. 2.5A). Strikingly, the interaction of Myc-DE-mTOR with HA-raptor was weak in the absence of serum growth factors, thus mimicking insulin stimulation; the interaction of Myc-AA-mTOR with HA-raptor was not appreciably strengthened (Fig. 2.5A). Importantly, the interaction of HA-GβL with Myc-mTOR-DE or -AA was unaltered, indicating that these amino acid substitutions do not adversely affect overall mTOR structure (Fig. 2.5A). These data additionally indicate that mutation of the kinase domain at S2159/T2164 does not alter the kinase domain structure, as GβL, which binds the mTOR kinase domain, interacts normally (169).

To examine the raptor-PRAS40 interaction, we cotransfected HEK293 cells with various AU1-mTOR alleles together with Myc-raptor and examined the levels of endogenous PRAS40 in Myc-raptor immunoprecipitates. As shown in Fig. 2.5B, insulin stimulation weakened the raptor-PRAS40 interaction, as expected (263, 308, 314). In cells expressing DE-mTOR, less PRAS40 associated with Myc-raptor than in cells expressing WT- and AA-mTOR under growth factor-deprived conditions. In cells expressing AA-mTOR, however, the raptor-PRAS40 interaction was stronger under both conditions. Our finding that phospho-mimetic substitutions at mTOR S2159/T2164 weaken while phospho-defective substitutions strengthen the inhibitory raptor-PRAS40 interaction provides an additional mechanism to explain the enhanced signaling capacity of DE-mTOR and decreased signaling capacity of AA-mTOR toward S6K1 and 4E-

BP1. By modulating inhibitory interactions of mTOR with mTORC1 components, mTOR S2159/T2164 phosphorylation shifts mTORC1 toward a more active conformation.

We have shown previously that insulin stimulation or Rheb overexpression promotes mTOR S2481 autophosphorylation within mTORC1, which monitors mTORC1 intrinsic catalytic activity in intact cells (285). Additionally, mTOR S1261 phosphorylation is required, at least in part, for the ability of Rheb to promote mTORC1-associated mTOR S2481 autophosphorylation (2). To investigate a role for mTOR S2159/T2164 phosphorylation in control of mTORC1 intrinsic kinase activity, we cotransfected HEK293 cells with various Myc-mTOR alleles (WT backbone) and HA-raptor in the absence or presence of Flag-Rheb. HA-raptor was immunoprecipitated to detect the autophosphorylation of mTORC1-associated mTOR S2481. As shown in Fig. 2.5C, mTORC1 that contained AA-mTOR displayed reduced S2481 autophosphorylation relative to mTORC1 that contained WT-mTOR, similar to S1261A-mTOR. Although mTORC1 that contained DE-mTOR rescued impaired S2481 autophosphorylation observed with AA-mTOR, it did not promote S2481 autophosphorylation further relative to WT-mTOR, which may possibly be due to maximal activation of mTORC1 intrinsic catalytic activity by Rheb overexpression. In the absence of Rheb overexpression, the DE-mTOR containing mTORC1 did not show increased S2481 autophosphorylation compared to WT-mTOR, indicating that mTOR S2159/T2164 phosphorylation is not sufficient to augment mTOR S2481 autophosphorylation under serum-deprived conditions (Fig. 2.5C). Additionally, this experiment confirmed that mTOR S2159/T2164 phosphorylation does not affect mTOR S1261 phosphorylation (Fig. 2.5C), consistent with Fig. 2.1B. Taken together, these data reveal that mTOR S2159/T2164 phosphorylation as well as S1261 phosphorylation contributes to the activation of intrinsic mTORC1 catalytic activity.

mTOR S2159/T2164 phosphorylation promotes mTORC1-mediated cell growth and G1-phase cell cycle progression

To facilitate analysis of mTOR S2159/T2164 phosphorylation in mTORC1-mediated cell growth and cell cycle progression, critical cellular functions of mTORC1, we employed stable HEK293 cells generated via the Flp-In system that express AU1-mTOR-WT, -RR, -RR/DE, -RR/AA, or -RR/KD. Upon chemical knockout of endogenous mTORC1 signaling with rapamycin, we found that RR/DE-mTOR mediated stronger mTORC1 signaling than RR- and RR/AA-mTOR under both basal and insulin-stimulated conditions, as assayed by the phosphorylation of endogenous S6K1, S6, and 4E-BP1 (Fig. 2.6A), similar to results from transient-transfection experiments. Additionally, RR/AA-mTOR signaled to S6K1, S6, and 4E-BP1 in a defective manner relative to RR- and RR/DE-mTOR (Fig. 2.6A).

We next used these stable cell lines to investigate a role for mTOR S2159/T2164 phosphorylation in control of cell growth and cell cycle progression. To assay cell growth, we cultured these stable cell lines in complete medium in the absence or presence of rapamycin for 4 days and used flow cytometry to measure the relative cell size of G1-phase cells by employing the parameter mean forward scatter height (FSC-H). As expected, rapamycin treatment of WT-mTOR-expressing cells decreased the mean FSC-H, while RR-mTOR, but not RR/KD-mTOR, rescued the rapamycin-mediated decrease in cell size, in agreement with earlier work (2, 95) (Fig. 2.6B). Consistent with the gain-of-function phenotype in our biochemical assays, RR/DE-mTOR-expressing cells exhibited a modest but statistically significant increase in cell size (Fig. 2.6B). It is important to note that when these stable cell lines were subjected to culture conditions identical to those employed for cell size analysis for Fig. 2.6B and were analyzed for biochemical signaling, RR/DE-mTOR mediated stronger phosphorylation of S6 than RR-mTOR, while RR/AA-mTOR mediated weaker phosphorylation of S6 than RR-mTOR, consistent with earlier results (Fig. 2.6C). Unexpectedly, RR/AA-mTOR-expressing cells also displayed an increased cell size (Fig. 2.6B).

To understand how both RR/DE- and RR/AA-mTOR increase cell size, we determined how expression of AA- and DE-mTOR affects cell cycle progression relative to RR-mTOR, as perturbation of cell cycle progression can result in indirect effects on cell size. We reasoned that if RR/AA-mTOR expressing cells were to spend an increased time in G1-phase but continued to grow, even at a reduced rate, they would display an increased cell size. To test this idea, stable cells were serum deprived for 24 hr to induce G1-phase accumulation and then stimulated with serum containing medium (DMEM-FBS) for 24 hr to promote cell cycle progression in the absence or presence of rapamycin. The percentage of cells in G1-phase was then determined on a flow cytometer by measuring DNA content after propidium iodide staining. As expected, WT-mTOR-expressing cells stimulated with serum in the presence of rapamycin displayed increased G1-phase content relative to cells stimulated in the absence of rapamycin, and expression of RR-mTOR completely rescued this G1-phase delay (Fig. 2.6D). Expression of RR/AA-mTOR, however, led to impaired rescue, while expression of RR/DE-mTOR conferred rescue (Fig. 2.6D). Thus, RR/AA-mTOR-expressing cells progress through G1-phase more slowly, thus having more time to grow, while RR/DE-mTOR-expressing cells progress through G1-phase normally with an augmented rate of cell growth; in the end, both situations lead to increased cell size.

To confirm the idea that cells expressing RR/AA-mTOR display a larger cell size due to delayed cell cycle progression, we repeated our cell size analysis with cells experiencing a cell cycle block. First, we treated WT-mTOR-expressing cells in the absence or presence of the DNA synthesis inhibitor 2-hydroxyurea (2-HU) for 4 days. 2-HU strongly reduced cell proliferation (data not shown) and perturbed cell cycle kinetics, causing significant accumulation in S-phase (Fig. 2.7A). 2-HU also increased cell size as expected, and importantly, this increased cell size occurred in an mTORC1-dependent manner, as rapamycin blocked the 2-HU-induced cell size increase (Fig. 2.7B). We next analyzed cell size in the stable lines expressing AU1-mTOR-WT, -RR, -RR/DE, -RR/AA, and -RR/KD after 4 days of culture with rapamycin and 2-HU. We found that when cell cycle

progression was eliminated as a complicating factor by using 2-HU, mTORC1 that contained mTOR-RR/DE promoted cell growth to a larger cell size while mTOR-RR/AA did not (Fig. 2.7C). Importantly, 2-HU caused similar S-phase accumulation in the mTOR-RR, -RR/DE, and -RR/AA cell lines (Fig. 2.7D). Thus, our original conclusion based on the cell size data in Fig. 2.6B was correct: AA-mTOR-expressing cells grow to a larger cell size than control cells under cycling conditions due to a primary defect in cell cycle progression, providing them more time to increase in mass and size prior to cell division.

Taken together, these data indicate that mTOR S2159/T2164 phosphorylation promotes both cell growth and cell cycle progression: constitutive phosphorylation on these sites promotes cell growth without appreciably accelerating cell cycle progression, while a lack of phosphorylation blunts cell cycle progression without appreciably reducing cell growth; both situations ultimately increase cell size. Moreover, they suggest the intriguing notion that mTOR S2159/T2164 phosphorylation differentially promotes cell growth and cell cycle progression in a manner dependent on relative stoichiometry of phosphorylation.

Discussion

To elucidate molecular mechanisms underlying mTORC1 regulation, we have investigated the role of site-specific mTOR phosphorylation in mTORC1 function. Among the mTOR phosphorylation sites characterized in the literature thus far (i.e., S2448, S2481, T2446, and S1261), only mTOR S1261 phosphorylation has been reported to regulate mTORC1 signaling (2, 51, 52, 135, 212, 237, 273). Here we identified and characterized two novel mTOR phosphorylation sites, S2159 and T2164, that localize to the N-terminus of the mTOR kinase domain. Mutational analysis demonstrates that mTOR S2159/T2164 phosphorylation promotes mTORC1-mediated signaling to S6K1 and 4E-BP1, similar to mTOR S1261 phosphorylation (2). Mechanistically, mTOR S2159/T2164 phosphorylation modulates the mTOR-raptor interaction and weakens the inhibitory raptor-PRAS40 interaction. These conformational

changes lead to increased mTORC1 intrinsic kinase activity, as monitored by mTOR S2481 autophosphorylation. Moving downstream, mTOR S2159/T2164 phosphorylation promotes both cell growth and cell cycle progression (Fig. 2.8). The rather weak signaling phenotypes conferred by phospho-mimetic and phosphorylation site-defective substitutions at mTOR S2159/T2164 suggest that these phosphorylation events modulate mTORC1 signaling rather than effect major regulatory function.

Tandem mass spectrometry suggested but did not unambiguously confirm dual mTOR phosphorylation on S2159/T2164 in intact cells. The generation of phospho-specific antibodies for P-S2159 and P-T2164 enabled us to confirm site-specific mTOR phosphorylation on S2159 and T2164 in intact cells. Using these antibodies, our data indicate that neither S2159 nor T2164 represents a site of mTOR autophosphorylation; moreover, two different kinases mediate mTOR S2159 versus T2164 phosphorylation: A staurosporine-sensitive kinase mediates P-S2159, while a staurosporine-insensitive kinase mediates P-T2164. In our HEK293 cell system, canonical mTORC1-regulating signals (e.g., insulin, amino acids, and glucose) did not modulate either phosphorylation event (data not shown), suggesting either that these events are constitutive or that we have uncovered a novel mTOR regulatory paradigm in which a currently unknown cellular signal regulates mTOR S2159 and T2164 phosphorylation. The rather weak affinity of the P-S2159 and P-T2164 antibodies for mTOR isolated from intact cells, coupled with our weak mass spectrometry data, suggest low stoichiometry of phosphorylation under steady-state conditions. That phospho-mimetic DE-mTOR mediates stronger substrate phosphorylation than wild-type mTOR supports this hypothesis. Low stoichiometry of phosphorylation may be explained by several potential reasons. As suggested above, an unknown cellular signal may regulate phosphorylation of these sites. Alternatively, mTOR S2159/T2164 phosphorylation may occur primarily in a specific subcellular compartment, on a fraction of total mTOR.

Insulin and nutrients (e.g., amino acids and glucose) weaken the mTOR-raptor interaction via an unknown mechanism(s), which correlates with active

mTORC1 signaling (2, 100, 168, 169). Raptor possesses dual functions in mTORC1 regulation, as it both suppresses and promotes mTORC1 signaling via a strong, inhibitory interaction and a weaker, required interaction, respectively (168). Although Kim et al. reported that insulin stimulation fails to destabilize the mTOR-raptor interaction (168), our results indicate otherwise. This discrepancy may result from the fact that Kim et al. employed an HEK293T cell line that likely possesses poor insulin responsiveness due to high basal signaling, whereas we employed an HEK293 cell line that possesses strong insulin responsiveness with low basal signaling. Indeed, our experience with an HEK293T cell line shows that these cells respond poorly to insulin. We find that mTOR S2159/T2164 phosphorylation weakens the strong mTOR-raptor interaction found in serum-deprived cells, as phospho-mimetic DE-mTOR bypasses the growth factor requirement. Phospho-defective AA-mTOR does not dominantly stabilize the mTOR-raptor interaction upon insulin stimulation, however, suggesting that mTOR S2159/T2164 phosphorylation is not absolutely required for insulin-induced destabilization. Collectively, these data suggest that mTOR S2159/T2164 phosphorylation releases an inhibitory interaction of raptor with mTOR.

mTOR S2159/T2164 phosphorylation additionally modulates the interaction of raptor with PRAS40, as expression of DE-mTOR weakens the raptor-PRAS40 interaction in the absence of serum growth factors while expression of AA-mTOR strengthens the raptor-PRAS40 interaction in both the absence and presence of insulin. Thus, similar to Akt- and mTOR-mediated phosphorylation of PRAS40 (222, 263, 308, 313) phosphorylation on mTOR itself weakens the raptor-PRAS40 interaction to promote mTORC1 signaling. As PRAS40 contains a TOS motif and may thus inhibit mTORC1 signaling by functioning as a competitive substrate (97, 222, 314), mTOR S2159/T2164 phosphorylation may facilitate S6K1 or 4E-BP1 docking and/or positioning by suppressing competitive PRAS40 action. mTOR S2159/T2164 phosphorylation also augments intrinsic mTORC1 catalytic activity, as expression of AA-mTOR reduces mTORC1-associated mTOR S2481 autophosphorylation that occurs

upon Rheb overexpression. Collectively, our work reveals several mechanisms that cooperate to increase mTORC1 signaling capacity upon mTOR S2159/T2164 phosphorylation: mTOR kinase domain phosphorylation weakens inhibitory mTORC1 component interactions and promotes mTORC1 intrinsic catalytic activity, which leads to increased S6K1 and 4E-BP1 phosphorylation.

Eukaryotic cells maintain a constant size over successive generations via the coordinated action of cell growth and cell cycle progression (94). Failure to couple these processes alters cell size homeostasis and can negatively affect development, tissue organization, and organismal physiology. For example, in *S. cerevisiae*, inactivation of the cyclin-dependent kinase Cdc28 causes cells to arrest in G1-phase at an abnormally large cell size because cell growth continues in the face of cell cycle arrest (154). Additionally, in mammals, overexpression of Cdk inhibitors induces cells to accumulate in G1-phase at a larger than normal cell size (95). These data indicate that while cell growth and cell cycle progression are generally coupled, they represent distinct processes (94, 95). mTORC1 functions as a critical controller of both cell growth and cell cycle progression via its positive control of anabolic metabolism (78, 93-95, 311). Coregulation of both cell growth and cell cycle progression by mTORC1 may thus represent a mechanism by which these processes are effectively coupled. We demonstrate here that mTOR S2159/T2164 phosphorylation promotes both mTORC1-mediated cell growth and G1-phase cell cycle progression. By expressing phospho-mimetic RR/DE-mTOR in the presence of rapamycin (to inhibit endogenous mTORC1 signaling), we find that constitutive mTOR S2159/T2164 phosphorylation is sufficient to promote cell growth to an increased cell size but insufficient to accelerate G1-phase progression. By expressing phospho-defective RR/AA-mTOR in the presence of rapamycin, we find that a complete lack of mTOR S2159/T2164 phosphorylation impairs G1-phase progression but does not strongly impair cell growth. Unexpectedly, we noted that cells expressing RR/AA-mTOR display an increased cell size relative to those expressing wild-type RR-mTOR. Our finding that mTORC1 signaling mediated by RR/AA-mTOR results in slower G1-phase progression resolves this

apparent paradox: as RR/AA-mTOR-expressing cells progress more slowly through G1-phase, they have more time to accumulate mass than wild type cells and therefore display an increased cell size due to an indirect effect. Importantly, we experimentally confirmed this notion by removing the effect of cell cycle progression on cell size. When cell cycle progression was blocked using the drug 2-hydroxyurea, RR/AA-mTOR-expressing cells no longer displayed an increased cell size relative to RR-mTOR-expressing cells, whereas RR/DE-mTOR-expressing cells still displayed an increase in cell size under these conditions. While a lack of mTOR S2159/T2164 phosphorylation did not produce a measurable defect in cell growth, it is reasonable to speculate that cells expressing RR/AA-mTOR indeed grow at a reduced rate relative to cells expressing wild-type mTOR.

Recent data suggest that mTORC1-mediated phosphorylation of 4E-BP1 preferentially promotes cell cycle progression over cell growth, while mTORC1-mediated phosphorylation of S6K1 preferentially promotes cell growth over cell cycle progression (78). Thus, not only do cell growth and cell cycle progression represent separable processes, but their regulation may occur via distinct mTORC1-controlled biochemical pathways. Interestingly, we find that under insulin-stimulated conditions, RR/AA-mTOR dominantly inhibits mTORC1 signaling to 4E-BP1 while RR-mTOR and RR/DE-mTOR signal to 4E-BP1 similarly; these biochemical data may explain why RR/AA-mTOR dominantly impairs G1-phase progression while RR/DE-mTOR does not accelerate G1-phase progression. Additionally, under insulin-stimulated conditions, RR/DE-mTOR augments mTORC1-mediated S6K1 but not 4E-BP1 phosphorylation; these data may explain why RR/DE-mTOR augments cell growth but does not accelerate G1-phase progression. Thus, it appears as though stoichiometry of phosphorylation on mTOR S2159/T2164 controls the strength of signaling along either the mTORC1/S6K1 or the mTORC1/4E-BP1 axes, which may explain our cell size phenotypes.

To fully understand the role of mTOR S2159/T2164 phosphorylation in mTORC1 regulation, it will be important to identify the mTOR S2159 and T2164

kinases and to understand their regulation in response to environmental cues. Additionally, it will be important to determine whether these phosphorylation events also occur on mTOR as part of mTORC2 and whether they control mTORC2 signaling. Emerging data indicate that complex multisite phosphorylation on Tsc2, raptor, and now mTOR underlies regulation of mTORC1 signaling in response to diverse environmental cues. A challenge for the future will be to identify the complete set of regulatory phosphorylation sites and understand how they cooperate to regulate mTORC1 signaling and its control of cellular and organismal physiology.

Experimental Methods

Materials

Reagents were obtained from the following sources. Protein A Sepharose CL4B, protein G-Sepharose Fast Flow, and 7-methyl-GTP-Sepharose 4E were from GE Healthcare; 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was from Pierce; Immobilon-P polyvinylidene difluoride (PVDF) membrane (0.45 μm) was from Millipore; autoradiography film (HyBlot CL) was from Denville Scientific; reagents for enhanced chemiluminescence. (ECL) were from Millipore (Immobilon Western chemiluminescent horseradish peroxidase [HRP] substrate); and all chemicals were from either Fisher Chemicals or Sigma.

Commercial antibodies

AU1, Myc (9E10), and HA.11 antibodies were from Covance. Flag-M2 antibody was from Sigma. HRP-conjugated donkey antirabbit and sheep anti-mouse secondary antibodies were from GE Healthcare. P-S6K1-T389 (rabbit monoclonal 108D2) (no. 9234), S6 (no. 2217), P-mTORS2481 (no. 2974), P-4E-BP1-S65 (no. 9451), 4E-BP1 (no. 9452), eIF4E (no. 9742), and glutathione *S*-transferase (GST) (no. 2622) antibodies were from Cell Signaling Technology. PRAS40 antibodies (05-998) were from Upstate.

Custom generation of antibodies to mTOR, P-S2159-mTOR, P-T2164-mTOR,

S6K1, and P-S6

Affinity-purified antipeptide antibodies to mTOR (amino acids 221 to 237; rat), P-mTOR-S1261 (amino acids 1256 to 1266; rat), S6K1 (C-terminal amino acids 485 to 502 of the 70-kDa isoform; rat), and P-S6 (amino acids 232 to 249) were generated as described previously (1). Phospho-specific antibodies against mTOR peptides phosphorylated at S2159 (catalog no. ABS79) or T2164 (catalog no. ABS88) were generated by EMD Millipore (serum-derived antibodies were used).

Plasmids

The pcDNA3/AU1-mTOR wild-type (WT), rapamycin-resistant (RR) (S2035I), kinase-dead (KD) (D2338A), and RR/KD (S2035I, D2338A) plasmids were generously shared by R. Abraham (Wyeth, Pearl River, NY); the pRK5/Myc-raptor plasmid was shared by D. Sabatini (MIT, Boston, MA). Myc-mTOR, pRK5/Myc-mTOR-KD, and pRK5/HA-raptor were obtained from D. Sabatini via Addgene (no. 1861, 8482, and 8513, respectively); the pRK7/HAS6K1, pKH3/HA-mLST8/GβL, and pRK7/Flag-Rheb plasmids were from J. Blenis (Harvard Medical School, Boston, MA); and pACTAG2/3HA-4E-BP1 was from N. Sonenberg (McGill University, Montreal, Canada).

Immunoaffinity purification of mTOR for analysis by MS

HEK293 cells (20 15-cm plates per condition; ~15 x10^6 cells/15-cm plate) were untransfected or transiently transfected with AU1-tagged mTOR (25 μg) and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). AU1-mTOR was immunoprecipitated overnight, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie blue R-250, excised from the gel, digested with trypsin, and analyzed by tandem mass spectrometry (MS/MS). Immunoisolated AU1-mTOR was prepared for analysis by MS/MS as described previously (2).

cDNA mutagenesis and sequencing

Site-directed mutagenesis was performed using QuikChange II XL (Stratagene), and mutated plasmids were fully sequenced. The following mutations were introduced in wild-type pcDNA3/AU1-mTOR: S2159D/T2164E (DE) and S2159A/T2164A (AA). The following mutations were introduced in the rapamycin-resistant (RR) backbone (S2035I) of plasmid pcDNA3/AU1-mTOR to generate the following plasmids: RR-S2159D/T2164E (RR/DE), RR-S2159A/T2164A (RR/AA), RR-S2159D, RR-S2159A, RR-T2164E, and RR-T2164A. A second rapamycin-resistant mutation, here designated RR-2 (S2035W), was introduced in pcDNA3/AU1-mTOR carrying WT or mutant mTOR alleles. The following mutations were introduced in pRK5/Myc-mTOR (wild-type backbone): S2159A, T2164A, S2159A/T2164A (AA), and S2159D/T2164E (DE). The following oligonucleotide primers were used to create point mutations in the rat mTOR cDNA (accession no. L37085) (capital letters indicate mismatches, and the three underlined nucleotides represent the codon mutated): for S2035I (RR), primer 1, 5'ggcctagaagaggccATtcgcttgtactttggg-3', and primer 2, 5'-cccaaagtacaagcgaATggcctcttctaggcc-3'; for S2035W (RR-2), primer 1, 5'-ggcctagaagaggcctGGcgcttgtactt-3', and primer 2, 5'-aagtacaagcgCCaggcctcttctaggcc-3'; for S2159A, primer 1, 5'-ccatagccccgGctttgcaagtcatc-3', and primer 2, 5'-gatgacttgcaaagCcggggctatgg-3'; for S2159D, primer 1, 5'-ccatagccccgGAtttgcaagtcatc-3', and primer 2, 5'-gatgacttgcaaaTCcggggctatgg-3'; for T2164A, primer 1, 5'-gcaagtcatcGcatccaagcagaggcc-3', and primer 2, 5'-ggcctctgcttggatgCgatgacttgc-3'; and for T2164E, primer 1, 5'-gcaagtcatcGAatccaagcagaggcc-3', and primer 2, 5'-ggcctctgcttggatTCgatgacttgc-3'.

Cell culture, drug treatment, and transfection

HEK293 cells were cultured in DMEM that contained high glucose (4.5 g/liter), glutamine (584 mg/liter), and sodium pyruvate (110 mg/liter) concentrations (Gibco/Invitrogen), supplemented with 10% fetal bovine serum (FBS) (HyClone), and were incubated at 37°C in a humidified atmosphere containing 5% CO2. Cells were serum deprived via incubation in DMEM supplemented with 20 mM

HEPES (pH 7.2) for ~20 hr. Insulin (100 nM) (Invitrogen) was added to serum-deprived cells and left for 30 min. Where indicated, serum-deprived cells were pretreated with rapamycin (20 ng/ml) (Calbiochem) for 30 min prior to the addition of insulin. For drug treatments under steady-state conditions (cycling in DMEM-FBS), cells were incubated in rapamycin (20 ng/ml) for 2 hr, staurosporine (1 μM) (Calbiochem) for 3 h, or Torin1 (50 nM) (a kind gift from S. Sabatini) for 3 h. For amino acid deprivation, HEK293 cells were incubated in Dulbecco's phosphate-buffered saline (PBS) containing D-glucose (1 g/liter), sodium pyruvate (36 mg/liter), and 10% dialyzed FBS for 60 min. Unless indicated otherwise in the figure legends, HEK293 cells on 60-mm plates were transfected according to the manufacturer's directions using TransIT-LT1 (Mirus) and a total of 5 μg of DNA per plate. The specific amounts of experimental plasmid transfected are stated in the figure legends. Cells were lysed at ~24 to 48 h posttransfection.

Cell lysis, immunoprecipitation, m^{7}GTP cap pulldown, and immunoblotting

Unless indicated otherwise, cells were washed twice with ice-cold PBS and scraped into ice-cold lysis buffer A containing NP-40 (0.5%) and Brij 35 (0.1%), as described previously (2). For Fig. 2.4, cells were lysed in buffer A containing CHAPS (0.3%). For Fig. 1 and in the mTOR-raptor coimmunoprecipitation experiment (see Fig. 2.5A), cells were lysed in buffer B (40 mM HEPES [pH 7.4], 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM β-glycerophosphate, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 40 μg/ml phenylmethylsulfonyl fluoride [PMSF]) containing CHAPS (0.3%), as originally described (168) (to maintain the mTOR-raptor interaction and to observe its prominent destabilization upon insulin stimulation). Lysates were spun at 13,200 rpm for 5 min at 4°C, and the postnuclear supernatants were collected. The Bradford assay was used to normalize protein levels for immunoprecipitation and immunoblot analysis. For immunoprecipitation, whole-cell lysate (WCL) was incubated with antibodies for ~2 hr or overnight at 4°C, incubated with protein G-Sepharose beads for 1 hr, washed three times in lysis buffer, and resuspended in

1x sample buffer, as described previously (2). For cap pulldown assays, WCLs were incubated with m^7GTP-Sepharose beads for 2 hr, washed three times in lysis buffer, and resuspended in 1x sample buffer. Samples were resolved by SDS-PAGE and transferred to PVDF membranes by using Towbin transfer buffer, as described previously (2). Immunoblotting was performed by blocking PVDF membranes in Tris-buffered saline containing Tween 20 (TBST) and 3% nonfat milk and incubating the membranes in TBST with 2% bovine serum albumin (BSA) containing primary antibodies or secondary HRP-conjugated antibodies, as described previously (2). Blots were developed by ECL and detected with autoradiographic film or digitally with a Chemi-Doc-It HR 410 system (UVP).

Generation of Flp-In stable cell lines

The Invitrogen Flp-In system was used to generate stable HEK293 cell lines that express various AU1-mTOR alleles. This system utilizes a genetically engineered HEK293 cell line (HEK293/FRT) that possesses an FLP recombination target (FRT) integrated into a transcriptionally active region of the genome; thus, after drug selection, each clone expresses AU1-mTOR at equivalent levels, thus allowing one to simply pool colonies for analysis. After subcloning of AU1-mTOR alleles into pcDNA5/FRT, HEK293/FRT cells (maintained in DMEM–10% FBS plus Zeocin [100 μg/ml]) were cotransfected with pcDNA5/FRT/AU1-mTOR (0.2 μg) and pOG44 (2 μg) (recombinase). At 3 days posttransfection, stable integrants were selected in DMEM–10% FBS containing hygromycin (200 μg/ml). (Note that the Invitrogen HEK293/FRT cell line is not as insulin sensitive as the HEK293 cell line employed throughout this work [originally from the lab of J. Blenis].)

Flow cytometry to measure cell size and DNA content

Flp-In HEK293 cells that stably express vector control or AU1-mTOR alleles were cultured in DMEM-FBS-hygromycin (100 μg/ml) in the absence or presence of rapamycin (20 ng/ml) and/or hydroxyurea (0.5 mM) for 96 hr. For cell size and

DNA content analysis, cells were harvested with PBS-EDTA (0.1%), fixed in 80% ethanol, and stained with propidium iodide (PI)-RNase A solution, as described previously (2). For cell size analysis, the mean forward scatter height (FSC-H) of ~3,000 single, unclumped G1-phase cells was determined by gating on PI fluorescence using a BD Biosciences FACSCalibur with CellQuest software. For cell cycle analysis, the PI fluorescence of ~10,000 single, unclumped cells was analyzed. Markers for each cell cycle phase (G0/G1, S, and G2/M) were set to quantitate percent G0/G1, S, and G2/M using CellQuest software.

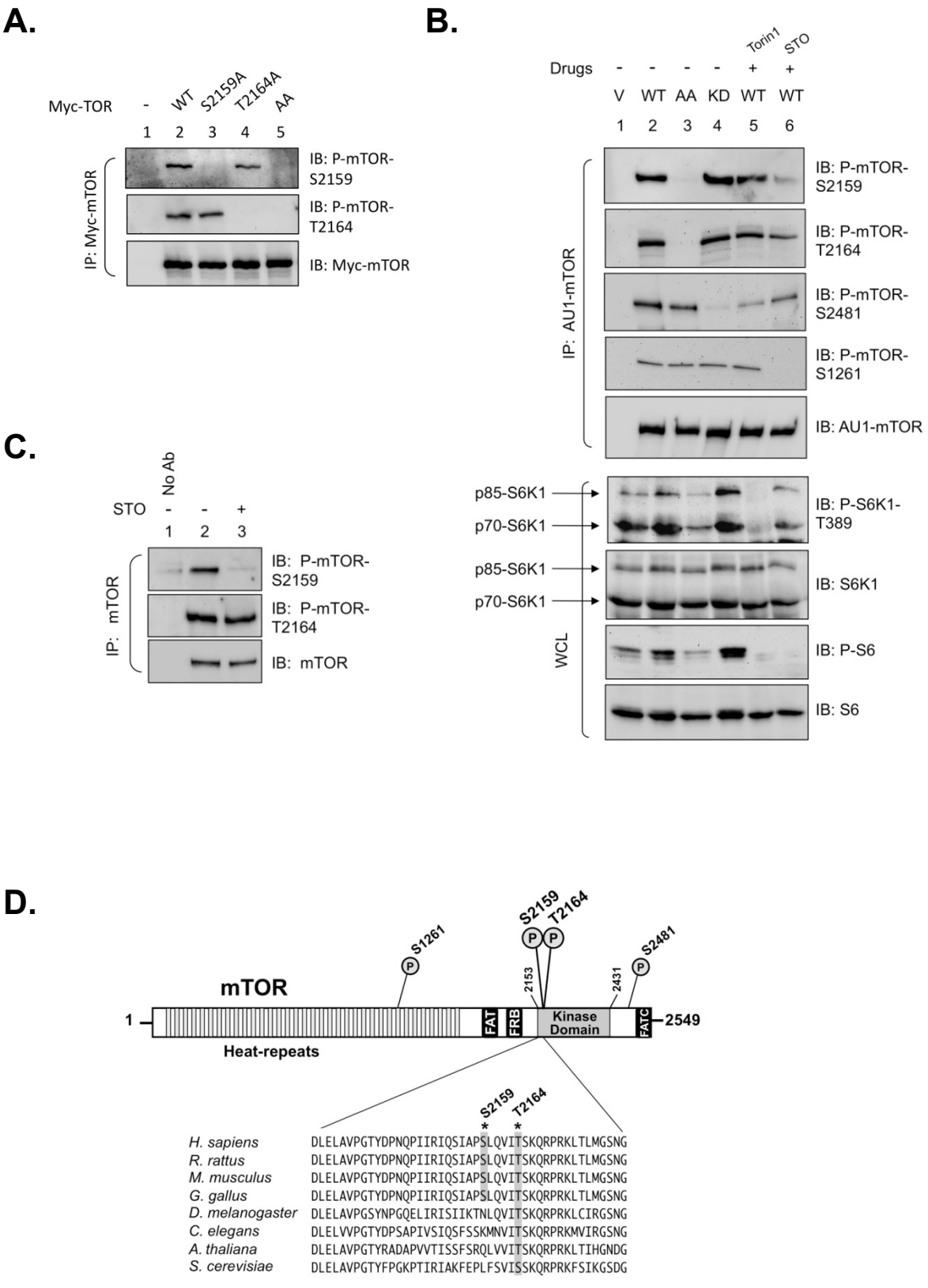
mTOR knockdown with lentivirally delivered shRNA

High-titer lentiviruses encoding scrambled or TOR_2 short hairpin RNAs (shRNAs) were generated by the University of Michigan Vector Core. These LKO-based plasmids were originally from the D. Sabatini lab but were obtained from Addgene (catalog no.1856 and 1864, respectively). HEK293 cells were infected with lentiviral supernatant in the presence of protamine sulfate (6 μg/ml) for 24 h and then selected in puromycin (2.5 μg/ml) for 5 days.

Image editing

For some figures, irrelevant lanes were removed from a scanned autoradiograph and flanking lanes juxtaposed using Adobe Photoshop. The presence of a thin, vertical black line indicates such a modification.

Figure 2.1 Identification of S2159 and T2164 as novel sites of mTOR phosphorylation.
A. P-S2159 and P-T2164 antibodies are site specific. HEK293 cells seeded on 10-cm plates were transiently transfected with vector control or various Myc-mTOR alleles (10 μg) and cultured in DMEM-FBS. Myc-mTOR was immunoprecipitated (IP) from whole-cell lysate (WCL) with Myc antibodies and immunoblotted (IB) with the indicated antibodies. Each lane represents an IP from WCL containing ~300 μg protein.
B. S2159 and T2164 are not sites of mTOR autophosphorylation. Flp-In HEK293 cells that stably express vector control or various AU1-mTOR alleles were cultured in DMEM-FBS containing hygromycin [200 μg/ml]. AU1-mTOR was immunoprecipitated from WCL with AU1 antibodies and immunoblotted with the indicated antibodies. Each lane represents an IP from WCL containing ~1,250 μg protein. WCLs were also immunoblotted directly.
C. Endogenous mTOR undergoes phosphorylation on S2159 and T2164. HEK293 cells were cultured in DMEM-FBS. Endogenous mTOR was immunoprecipitated from WCL with mTOR antibodies and immunoblotted with the indicated antibodies (Ab). Each lane represents an IP from WCL containing ~5 mg protein.
D. Localization of S2159/T2164, as well as S1261 and S2481, within the mTOR domain structure and alignment of mTOR S2159/T2164 from various organisms using the algorithm Clustal W. S2159 and T2164 lie at the beginning of the mTOR kinase domain (positions 2153 to 2431).


A.
Myc-TOR
-
WT
S2159A
T2164A
AA
1 2 3 4 5
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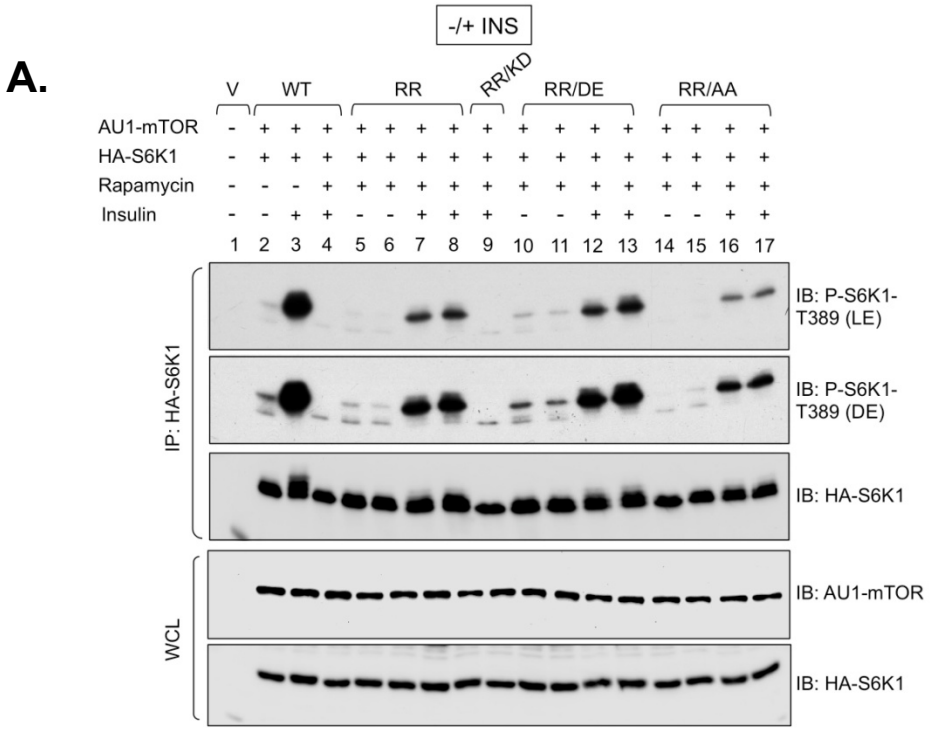
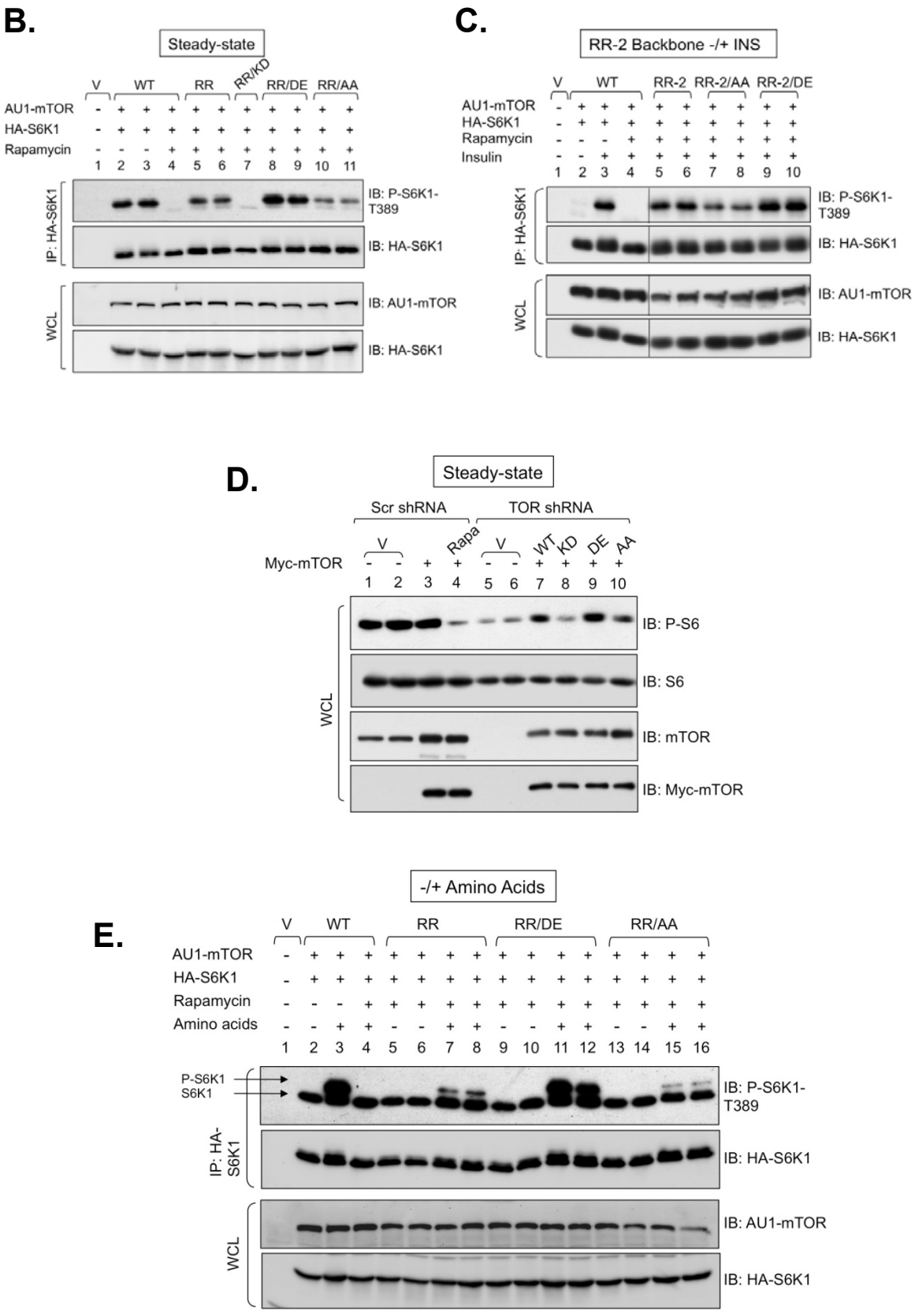
IB: P-mTOR-S2159
IB: P-mTOR-T2164
IB: Myc-mTOR
B.
Drugs
-
-
-
-
Torin1 +
STO +
V WT AA KD WT WT
1 2 3 4 5 6
IP: AU1-mTOR
IB: P-mTOR-S2159
IB: P-mTOR-T2164
IB: P-mTOR-S2481
IB: P-mTOR-S1261
IB: AU1-mTOR
p85-S6K1
p70-S6K1
IB: P-S6K1-T389
p85-S6K1
p70-S6K1
IB: S6K1
WCL
IB: P-S6
IB: S6
C.
STO
No Ab
-
-
+
1 2 3
IP: mTOR
IB: P-mTOR-S2159
IB: P-mTOR-T2164
IB: mTOR
D.
mTOR
S1261
S2159
T2164
S2481
2153
2431
1
FAT
FRB
Kinase Domain
FATC
2549
Heat-repeats
S2159
T2164
H. sapiens DLELAVPGTYDPNQPIIRIQSIAPSLQVITSKQRPRKLTLMGSNG
R. rattus DLELAVPGTYDPNQPIIRIQSIAPSLQVITSKQRPRKLTLMGSNG
M. musculus DLELAVPGTYDPNQPIIRIQSIAPSLQVITSKQRPRKLTLMGSNG
G. gallus DLELAVPGTYDPNQPIIRIQSIAPSLQVITSKQRPRKLTLMGSNG
D. melanogaster DLELAVPGSYNPGQELIRISIIKTNLQVITSKQRPRKLCIRGSNG
C. elegans DLELVVPGTYDPSAPIVSIQSFSSKMNVITSKQRPRKMVIRGSNG
A. thaliana DLELAVPGTYRADAPVVTISSFSRQLVVITSKQRPRKLTIHGNDG
S. cerevisiae DLELAVPGTYFPGKPTIRIAKFEPLFSVISSKQRPRKFSIKGSDG

Figure 2.2 mTOR S2159/T2164 phosphorylation promotes mTORC1 signaling to S6K1.
A. mTOR S2159/T2164 phosphorylation promotes S6K1 phosphorylation in the absence and presence of insulin (INS). HEK293 cells were transiently transfected with vector control or cotransfected with various AU1-mTOR alleles (5 μg) together with HA-S6K1 (0.5 μg) and cultured in DMEM-FBS. Transfected cells were serum deprived (20 hr), pretreated without or with rapamycin (20 ng/ml) for 30 min, incubated in the absence or presence of insulin (100 nM) for 30 min, and then lysed. HA-S6K1 was immunoprecipitated from WCL with HA antibodies and immunoblotted with the indicated antibodies (upper panels). WCLs were also immunoblotted directly (lower panels). LE, light exposure; DE, dark exposure.
B. mTOR S2159/T2164 phosphorylation promotes S6K1 phosphorylation under steady-state conditions. The procedure was similar to that described for panel A except that cycling cells were cultured in DMEM-FBS and pretreated without or with rapamycin (20 ng/ml) for 2 hr prior to lysis.
C. Within the mTOR RR-2 backbone, mTOR S2159/T2164 phosphorylation promotes S6K1 phosphorylation in response to insulin. The procedure was similar to that described for panel A, except that an alternate rapamycin-resistant mTOR allele was employed, which contains S2035W (RR-2) rather than the more commonly used S2035I (RR).
D. Phospho-mimetic DE-mTOR (S2159D/T2164E) rescues mTORC1 signaling better than phospho-defective AA-mTOR (S2159A/T2164A) after mTOR knockdown. Cells infected with lentiviruses encoding scrambled shRNA (Scr) or shRNA engineered to knock down endogenous mTOR (human) but not exogenous AU1-mTOR (rat) were transfected with various Myc-mTOR alleles (5 μg). WCLs were immunoblotted directly with the indicated antibodies.
E. mTOR S2159/T2164 phosphorylation promotes S6K1 phosphorylation in response to amino acids. The procedure was similar to that described for panel A except that prior to lysis, transfected cells were pretreated without or with rapamycin (20 ng/ml) for 30 min, amino acid deprived (60 min), and then stimulated with amino acids (30 min) via incubation in DMEM-FBS in the continuous absence or presence of rapamycin.




B.
Steady-state
V
WT
RR
RR/KD
RR/DE
RR/AA
AU1-mTOR
HA-S6K1
Rapamycin
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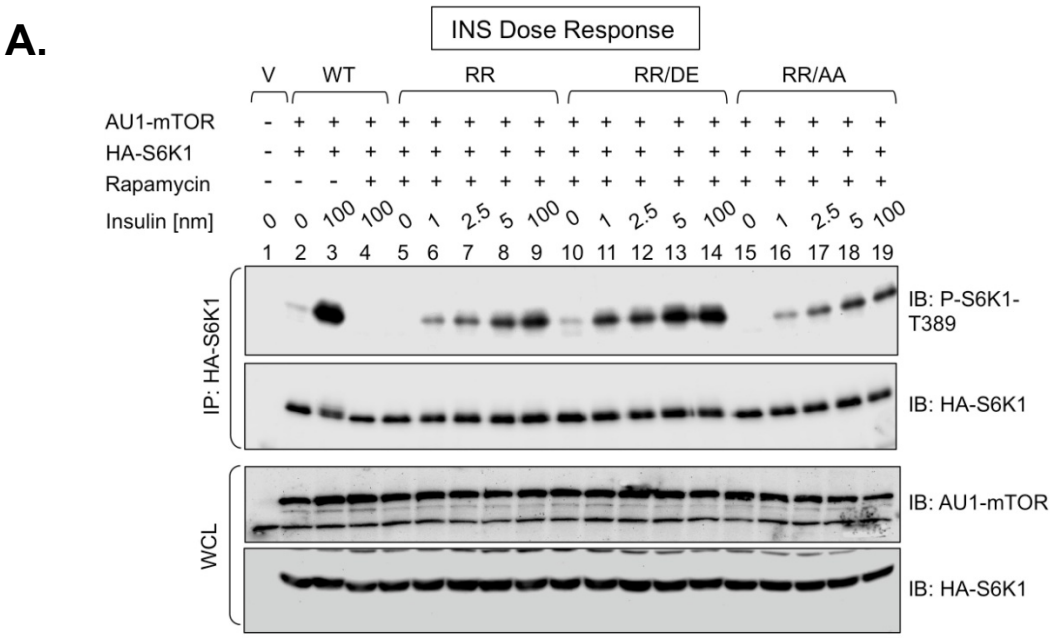
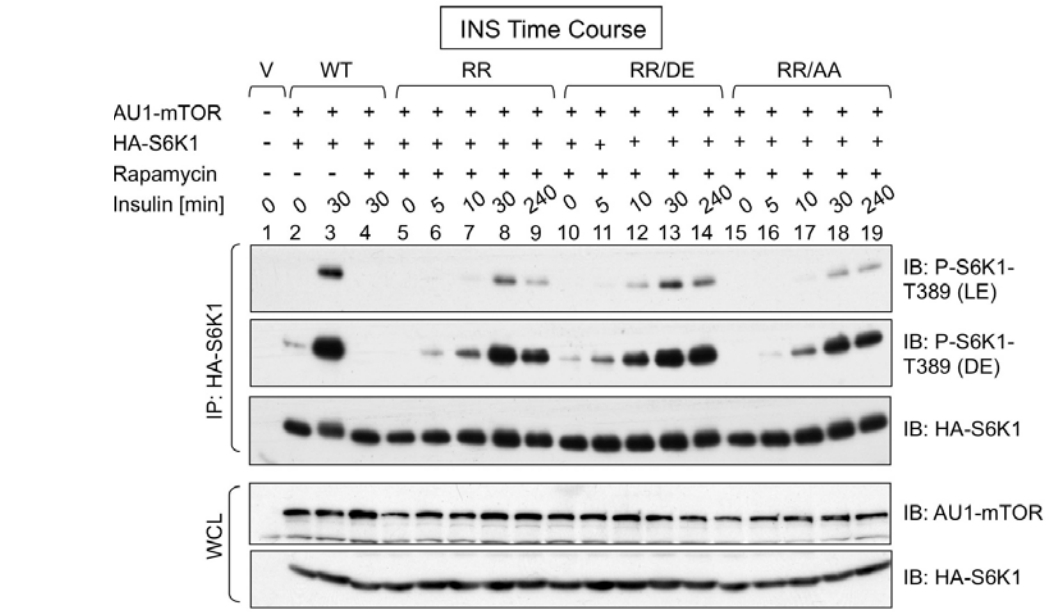
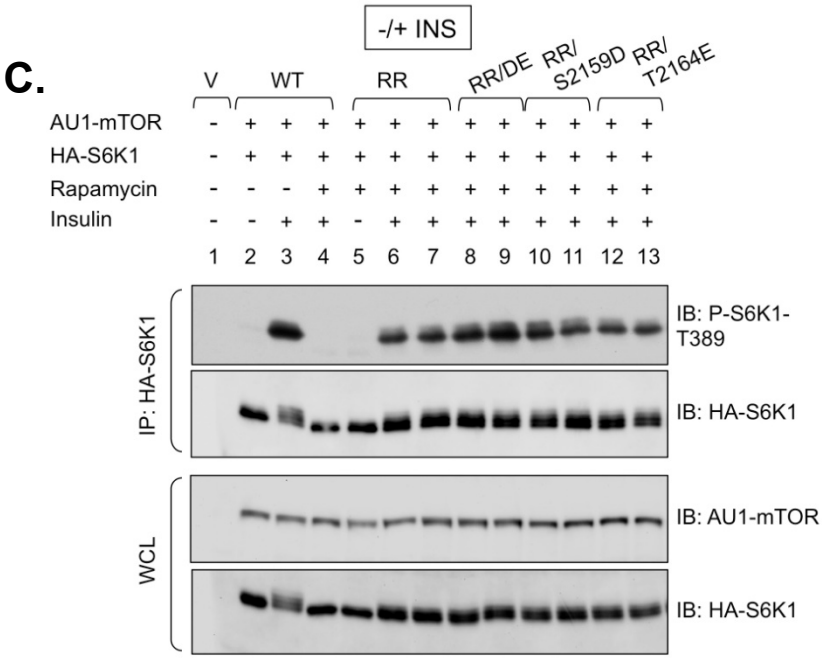
IP: HA-S6K1
IB: P-S6K1-T389
IB: HA-S6K1
WCL
IB: AU1-mTOR
IB: HA-S6K1
C.
RR-2 Backbone -/+ INS
V
WT
RR-2
RR-2/AA
RR-2/DE
AU1-mTOR
HA-S6K1
Rapamycin
Insulin
1 2 3 4 5 6 7 8 9 10
IP: HA-S6K1
IB: P-S6K1-T389
IB: HA-S6K1
WCL
IB: AU1-mTOR
IB: HA-S6K1
D.
Steady-state
Scr shRNA
TOR shRNA
V
Rapa
V
WT
KD
DE
AA
Myc-mTOR
1 2 3 4 5 6 7 8 9 10
WCL
IB: P-S6
IB: S6
IB: mTOR
IB: Myc-mTOR
E.
-/+ Amino Acids
V
WT
RR
RR/DE
RR/AA
AU1-mTOR
HA-S6K1
Rapamycin
Amino acids
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
P-S6K1
S6K1
IP: HA-S6K1
IB: P-S6K1-T389
IB: HA-S6K1
WCL
IB: AU1-mTOR
IB: HA-S6K1

Figure 2.3 mTOR S2159/T2164 phosphorylation increases the sensitivity and duration of insulin-stimulated mTORC1 signaling.
A. Insulin dose response. The procedure was similar to that described for Fig. 2.2A except that cells were stimulated with various concentrations of insulin for 30 min as indicated.
B. Insulin time course. The procedure was similar to that described for Fig. 2.2A except that cells were stimulated with insulin (100 nM) for various amounts of time as indicated. LE, light exposure; DE, dark exposure.
C and D. Phosphorylation on mTOR S2159 and T2164 each contributes to insulin-stimulated mTORC1 signaling. The procedure was similar to that described for Fig. 2.2A.






C.
-/+ INS
V
WT
RR
RR/DE
RR/ S2159D
RR/ T2164E
AU1-mTOR
HA-S6K1
Rapamycin
Insulin
1 2 3 4 5 6 7 8 9 10 11 12 13
IP: HA-S6K1
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IB: HA-S6K1
WCL
IB: AU1-mTOR
IB: HA-S6K1

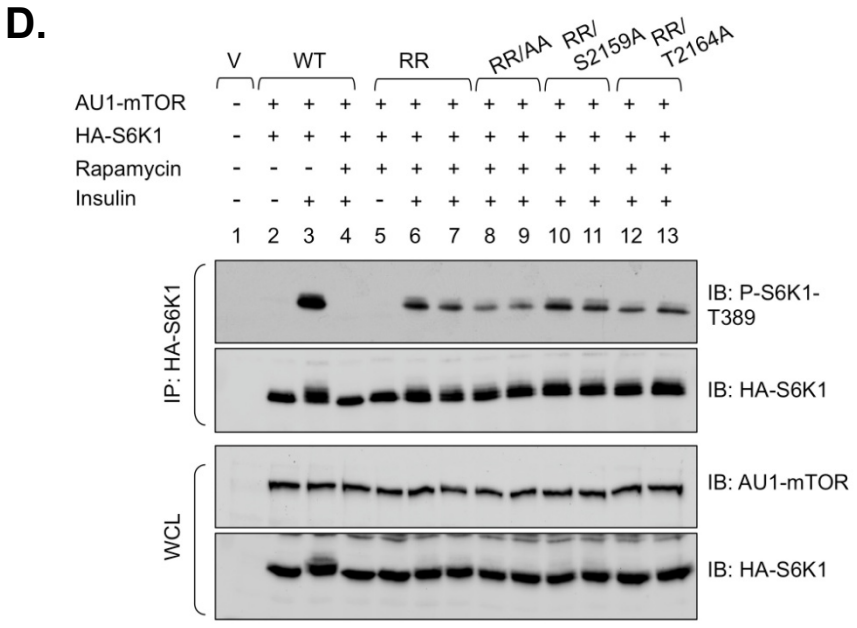

D.
V
WT
RR
RR/AA
RR/ S2159A
RR/ T2164A
AU1-mTOR
HA-S6K1
Rapamycin
Insulin
1 2 3 4 5 6 7 8 9 10 11 12 13
IP: HA-S6K1
IB: P-S6K1-T389
IB: HA-S6K1
WCL
IB: AU1-mTOR
IB: HA-S6K1

Figure 2.4 Phosphorylation of mTOR S2159/T2164 promotes mTORC1 signaling to 4E-BP1. HEK293 cells on 10-cm plates were transfected with various AU1-mTOR alleles (7 μg) together with 3HA-4E-BP1 (2 μg) and Myc-raptor (1 μg), deprived of serum (20 hr), incubated in the absence or presence of insulin (100 nM) for 30 min, and lysed in buffer A containing CHAPS. HEK293 lysate was incubated with m^7GTP-Sepharose beads to pull down eIF4E and associated 4E-BP1. m^7GTP pulldowns and WCLs were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. LE, light exposure; DE, dark exposure. The P-4E-BP1-S65 signal represents endogenous 4E-BP1, as it is much stronger than the P-S65 signal on exogenous HA-4E-BP1.

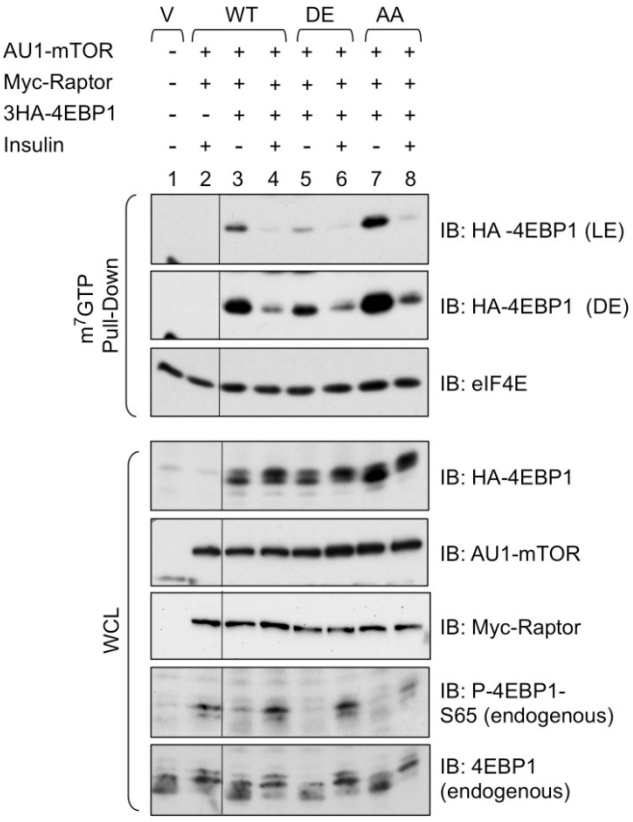
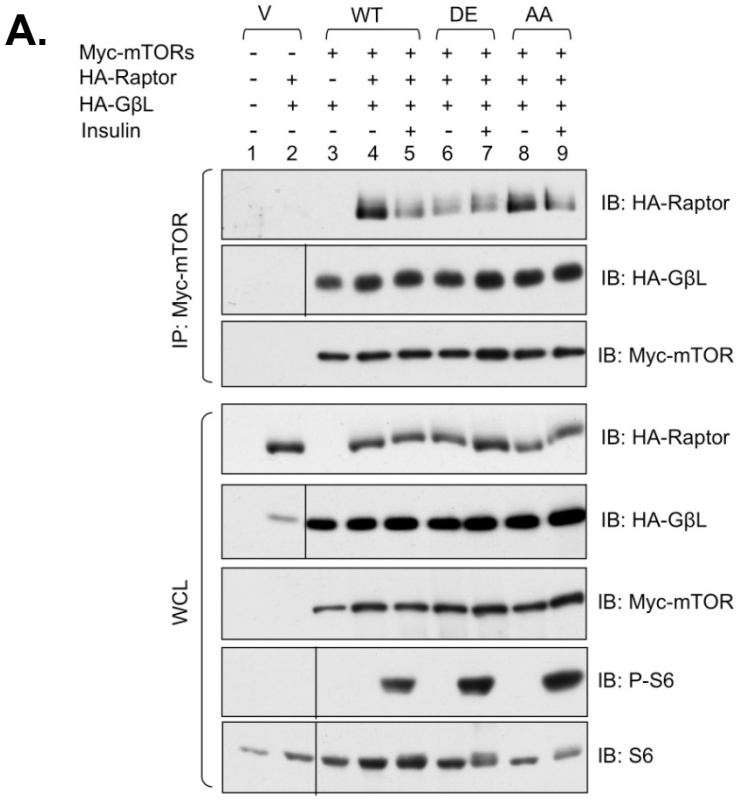
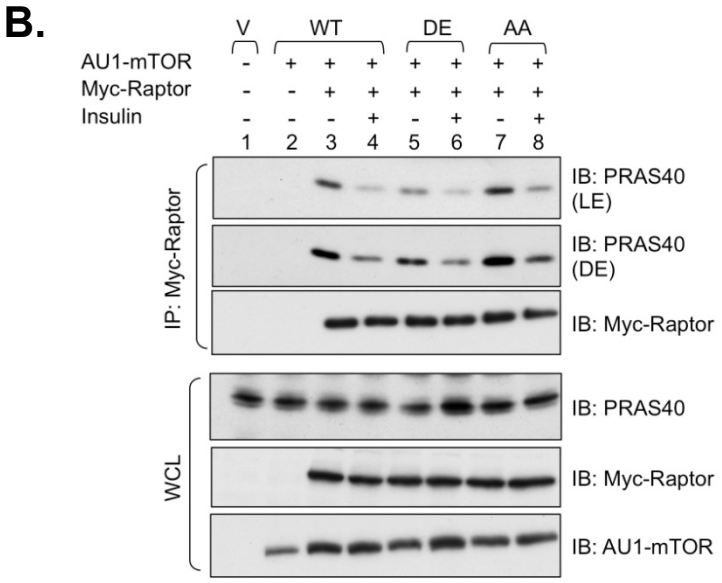


Figure 2.5 mTOR S2159/T2164 phosphorylation weakens the mTOR-raptor and raptor-PRAS40 interactions and augments mTORC1 intrinsic kinase activity.
A. mTOR S2159/T2164 phosphorylation alters the interaction of mTOR with raptor but not mLST8/GβL. HEK293 cells were cotransfected with HA-mLST8/GβL (0.5 μg) and HA-raptor (0.5 μg) together with various Myc-mTOR alleles (4 μg), cultured in DMEM-FBS, serum deprived (20 hr), incubated in the absence or presence of insulin (100 nM) for 30 min, and lysed in buffer B containing CHAPS. Myc-mTOR was immunoprecipitated from WCL with Myc antibodies and immunoblotted as indicated (upper panels). WCLs were also immunoblotted directly (lower panels). The phosphorylation of endogenous S6 confirmed cellular insulin stimulation.
B. mTOR S2159/T2164 phosphorylation alters the interaction of raptor with PRAS40. HEK293 cells seeded on 10-cm plates were cotransfected with various AU1-mTOR alleles (10 μg) together with Myc-raptor (0.5 μg), cultured in DMEM-FBS, serum deprived (20 hr), incubated in the absence or presence of insulin (100 nM), and lysed in buffer A containing CHAPS. Myc-raptor was immunoprecipitated from WCL with Myc antibodies and immunoblotted as indicated (upper panels). WCLs were also immunoblotted directly (lower panels). LE, light exposure; DE, dark exposure.
C. mTOR S2159/T2164 phosphorylation is required for mTORC1-associated mTOR S2481 autophosphorylation. HEK293 cells were cotransfected with various Myc-mTOR alleles (2.5 μg) together with HA-raptor (0.5 μg) and Flag-Rheb (2.5 μg), as indicated. Cells were serum deprived (20 hr) and lysed. HA-raptor was immunoprecipitated from WCL with HA antibodies to immunoisolate mTORC1 and immunoblotted as indicated (upper panels). WCLs were also immunoblotted directly (lower panels).




B.
V
WT
DE
AA
AU1-mTOR
Myc-Raptor
Insulin
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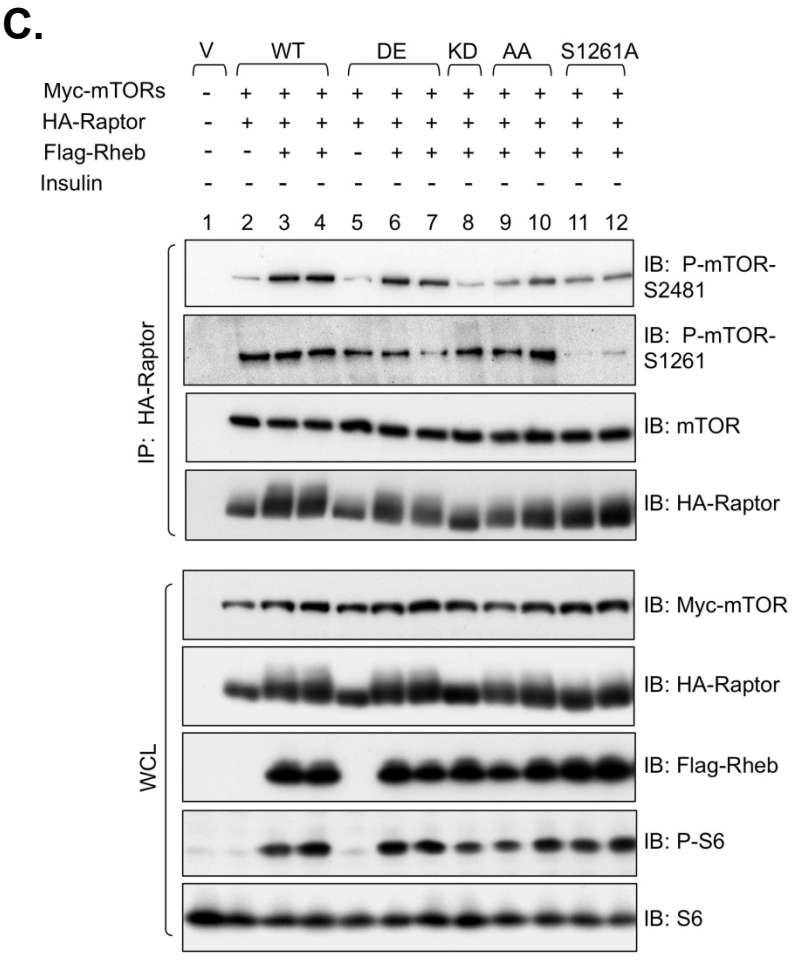
IB: PRAS40 (DE)
IB: Myc-Raptor
WCL
IB: PRAS40
IB: Myc-Raptor
IB: AU1-mTOR


C.
V
WT
DE
KD
AA
S1261A
Myc-mTORs
HA-Raptor
Flag-Rheb
Insulin
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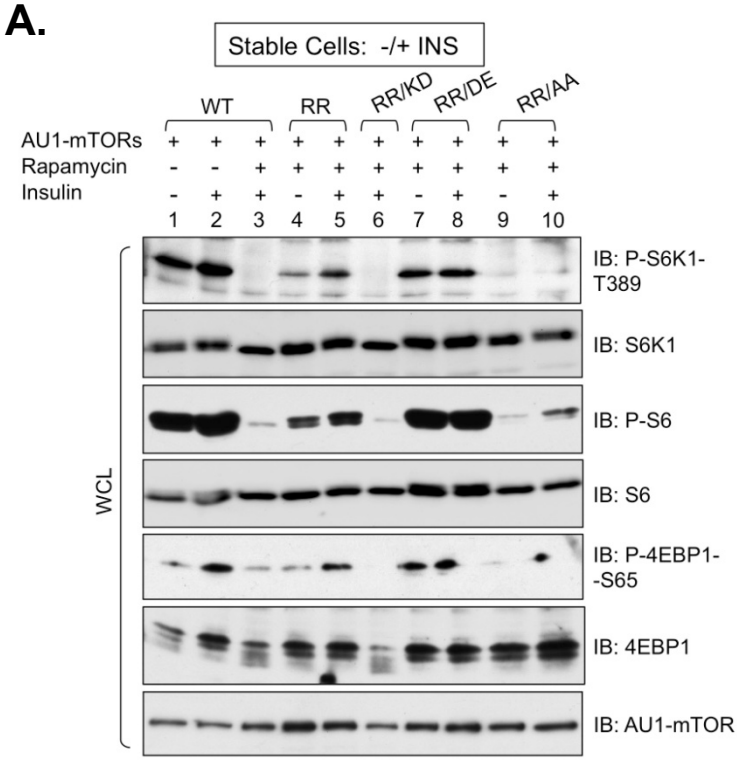
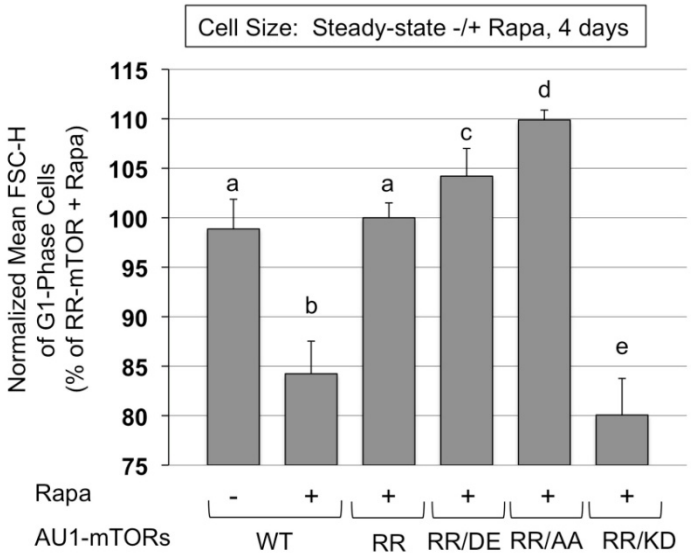
IP: HA-Raptor
IB: P-mTOR-S2481
IB: P-mTOR-S1261
IB: mTOR
IB: HA-Raptor
WCL
IB: Myc-mTOR
IB: HA-Raptor
IB: Flag-Rheb
IB: P-S6
IB: S6

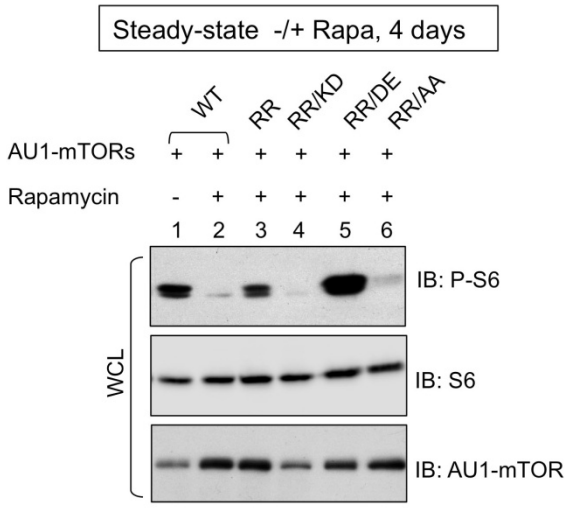
Figure 2.6 mTOR S2159/T2164 phosphorylation promotes mTORC1-mediated cell growth and G1-phase cell cycle progression.
A. Biochemical analysis of stable HEK293 Flp-In cell lines. mTOR S2159/T2164 phosphorylation promotes S6K1 and 4E-BP1 phosphorylation in response to insulin. Cells expressing various AU1-mTOR alleles were serum deprived (20 hr), pretreated without or with rapamycin (20 ng/ml) for 30 min, incubated in the absence or presence of insulin (100 nM) for 30 min, and lysed. WCLs were immunoblotted directly.
B. mTOR S2159/T2164 phosphorylation promotes cell growth to increased cell size. Cells stably expressing various AU1-mTOR alleles were cultured for 96 hr in the absence or presence of rapamycin. The relative size of subconfluent cells was determined using a flow cytometer via the parameter mean FSC-H. The graph shows mean FSC-H (± standard deviation [SD]) of G1-phase cells from three experiments, two performed in quadruplicate and one in triplicate (n = 11). The size of cells expressing RR-mTOR and cultured in the presence of rapamycin was set to 100%. All other samples were normalized to this value. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* tests. The letters (a to e) indicate significance at a P value of <0.05.
C. Expression of the various AU1-mTOR alleles and phosphorylation of S6 from one representative cell size experiment.
D. mTOR S2159/T2164 phosphorylation promotes G1-phase cell cycle progression. Flp-In HEK293 cells stably expressing various AU1-mTOR alleles were serum deprived for 24 hr and then stimulated with serum-containing medium (DMEM-FBS) in the absence or presence of rapamycin (20 ng/ml) for an additional 24 hr. DNA content was determined on a flow cytometer after propidium iodide staining. The graph shows the percentage of cells in G1 phase following serum stimulation in the absence or presence of rapamycin. Mean values (± SD) from one representative experiment performed in quadruplicate are shown
(n = 4). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* tests. The letters (a to c) indicate that the G1-phase percentage means are significantly different at a P value of <0.05.



B.


Cell Size: Steady-state -/+ Rapa, 4 days
Normalized Mean FSC-H of G1-Phase Cells (% of RR-mTOR + Rapa)
115
110
105
100
95
90
85
80
75
a
b
a
c
d
e
Rapa
- + + + + +
AU1-mTORs
WT
RR
RR/DE
RR/AA
RR/KD

C.


Steady-state -/+ Rapa, 4 days
WT
RR
RR/KD
RR/DE
RR/AA
AU1-mTORs
+ + + + + +
Rapamycin
- + + + + +
1 2 3 4 5 6
WCL
IB: P-S6
IB: S6
IB: AU1-mTOR

D.

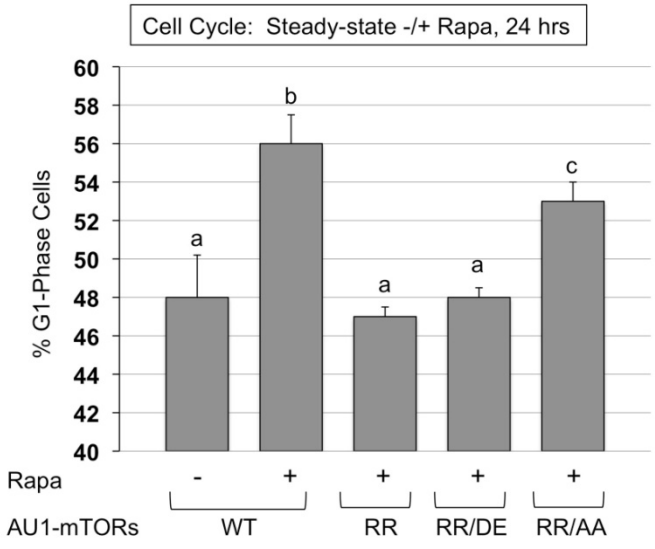
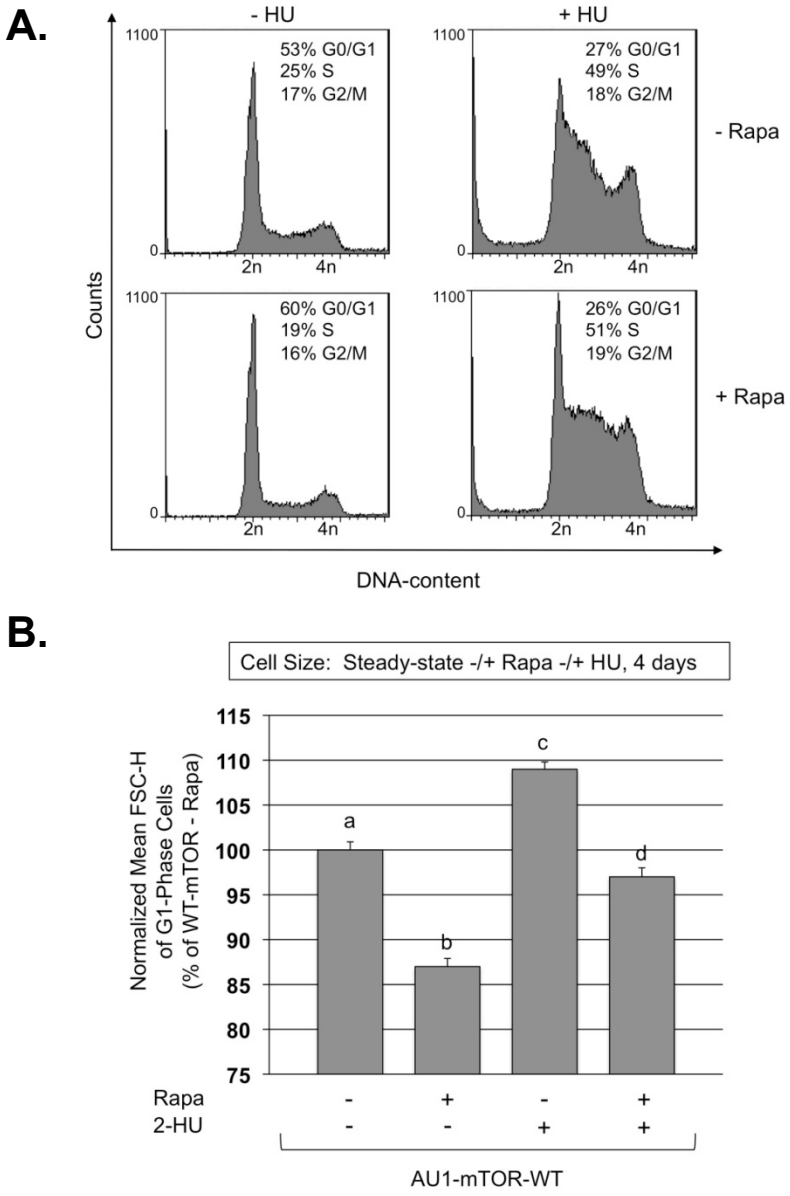
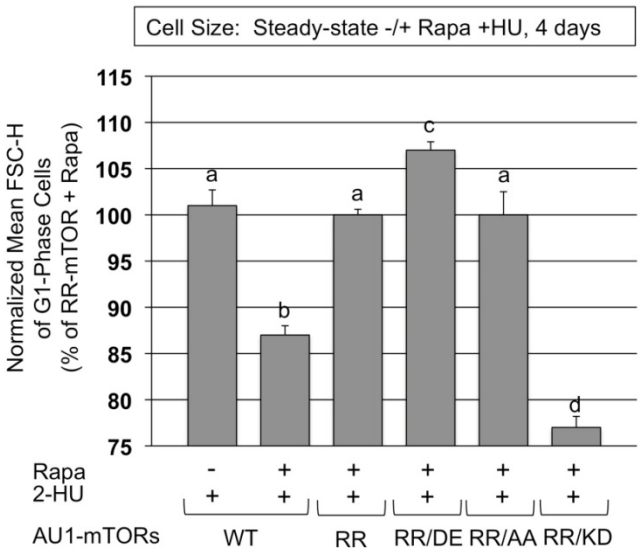

Cell Cycle: Steady-state -/+ Rapa, 24 hrs
% G1-Phase Cells
60
58
56
54
52
50
48
46
44
42
40
a
b
a
a
c
Rapa
- + + + +
AU1-mTORs
WT
RR
RR/DE
RR/AA

Figure 2.7 mTOR-S2159D/T2164E (DE) but not S2159A/T2164A (AA) promotes cell growth to an increased cell size during a cell cycle block.
A and B. 2-Hydroxyurea (2-HU) treatment induces an S-phase cell cycle block, which results in increased cell size in an mTORC1-dependent manner. Stable HEK293 Flp-In cell lines expressing AU1-mTOR-WT were cultured for 96 hr in the absence or presence of rapamycin (20 ng/ml) and/or hydroxyurea (0.5 mM). DNA content (A) or cell size (B) was determined on a flow cytometer after propidium iodide staining. **A.** Representative histogram from an experiment performed in quadruplicate. The inset shows the percentage of cells in the various cell cycle phases (mean for quadruplicate samples). **B.** Mean FSC-H (± SD) of G1-phase cells from quadruplicate samples (*n* = 4). The size of WT-mTOR-expressing cells cultured in the absence of rapamycin and hydroxyurea was set to 100%. All other samples were normalized to this value. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* tests. The letters (a to d) indicate significance at a *P* value of <0.05.
C and D. During a cell cycle block, RR/DE- but not RR/AA-mTOR-expressing cells display increased cell size. HEK293 Flp-In cells stably expressing various AU1-mTOR alleles were cultured and analyzed as for panels A and B. **C.** Cell size; **D.** DNA content. Results from a representative experiment performed in quadruplicate are shown (*n* = 4)



C.


Cell Size: Steady-state -/+ Rapa +HU, 4 days
Normalized Mean FSC-H of G1-Phase Cells (% of RR-mTOR + Rapa)
115
110
105
100
95
90
85
80
75
a
b
a
c
a
d
Rapa
2-HU
AU1-mTORs
WT
RR
RR/DE
RR/AA
RR/KD

D.

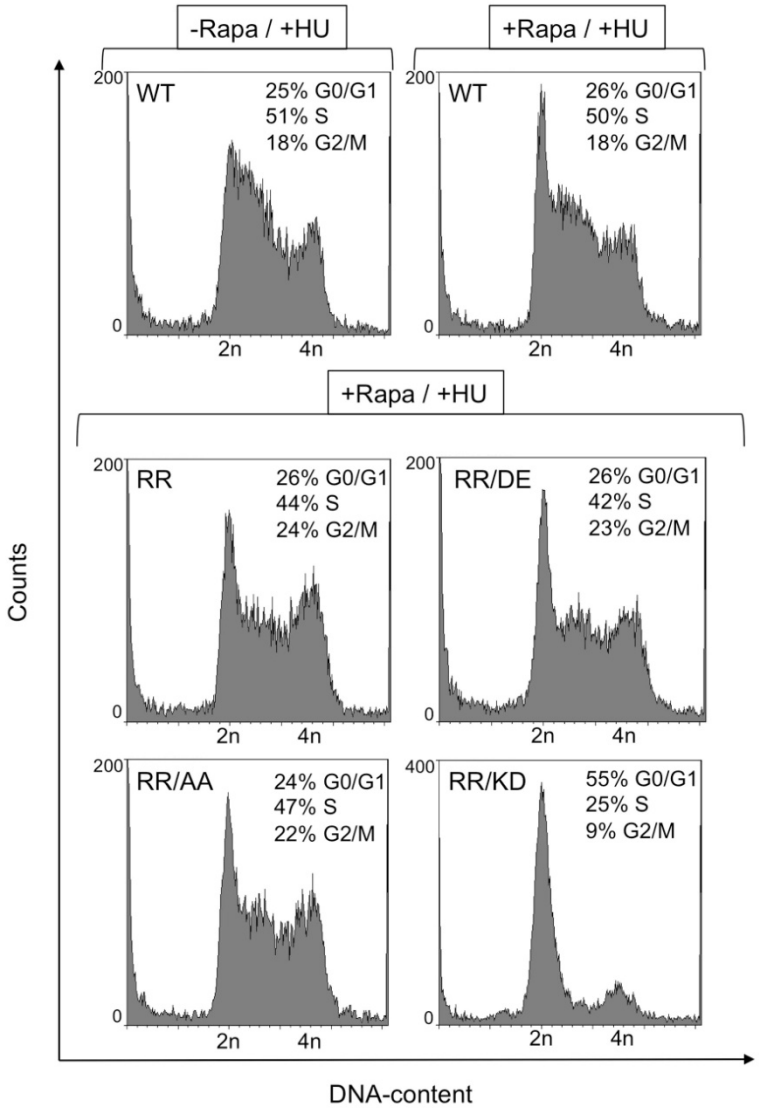
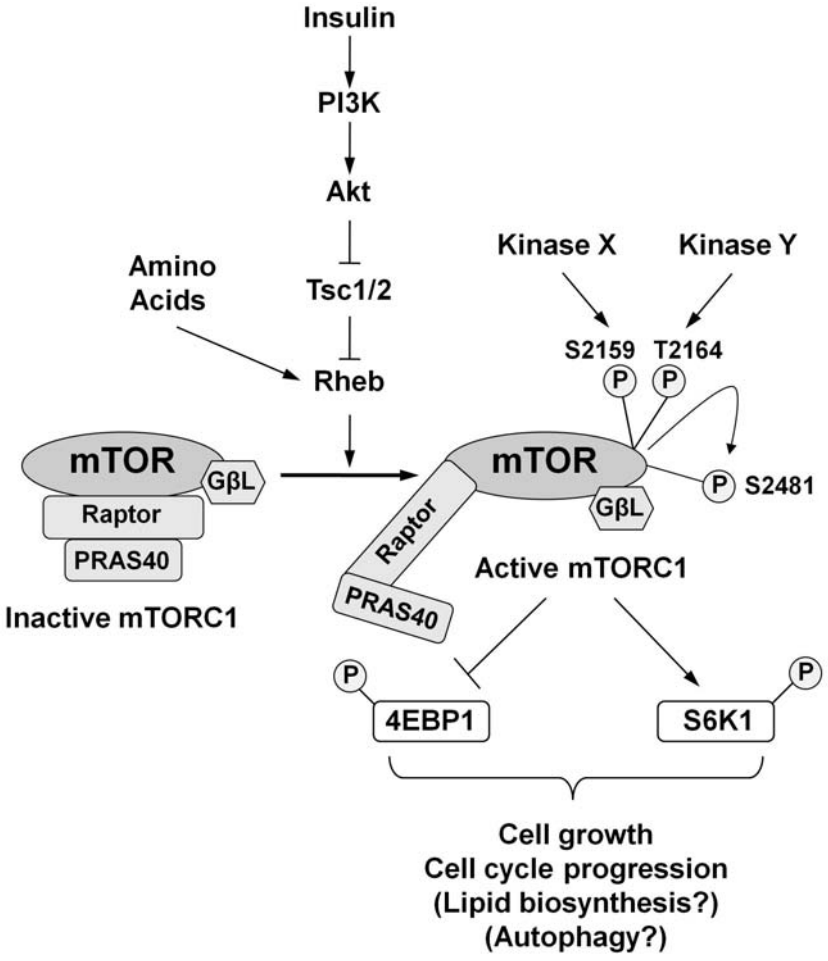

-Rapa / +HU
+Rapa / +HU
+Rapa / +HU
WT
25% G0/G1
51% S
18% G2/M
WT
26% G0/G1
50% S
18% G2/M
RR
26% G0/G1
44% S
24% G2/M
RR/DE
26% G0/G1
42% S
23% G2/M
RR/AA
24% G0/G1
47% S
22% G2/M
RR/KD
55% G0/G1
25% S
9% G2/M
200
400
0
2n
4n
Counts
DNA-content

Figure 2.8 Model (see Discussion).



Chapter 3

Novel Regulation of mTORC1 by TBK1 and IKKε

Introduction

The mammalian or mechanistic target of rapamycin (mTOR), an evolutionarily conserved serine threonine protein kinase, senses and integrates signals from diverse environmental cues to promote protein synthesis, cell growth, and cell proliferation (81, 100, 150, 153, 198, 247, 324). Aberrant regulation of mTOR contributes to myriad of diseases including cancer, type II diabetes, and cardiovascular diseases (70, 113, 144, 198). mTOR acts in at least two functionally distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (7, 18, 100). Acute rapamycin treatment inhibits mTORC1, which contains mTOR, raptor, mLST8/GβL, PRAS40, and deptor (114, 119, 168, 169, 188, 238, 263, 308). Acute rapamycin treatment fails to inhibit mTOR complex 2 (mTORC2), which contains mTOR, rictor, mSin1, mLST8/GβL, deptor, and PPR5/protor (7, 101, 152, 188, 238, 264). Anabolic cellular signals such as growth factors (i.e. insulin) and nutrients (i.e. amino acids, glucose) promote mTORC1 signaling, while catabolic signals such as energy depletion and various cellular stresses suppress mTORC1 signaling (81, 100, 247, 324). mTORC1 phosphorylates ribosomal protein S6 kinase 1 (S6K1) and the translational repressor eIF4E-binding protein 1 (4E-BP1), which increases the rate of cap-dependent protein translation, promotes cell growth (increase in cell size), and drives cell proliferation (93, 100, 181, 194). While mTORC2 remains relatively less well characterized compared to mTORC1, it is established that PI3K/insulin signaling promotes mTORC2 signaling via poorly defined signaling intermediates. Tsc1/Tsc2 may also function as a positive upstream regulator of

mTORC2, although this notions was recently questioned (66, 139, 140). Once activated, mTORC2 phosphorylates a subset of AGC family kinases including Akt, SGK1, and PKCα on their hydrophobic motif site to promote their activity (18, 115, 217, 229, 265, 266, 340).

Rheb is a small GTP binding protein and represents the most proximal positive regulator of mTORC1 (12, 103, 146, 298). Tsc1 and Tsc2 form a complex and act as a GTPase activating protein towards Rheb to downregulate mTORC1 signaling (139, 146, 179, 298). Many different signaling pathways converge on Tsc1/Tsc2 to control mTORC1 function. For instance, insulin/PI3K signaling activates Akt, which in turn phosphorylates Tsc2 to inhibit Tsc1/Tsc2 function and promote mTORC1 signaling (141, 146, 200, 297). During energy depletion, activated AMPK phosphorylates Tsc2 and raptor to downregulate mTORC1 function (117, 148). Nutrients such as amino acids also regulate mTORC1 signaling. During amino acid deprivation, mTORC1 resides in the cytoplasm. Upon stimulation with amino acids, the multi-protein ragulator complex localizes Rag GTPases and mTORC1 to the late endosomal/lysosomal surface, where Rheb is available to promote mTORC1 signaling (261, 262).

Toll-like receptors (TLRs) are transmembrane proteins expressed by cells of the immune system. They detect a variety of pathogen-associated molecular patterns (PAMPs) such as lipids, proteins, lipoproteins, and nucleic acids to activate innate immune responses (27, 166, 176, 177). In humans, ten different TLRs responsible for recognition of pathogens (i.e. viruses, bacteria, fungi, and parasites) exist (27, 166, 176, 177). The unique extracellular leucine-rich repeat (LRL) domain of TLRs defines their agonist specificity. Their intracellular TIR (toll/interleukin-1 [IL-1] receptor) domain, on the other hand, plays a role in the recruitment of adaptor proteins, which rapidly initiate a cascade of signaling events to activate multiple transcription factors (i.e. NF-κB, AP-1 [activator protein 1] and the interferon [IFN] regulatory factors [IRFs]) to initiate innate immune responses (27, 165, 166, 176, 177). Upon activation, these transcription factors induce the expression of a set of genes that produce cytokines (e.g. type I IFNs: IFN-α and IFN-β). Cytokines act in an autocrine and paracrine manner to

inhibit cell growth and viral replication and promote apoptosis (110, 199). In resting cells, IκB proteins sequester NF-κB members in the cytoplasm by masking their NLS (127, 234). Upon activation of TLRs, the Iκβ kinase (IKK) complex phosphorylates IκBα, causing its ubiquitin-mediated degradation, and resulting in activation and nuclear translocation of NF-κB (127, 234). Intensive research on this IKK complex led to the discovery of the IKK-related kinases TBK1 and IKKε, which are structurally similar to IKK complex catalytic components IKKα and IKKβ. TBK1 and IKKε share 64% sequence identity and overlapping functions with each other (22, 236, 240, 278, 303). Although most tissues express TBK1 ubiquitously, only a subset of organs such as pancreas, spleen, thymus, and leukocytes express IKKε (278). Similar to IKKα and IKKβ, IKK-related kinases contain an N-terminal kinase domain and a leucine zipper (LZ) domain that likely plays a role in homo- or hetero-dimerization. The putative helix loop helix (HLH) motif at the C-terminal end may regulate the kinase activity of TBK1 and IKKε (59, 60, 71). T-loop site (S172) phosphorylation of TBK1 and IKKε within their kinase domain is required for their catalytic activity (171, 236, 278).

Exposure of immune cells to dsRNA (double stranded RNA) and bacterial lipopolysaccharide (LPS) activates TLR3 and TLR4, respectively. Downstream of TLR3 and TLR4, the adaptor molecule TRIF (Toll/IL-1 Receptor Domain-Containing Adaptor Inducing IFN-β) directly interacts with TBK1 and IKKε, activating downstream signaling. (165, 176, 177, 326). Activated TBK1 and IKKε promote type I IFN production by phosphorylating and activating IRF-3 and IRF-7 at key C-terminal residues (S385/S386; S396; S398; S402; T404; S405 on IRF3 and S429-S431; S437; S438; S441; S477; S479 on IRF-7) (34, 59, 60, 96, 207, 226, 276, 300). Upon phosphorylation, IRF-3 homodimerizes or heterodimerizes with IRF-7 and translocates to the nucleus to activate the transcription of type I IFNs and initiate innate immune responses (293). Although overexpression of TBK1 or IKKε promotes NF-κB signaling, $TBK1^{-/-}$ or $IKK\varepsilon^{-/-}$ mouse embryonic fibroblasts (MEFs) do not show defects in NF-κB pathway, indicating that the

physiological role of IKK-related kinases in NF-κB activation requires further investigation (3, 130, 236, 240, 278).

Studies from three different labs established a direct link between IKK-related kinases and Akt and showed that upon growth factor stimulation TBK1 and IKKε phosphorylate Akt on both its hydrophobic motif (HM) site (S473) and T-loop site (T308) to activate Akt (155, 224, 325). Thus, in addition to mTORC2, DNA-PK, ILK, PAK1, MAPKAP2, ATM, and PKC, TBK1 and IKKε emerge as *bona fide* kinases for Akt S473 phosphorylation site (24, 76, 89, 115, 151, 265). Recent data also suggest that TBK1 and IKKε play roles in oncogenic Ras-induced tumorigenesis and tumor cell survival (14, 54, 224, 325). Two thirds of breast cancers cells overexpress IKKε (21, 142). Moreover, IKKε also regulates energy balance in mice as IKKε$^{-/-}$ mice are protected from diet-induced obesity, type II diabetes, and chronic inflammation in liver and adipose tissue (53).

Previously, by employing liquid chromatography tandem mass spectrometry (LC-MS/MS) and phospho-specific antibodies, we identified two novel phosphorylation sites on mTOR, S2159 and T2164, which lie at the extreme N-terminus of the mTOR kinase domain (84) (Chapter 2). Our work on S2159 and T2164 phosphorylation revealed that they act in a coordinative manner to promote mTORC1 function (84) (Chapter 2). S2159 and T2164 phosphorylation modulates the interaction of mTOR with its partner proteins raptor and PRAS40 and increase mTORC1 intrinsic catalytic activity, which in turn promotes biochemical mTORC1 signaling, cell growth, and cell cycle progression (84) (Chapter 2).

Moving forward, we performed an *in vitro* kinome screen and identified TBK1 and IKKε as candidate kinases for mTOR S2159 phosphorylation. Here, we show that IKK-related kinases TBK1 and IKKε interact with mTOR and phosphorylate it on S2159 both *in vitro* and in intact cells. Overexpression of TBK1 and IKKε not only increases mTOR S2159 phosphorylation but also promotes mTOR intrinsic catalytic activity, as monitored by mTOR S2481 autophosphorylation (284). TBK1 and IKKε overexpression also induces raptor and rictor phosphorylation. Stimulation of TBK1/IKKε activators, TLR3 and TLR4,

promotes downstream mTORC1 and mTORC2 signaling. Overall, our findings indicate that TBK1 and IKKε play an extensive role in regulating mTORC1 and mTORC2 signaling via multiple mechanisms.

Results

TBK1 and IKKε phosphorylate mTOR on S2159

To identify potential mTOR S2159 kinases, in collaboration with Steve Riddle from Life Technologies, we performed an *in vitro* kinome screen and tested ~300 recombinant kinases on a 384-well plate system. As a substrate, we used a recombinant GST-mTOR fragment containing amino acids 2144-2175 wild type (WT) or S2159A/T2164A (AA) phospho-deficient substitutions. We analyzed S2159 phosphorylation by performing dot-blot analysis with the phospho-specific mTOR S2159 (P-S2159) antibody generated previously (84) (Chapter 2) (Fig. 3.1A). We also employed an alternative approach by measuring site-specific ^{32}P incorporation in *in vitro* kinase reactions containing [γ-^{32}P] ATP and GST-mTOR-WT or -AA together with the recombinant kinases (Fig. 3.1B). TBK1, IKKε, and STK22D (a.k.a Tssk3 [testis-specific serine/threonine-protein kinase 3]) gave the strongest site-specific P-S2159 signals among the kinases tested (Fig. 3.1A and B). Since STK22D is reported to be testis specific (312, 342), we decided to focus on TBK1 and IKKε as potential mTOR S2159 kinases.

To confirm the multi-plexed kinome screen, we performed *in vitro* kinase assays by employing recombinant TBK1/IKKε kinases and the GST-mTOR substrate. We ran the *in vitro* kinase reactions on SDS-PAGE and immunoblotted with P-S2159-mTOR antibody. As shown in Fig. 3.2A, both TBK1 and IKKε phosphorylated GST-mTOR on S2159 *in vitro*. When pretreated with the recently described dual TBK1/IKKε pharmacologic inhibitor BX795, both TBK1 and IKKε failed to phosphorylate mTOR S2159 (58). In addition to BX795, we tested a panel of other TBK1 inhibitors in our *in vitro* kinase reactions including CYT, MRT67307, and KBS. As shown in Fig. 3.2B, pretreatment of TBK1 with these drugs inhibited mTOR S2159 phosphorylation across the board. These data indicate that mTOR S2159 phosphorylation requires TBK1 and IKKε catalytic

activities. Next, we asked whether TBK1 and IKKε phosphorylate full-length mTOR. In order to obtain full-length mTOR substrate, we transfected HEK293 cells with Myc-mTOR-WT (wild type) or a phospho-site defective Myc-mTOR-S2159A mutant, lysed the cells 24 hr post-transfection, and immunoprecipitated Myc-mTOR with Myc antibodies. Both recombinant TBK1 and IKKε phosphorylated full-length Myc-mTOR-WT on S2159. The P-S2159 antibody failed to detect signal on Myc-mTOR-S2159A, demonstrating that the P-S2159 antibody is site-specific, as we have shown previously (Fig. 3.2C) (84). In order to rule out the possibility that a contaminating kinase in recombinant TBK1/IKKε may phosphorylate mTOR S2159, we employed an alternative *in vitro* kinase assay approach. Rather than employing recombinant TBK1/IKKε, we immunoprecipitated overexpressed Flag-TBK1/IKKε-WT (wild-type) or -KD (kinase dead) from HEK293 cells as kinase source for the *in vitro* kinase reactions along with the GST-mTOR substrate. As shown in Fig. 3.2D, WT- but not KD-Flag-TBK1/IKKε phosphorylated mTOR S2159, indicating that TBK1- or IKKε-mediated mTOR S2159 phosphorylation indeed requires functional TBK1/IKKε catalytic activity. In support of these data, the mTOR amino acid sequence that surrounds S2159 fits well with the TBK1 target phosphorylation motif and partially fits with the IKKε target phosphorylation motif (Fig. 3.2E) (142, 236, 278). Overall, these data indicate that both TBK1 and IKKε directly phosphorylate mTOR S2159 *in vitro*.

TBK1/IKKε and mTOR interact

As physical interactions between kinases and their substrates can be often detected by classic coimmunoprecipitation assays, we investigated whether TBK1 and IKKε interact with mTOR. We therefore employed Flp-In-HEK293 cells that stably express vector control or AU1-mTOR, as generated previously (84) (Chapter 2). We transiently transfected these cells with vector control, Flag-TBK1/IKKε-WT or –KD to perform coimmunoprecipitation (coIP) experiments. We immunoprecipitated AU1-mTOR and examined the levels of Flag-TBK1/IKKε-WT or -KD in the immunoprecipitates by immunoblotting with Flag antibodies. As

shown in Fig. 3.3, both Flag-TBK1 and Flag-IKKε interacted with mTOR. Although not visible in Fig. 3.3, we can detect Flag-TBK1-WT signal with AU1-mTOR immunoprecipitates on darker exposures. Despite the background Flag-TBK1/ IKKε signal in control immunoprecipitates from vector expressing cells, the Flag signal is stronger in immunoprecipitates from Flp-In-AU1-mTOR cells, suggesting that TBK1 and IKKε may interact with mTOR. Interestingly, kinase dead TBK1/IKKε interacted with AU1-mTOR more strongly than WT-TBK1/IKKε (Fig. 3.3), suggesting that the interaction between mTOR and TBK1/IKKε may take place in a hit and run manner, in which substrate phosphorylation triggers rapid kinase release. Thus lack of TBK1/IKKε-mediated S2159 phosphorylation upon cotransfection of kinase dead TBK1/IKKε alleles enables the mTOR-TBK1/IKKε interaction to be more readily detectable (Fig. 3.3). This relationship between kinases and their substrates is common. For example, catalytically inactive MEK interacts with its substrate ERK more robustly compared to wild type MEK (251). Overall, our data indicate that TBK1 and IKKε kinases interact with their substrate mTOR, supporting our findings further that TBK1 and IKKε directly phosphorylate mTOR S2159.

TBK1 and IKKε phosphorylate mTOR S2159 in intact cells

After demonstrating that TBK1 and IKKε interact with mTOR and directly phosphorylate mTOR S2159 *in vitro*, we employed $TBK1^{+/+}$ vs. $TBK1^{-/-}$ and $IKK\varepsilon^{+/+}$ vs. $IKK\varepsilon^{-/-}$ single knockout mouse embryonic fibroblast (MEFs) to investigate whether TBK1 and IKKε mediate mTOR S2159 phosphorylation in intact cells. We immunoprecipitated endogenous mTOR from these cells and immunoblotted with mTOR P-S2159 antibodies. As shown in Fig. 3.4A and B, mTOR S2159 phosphorylation is significantly reduced in $TBK1^{-/-}$ and $IKK\varepsilon^{-/-}$ MEFs relative to littermate-matched wild type MEFs ($TBK1^{+/+}$ and $IKK\varepsilon^{+/+}$ MEFs, respectively). mTOR S2159 phosphorylation is not completely abolished in $TBK1^{-/-}$ and $IKK\varepsilon^{-/-}$ single knockout MEFs, most likely due to the expression of IKKε in $TBK1^{-/-}$ MEFs and TBK1 in $IKK\varepsilon^{-/-}$ MEFs. Overall, these data indicate that both

TBK1 and IKKε represent physiological kinases for mTOR S2159 phosphorylation.

In our previous study (84) (Chapter 2), we demonstrated that mTOR S2159 and T2164 phosphorylation cooperate with each other to promote mTORC1 intrinsic catalytic activity (as monitored by mTOR S2481 autophosphorylation) and downstream signaling to S6K1, S6, and 4E-BP1 (84, 284). Thus, we examined mTOR S2481 autophosphorylation to monitor mTOR intrinsic catalytic activity and downstream mTORC1 signaling in $TBK1^{+/+}$ vs $TBK1^{-/-}$ MEFs. As shown in Fig. 3.4A, in $TBK1^{-/-}$ MEFs, mTOR displayed reduced S2481 autophosphorylation relative to $TBK1^{+/+}$ MEFs, indicating that TBK1 contributes to the activation of intrinsic mTOR catalytic activity under steady-state conditions (284). $TBK1^{-/-}$ MEFs also displayed reduced downstream mTORC1 signaling towards S6K1, S6, and 4E-BP1 (subtle reduction in 4E-BP1 phosphorylation is evident by the appearance of the hypophosphorylated alpha band). These data indicate that TBK1 promotes mTOR intrinsic catalytic activity and downstream mTORC1 signaling under steady-state conditions.

As described in Chapter 1, mTORC2 phosphorylates Akt on its HM site S473 and PDK1 phosphorylates Akt on its T-loop site T308 to activate Akt (5, 87, 115, 138, 143, 151, 266). Although Akt S473 phosphorylation is dispensible for mTORC1 function, mTORC1 signaling requires Akt T308 phosphorylation (115, 151). Recently three groups independently reported that upon growth factor stimulation, TBK1 and IKKε phosphorylate Akt on its both HM motif (S473) and T-loop site (T308) to promote its activity (155, 224, 325). Based on these recently published studies, we investigated whether reduced mTORC1 signaling in $TBK1^{-/-}$ MEFs may be due to reduced Akt T308 phosphorylation. $TBK1^{-/-}$ MEFs, however, did not display defective Akt phosphorylation on either S473 or T308 (Fig. 3.4A), indicating that TBK1 promotes mTORC1 signaling independently of Akt T308 phosphorylation under steady-state conditions. We also observed a modest increase in Akt 308 phosphorylation in $TBK1^{-/-}$ MEFs, which may be due to reduced S6K1 phosphorylation and thus reduced action of the negative feedback loop that suppresses insulin/PI3K signaling via inhibitory IRS

phosphorylation. Reduced action of the negative feedback loop would therefore augment PI3K activity, which would enhance PDK1-mediated Akt T308 phosphorylation. We also examined mTOR S2481 autophosphorylation and downstream mTORC1 signaling in IKKε$^{-/-}$ vs. IKKε$^{+/+}$ MEFs. Unlike TBK1$^{-/-}$ MEFs, IKKε$^{-/-}$ MEFs did not display impaired mTOR S2481 autophosphorylation or downstream mTORC1 signaling relative to IKKε$^{+/+}$ MEFs, indicating that although loss of IKKε expression reduces mTOR S2159 phosphorylation (Fig. 3.4B), it is not sufficient to blunt mTOR autophosphorylation or mTORC1 signaling under steady state conditions (data not shown).

TBK1 and IKKε promote mTOR S2159 phosphorylation and S2481 autophosphorylation in intact cells

In addition to the loss-of-function approach in which we demonstrated that TBK1$^{-/-}$ and IKK$^{-/-}$ MEFs show defective mTOR S2159 phosphorylation (Fig. 3.4), we employed a gain-of-function approach in which we tested whether TBK1 or IKKε overexpression regulates mTOR S2159 phoshorylation in HEK293 cells that stably express TLR3 (hereafter referred to as HEK293-TLR3 cells). To test this, we transiently transfected HEK293-TLR3 cells with Myc-mTOR and Flag-TBK1/IKKε-WT (wild type) or –KD (kinase dead) plasmids. We immunoprecipitated Myc-mTOR with Myc antibodies and immunoblotted with P-S2159 antibodies. As shown in Fig. 3.5A, overexpression of Flag-TBK1/IKKε-WT but not -KD promoted mTOR S2159 phosphorylation in intact cells. Furthermore, pretreatment of cells with BX795 drug for 2 hr completely abolished TBK1/IKKε-induced phosphorylation of mTOR on S2159 (Fig. 3.5A). We also asked whether TBK1/IKKε overexpression is sufficient to increase mTOR intrinsic catalytic activity by monitoring S2481 autophosphorylation. As shown in Fig. 3.5A, WT- but not KD-TBK1/IKKε increased mTOR S2481 autophosphorylation in BX795 sensitive manner. Overall, these data indicate that TBK1 and IKKε overexpression is sufficient to augment mTOR S2159 phosphorylation and mTOR intrinsic catalytic activity.

To determine whether mTOR S2159 phosphorylation occurs exclusively in mTORC1 or mTORC2 or occurs in both complexes, we examined mTOR S2159 phosphorylation in raptor and rictor immunoprecipitates. We co-transfected HEK293-TLR3 cells with Myc-mTOR and Flag-TBK1/IKKε along with the mTORC1 component HA-raptor or the mTORC2 components HA-rictor and Myc-Sin1. 24 hr post-transfection, we lysed the cells and immunoprecipitated HA-raptor or HA-rictor to pull down mTORC1 or mTORC2, respectively. As shown in Fig. 3.5B (left panel), TBK1 and IKKε promoted mTOR S2159 phosphorylation within mTORC1. Consistently, upon TBK1 or IKKε overexpression, mTORC1-associated mTOR S2481 autophosphorylation increased, as well (Fig. 3.5B - left panel). Interestingly, IKKε but not TBK1 overexpression also increased mTOR S2159 phosphorylation within mTORC2 (Fig. 3.5B - right panel). This experiment has only been performed once, thus we need to do further experiments to confirm whether it is indeed only IKKε but not TBK1 that promotes mTOR S2159 phosphorylation within mTORC2 (Fig. 3.5B - right panel). In addition, TBK1 or IKKε overexpression increased mTORC2-associated mTOR S2481 autophosphorylation modestly relative to the mTORC1-associated S2481 autophosphorylation (Fig. 3.5B). Furthermore, expression of TBK1 and IKKε induced HA-raptor and HA-rictor to undergo a shift in electrophoretic mobility on SDS-PAGE, suggesting that TBK1 and IKKε expression also promotes raptor and rictor phosphorylation (Fig. 3.5B). We next investigated whether overexpression of TBK1 or IKKε promotes raptor S863 phosphorylation, an mTORC1- and ERK-mediated phosphorylation event that promotes mTORC1 function (40, 99, 315). To test this, we transiently transfected HEK293-TLR3 cells with Flag-TBK1 and HA-raptor with or without Myc-mTOR. As shown in Fig. 3.5C, overexpression Flag-TBK1 greatly increased S863 phosphorylation on HA-raptor in a BX795 sensitive manner (co-expression with Myc-mTOR was inconsequential). Overexpression of IKKε also increased raptor S863 phosphorylation (data not shown). Overall, these data indicate that TBK1 and IKKε phosphorylate mTOR S2159 within mTORC1 and promote mTOR intrinsic catalytic activity within both mTORC1 and mTORC2. In addition, overexpression

of TBK1 induces phosphorylation of raptor and rictor, indicating that TBK1 and IKKε employ several mechanisms to regulate mTORC1 and mTORC2.

Poly (I:C) (polyinosinic:polycytidylic acid), a synthetic dsRNA analog that binds to activates TLR3, activates TBK1 and IKKε to initiate innate immune signaling (8). Thus, we investigated whether poly(I:C) promotes mTOR S2159 phosphorylation. To test this, we employed poly(I:C) responsive HEK293-TLR3 cells. We co-transfected HEK293-TLR3 cells with Flag-TBK1 and Myc-mTOR, and ~24 hr post-transfection, we serum-deprived cells in DMEM-FBS [0.5%] for ~20 hr. Prior to lysis, we stimulated cells with or without poly(I:C) for 2 hr. After immunoprecipitating Myc-mTOR with Myc antibodies, we analyzed mTOR S2159 phosphorylation by immunoblotting. Consistent with the data shown in Fig. 3.5A, overexpression of Flag-TBK1 promoted mTOR S2159 phosphorylation and upon poly(I:C) stimulation, mTOR S2159 phosphorylation increased further (Fig. 3.5D). In the absence of Flag-TBK1, we failed to observe an increase in mTOR S2159 phosphorylation upon poly(I:C) stimulation (data not shown) possibly because HEK293-TLR3 cells express TBK1 at relatively low levels. These data suggest that TBK1 functions as an important signaling intermediate for TLR3-induced mTOR S2159 phosphorylation.

While overexpression of transiently transfected TBK1 and IKKε at high levels promoted mTOR S2159 phosphorylation, it inhibited downstream mTORC1 signaling. (Fig. 3.5D, compare lanes 2 and 3). Poly (I:C) stimulation, however, rescued both S6K1 and 4E-BP1 phosphorylation. Since the interaction between overexpressed TBK1/IKKε and mTOR can be detected by coimmunoprecipitation (Fig. 3.3), it is possible that when overexpressed above physiological levels in the absence of an appropriate activating signal such as poly(I:C), TBK1/IKKε binds to and sequesters mTORC1, resulting in suppression of mTORC1-mediated phosphorylation of S6K1 and 4E-BP1 (Fig. 3.5D, compare lanes 2 and 3). Our data in Fig. 3.3 suggest that phosphorylation of mTOR S2159 accelerates the release of TBK1/IKKε from mTOR. Thus, in TBK1/IKKε overexpressing cells, poly(I:C) activates TBK1/IKKε, leading to an increase in mTOR S2159 phosphorylation and a weakening of TBK1/IKKε–mTOR

interaction, thus promoting downstream mTORC1 signaling (Fig. 3.5D, compare lanes 3 and 4). Overall these data indicate that activation of TBK1 by poly(I:C)-induced TLR3 signaling promotes mTOR S2159 phosphorylation as well as downstream mTORC1 signaling towards S6K1 and 4E-BP1.

Activation of TLR3 and TLR4 promotes mTORC1 signaling

Upon stimulation with poly(I:C) and LPS, TLR3 and TLR4, respectively promote TBK1/IKKε signaling to induce the production of type I IFNs (96, 130, 276). Thus, we investigated whether activation of TLR3 and TLR4 in poly(I:C)- and LPS-responsive RAW264.7 murine macrophages regulates downstream mTORC1 signaling to S6K1, S6, and 4E-BP1. We pretreated RAW264.7 cells grown under steady-state conditions with or without mTOR catalytic site inhibitor KU-0063794 for 1 hr and then stimulated the cells with either poly (I:C) or LPS for 2 hr. We lysed the cells and immunoblotted the lysates with P-S6K1 (T389), P-S6 (240/244), and P-4E-BP1 (T37/46) antibodies to examine downstream mTORC1 signaling. As shown in Fig. 3.6A, poly(I:C) and LPS promoted S6K1, S6, and 4E-BP1 phosphorylation in an mTOR-dependent manner, as the mTOR catalytic inhibitor KU-0063794 treatment abolished S6K1, S6, and 4E-BP1 phosphorylation. Poly(I:C) and LPS stimulation also increased the phosphorylation of TBK1 on its activation loop site, T172, indicating that poly(I:C) and LPS activated TBK1. Overall, these data indicate that activation of TLR3 and TLR4 in RAW264.7 macrophages activates TBK1 and promotes downstream mTORC1 signaling towards S6K1, S6, and 4E-BP1.

Since TBK1 and IKKε promote mTOR intrinsic catalytic activity (Fig. 3.4A and Fig. 3.5A), we investigated whether TLR3 and TLR4 activation in RAW264.7 macrophages regulates mTOR S2481 autophosphorylation, as well. As shown in Fig. 3.5B, both poly(IC) and LPS increased mTOR S2481 autophosphorylation in a KU-0063794 sensitive manner, indicating that TLR3 and TLR4 indeed promote mTOR intrinsic catalytic activity.

Recently, three different groups reported that TBK1 and IKKε phosphorylate Akt on both S473 and T308 sites to activate Akt upon EGF and

LPS stimulation of MEFs (155, 224, 325). Thus, we examined Akt S473 and T308 phosphorylation in RAW264.7 macrophages stimulated with poly(I:C) or LPS. As shown in Fig. 3.6B, poly(I:C) and LPS increased Akt S473 phosphorylation in an mTOR-dependent manner, as KU-0063794 treatment completely abolished poly(I:C)- or LPS-induced Akt S473 phosphorylation. These data indicate that in poly(I:C)- or LPS-stimulated RAW264.7 cells, the major kinase for Akt S473 phoshorylation is mTORC2 but not TBK1 or IKKε. Moreover, these data indicate that activation of TLR3 and TLR4 not only promotes mTORC1 but also mTORC2 signaling. Stimulation of RAW264.7 macrophages with poly(I:C) or LPS also promoted Akt T308 phosphoryation, which may contribute to increased mTORC1 signaling (Fig. 3.6A and B) (115, 151). Nevertheless, activation of TBK1 by poly(I:C) or LPS may also increase mTOR S2159 phosphorylation, which may promote mTORC1 signaling independently of Akt T308 phosphorylation (84) (Chapter 2), which we plan to investigate in the future.

Taken together, these data indicate that activation of TLR3 and TLR4 in RAW264.7 murine macrophages increases mTOR intrinsic catalytic activity and promotes downstream mTORC1 and mTORC2 signaling. In the future, we plan to test whether TBK1 and TBK1-dependent mTOR S2159 phosphorylation mediate the TLR3- and TLR4- induced increase in mTOR intrinsic catalytic activity as well as downstream mTORC1 and mTORC2 signaling.

Discussion

In this study, we identified a novel link between mTOR and IKK-related kinases, TBK1 and IKKε. We previously identified S2159 as a novel mTOR phosphorylation site and demonstrated that mTOR S2159 phosphorylation promotes mTORC1 signaling, cell growth, and cell cycle progression (84) (Chapter 2). The kinase(s) responsible for mTOR S2159 phosphorylation, however, remained elusive. In our previous study (84) (Chapter 2), we reported that canonical signals that regulate mTORC1 signaling (e.g., insulin, amino acids, and glucose) do not modulate mTOR S2159 phosphorylation. These

observations suggested that there may be a novel mTOR regulatory paradigm in which an unknown cellular signal regulates mTOR S2159 phosphorylation. In the study presented here, we demonstrate that two related kinases with roles in innate immune signaling, TBK1 and IKKε, physically interact with and phosphorylate mTOR S2159 both *in vitro* and in intact cells. Consistently, MEFs lacking either TBK1 or IKKε display reduced mTOR S2159 phosphorylation, indicating that TBK1 and IKKε represent physiological kinases for this site. In addition, stimulation of TBK1/IKKε upstream activators, TLR3 and TLR4, with poly(I:C) and LPS, respectively, promotes mTOR intrinsic catalytic activity as well as downstream mTORC1 signaling towards S6K1 and 4E-BP1. Overall, our findings indicate that we have indeed discovered a novel mTOR regulatory paradigm.

Our *in vitro* kinome screen revealed TBK1 and IKKε as two of three candidates that phosphorylate mTOR S2159. STK22D, a member of CaMK (Ca^{+2}/calmodulin-dependent protein kinase) family, was the third candidate. STK22D is only expressed in testis, but not in brain, ovary, kidney, liver or early embryonic cells, suggesting that it may play role in signal transduction events during male germ cell development or mature sperm function (312, 342). Since STK22D has a very limited expression profile and poorly understood function, we focused on TBK1 and IKKε and investigated their roles as potential mTOR S2159 kinases. We confirmed the kinome screen results by performing *in vitro* kinase reactions and showed that TBK1 and IKKε directly phosphorylate mTOR S2159 (Fig. 3.2). In support of these data, we also showed that TBK1 and IKKε interact with mTOR in intact cells (Fig. 3.3). The catalytically inactive mutants of TBK1 and IKKε interact with mTOR more strongly relative to wild type kinases, suggesting that phosphorylation of mTOR S2159 (and possibly other residues) by TBK1 and IKKε promotes their release from mTOR. In the literature, there are several examples of such kinase-substrate interactions. Catalytically inactive MEK, for instance, interacts with its substrate ERK more strongly relative to wild type MEK (251)

Both TBK1$^{-/-}$ and IKKε$^{-/-}$ single knockout MEFs display impaired mTOR S2159 phosphorylation relative to the TBK1$^{+/+}$ and IKKε$^{+/+}$ MEFs, respectively (Fig. 3.4). These data indicate that both TBK1 and IKKε phosphorylate mTOR on S2159 under physiological conditions. mTOR S2159 phosphorylation, however, is not completely abolished in these single knockout MEFs, most likely due to the expression of IKKε in TBK1$^{-/-}$ MEFs and TBK1 in IKKε$^{-/-}$ MEFs. In addition to the experiments performed in TBK1$^{-/-}$ and IKKε$^{-/-}$ MEFs, we also employed gain-of-function experiments to examine mTOR S2159 phosphorylation upon TBK1 or IKKε overexpression. Overexpression of either TBK1 or IKKε boosts mTOR S2159 phosphorylation under both steady-state and serum deprived conditions (Fig. 3.5A and D). TBK1 and IKKε also increase mTOR S2159 phosphorylation within mTORC1. Interestingly, overexpression of TBK1 or IKKε also promotes mTORC1 and mTORC2 intrinsic catalytic activity, as monitored by increased mTOR S2481 autophosphorylation (Fig. 3.5B) (284).

The mTORC1 component raptor and the mTORC2 component rictor undergo phosphorylation on several sites to regulate mTORC1 and mTORC2 function (39, 40, 74, 99, 117, 156, 305, 315). Importantly, overexpression of TBK1 and IKKε induces raptor and rictor phosphorylation possibly on multiple sites due to their striking shift in electophoretic mobility. TBK1 and IKKε overexpression leads to increased raptor S863 phosphorylation which promotes mTORC1 function (Fig. 3.5C) (99, 315). As mTOR phosphorylates raptor S863, these data suggest that TBK1/IKKε-mediated phosphorylation of mTOR S2159 contributes to activation of mTOR intrinsic catalytic activity, which then leads to mTOR-mediated raptor S863 phosphorylation, a chain of events that lead to increased mTORC1 signaling. Overall, our findings indicate that TBK1 and IKKε regulate both mTORC1 and mTORC2 by inducing multiple phosphorylation events on mTOR, raptor, and rictor.

Several pieces of data presented in this study suggest that the TLR/TBK1 signaling pathway promotes mTORC1 function. Firstly, under steady state conditions, TBK1$^{-/-}$ MEFs display impaired mTOR intrinsic catalytic activity and reduced mTORC1 signaling towards S6K1, S6, and 4E-BP1, without any

decrease in Akt T308 or S473 phosphorylation (Fig. 3.4A). These data indicate that TBK1 promotes mTORC1 signaling independently of its recently identified role as an Akt T308 or S473 kinase (224, 325). In addition, stimulation of TLR3 and TLR4 in RAW264.7 macrophages also activates TBK1, increases mTORC1 intrinsic catalytic activity, and promotes downstream mTORC1 signaling towards S6K1, S6 and 4E-BP1 (Fig. 3.5D and Fig. 3.6A and B). Based on these data and our previously published study (84) (Chapter 2), one may hypothesize that it is TBK1-dependent mTOR S2159 phosphorylation that promotes mTOR intrinsic catalytic activity and downstream mTORC1 signaling. In addition, poly(I:C) and LPS stimulation of RAW264.7 macrophages modestly increases Akt T308 phosphorylation, which we acknowledge may also contribute to the increase in mTORC1 signaling (Fig. 3.6A and B). In RAW264.7 macrophages, poly(I:C) and LPS promote Akt S473 phosphorylation in an mTOR-dependent manner, indicating that under these conditions, mTORC2 rather than TBK1 or IKKε phosphorylates Akt S473. In the future, we would like to identify the signaling intermediates that lie downstream of TLR3 and TLR4 that participate in mTORC1 and mTORC2 regulation. One candidate to investigate may be TRIF, a TLR3 and TLR4 adaptor molecule that functions in activation of TBK1/IKKε signaling (326).

Overall, these data indicate that activation of TLR3 and TLR4 signaling pathways promotes biochemical mTORC1 and mTORC2 function. Based on these observations, one would expect that TBK1 or IKKε overexpression would also increase mTORC1 signaling. However, as seen in Fig. 3.5D, overexpression of transiently transfected TBK1 promotes mTOR S2159 phosphorylation but blunts S6K1 and 4E-BP1 phosphorylation (compare lanes 2 and 3), suggesting that TBK1 overexpression may result in a dominant negative effect on downstream mTORC1 signaling. Since the interaction between IKK-related kinases and mTOR is strong enough to detect by coimmunoprecipitation experiments (Fig. 3.3), it is possible that when overexpressed, TBK1 or IKKε binds to and sequesters mTOR to blunt mTORC1 downstream signaling (Fig. 3.5D, compare lanes 2 and 3). In Fig. 3.3, we demonstrate that WT-TBK1 or -IKKε interact with mTOR more weakly/transiently relative to catalytically inactive

mutants, suggesting that phosphorylation of mTOR S2159 accelerates the release of TBK1/IKKε from mTOR. Thus, upon poly(I:C) stimulation, the increase in mTOR S2159 phosphorylation may very well relieve the inhibitory TBK1-mTORC1 interaction, promoting downstream mTORC1 signaling (Fig. 3.5D, compare lanes 3 and 4). Generating stable cell lines that overexpress TBK1 and IKKε at more physiological levels may help us further investigate the functional role of IKK-related kinases in mTORC1 signaling.

During viral or bacterial infection, the transcription of several genes such as type I IFNs that regulates innate immune responses are upregulated (176, 177, 293). While interferons inhibit global mRNA translation to block cell growth and viral replication, they promote translation of interferon-regulated mRNAs to establish an antiviral state in the cell (61). Recent data suggest that mTORC1 mediates the role of interferons in translation of interferon regulated mRNAs to enhance innate immune function. For instance, the Tsc1/2-mTORC1-4E-BP axis regulates IRF7, ISG15 (interferon-stimulated gene 15), and CXCL10 (C-X-C motif chemokine 10) mRNA translation upon virus infection to promote innate immunity. The 4E-BP1/2$^{-/-}$, TSC1$^{-/-}$, and TSC2$^{-/-}$ MEFs and 4E-BP1/2$^{-/-}$ mice display stronger antiviral responses due to increased cap-dependent translation of these transcripts (61, 163). Furthermore, inhibition of mTORC1 and S6K1 suppresses type I IFN production in plasmacytoid dendritic cells (pDCs) (35). These studies indicate that mTORC1 positively regulates innate immune signaling through its well-characterized substrates 4E-BP1 and S6K1 (35, 61, 163). Thus, upon viral or bacterial infection, TBK1 and IKKε may employ two distinct mechanisms to promote innate immune function at both transcriptional and translational levels: First, they may phosphorylate and activate IRF3 and IRF7 to induce the transcription of type I IFNs, which is a well established phenomenon. Second, they may phosphorylate mTOR S2159 to activate cap-dependent translation of IFN-regulated mRNAs such as IRF7, ISG15, and CXCL10. We plan to test this hypothesis in the future.

Chronic inflammation induces aberrant NF-κB signaling, which promotes tumorigenesis (162, 233). Ras-induced increases in TBK1 and IKKε activity also

augments aberrant Akt and NF-κB signaling, which promotes cellular transformation and cancer cell survival (14, 21, 224, 325). In addition, IKKε$^{-/-}$ mice are protected from diet-induced obesity and insulin resistance associated with type II diabetes as well as chronic inflammation in liver and adipose tissue (53). Interestingly, hyperactive mTORC1 signaling contributes to progression of these disease states (70, 133, 341). Thus, it is tempting to speculate that deregulated phosphorylation of mTOR S2159 by TBK1 and IKKε may contribute to these diseases. Thus, our findings may facilitate the development of novel therapeutics to treat these prevalent human disorders.

Experimental Methods

Materials

Reagents were obtained from the following sources. Protein A- and G-Sepharose Fast Flow and Glutathione-Sepharose beads were from GE Healthcare; 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) was from Pierce; Immobilon-P polyvinylidene difluoride (PVDF) membrane (0.45 μm) was from Millipore, reagents for enhanced chemiluminescence (ECL) were from Millipore (Immobilon Western chemi-luminescent horseradish peroxidase [HRP] substrate); all chemicals were from either Fisher Chemicals or Sigma.

Commercial antibodies

AU1, Myc (9E10) and HA.11 (for immunoprecipitation) antibodies were from Covance while HA.12CA5 antibody (for immunoblot) was generated by the UM Hybridoma Core. Flag-M2 antibody was from Sigma. Donkey anti-rabbit-HRP and sheep anti-mouse-HRP secondary antibodies were from GE Healthcare. P-S6K1-T389 (rabbit monoclonal 108D2) (no. 9234), P-S6-240/244 (no. 5364), S6 (no. 2217), P-mTOR-S2481 (no. 2974), mTOR (no. 2972), P-4E-BP1-T37/46 (no. 9459), 4E-BP1 (no. 9452), GST (no. 2622), TBK1 (no. 3504), P-TBK1 (no. 5483), IKKε (no. 3416) antibodies were from Cell Signaling Technology.

Custom generation of antibodies to mTOR, P-S2159-mTOR, S6K1, P-S863-raptor

Affinity purified, anti-peptide antibodies to mTOR (amino acids 221-237; rat), S6K1 (amino acids 485-502 of the 70 kDa rat isoform), P-S2159-mTOR (amino acids 2154-2163; rat), and P-S863-raptor (amino acids 860-868; human) were generated as described previously (2, 84, 99).

Plasmids

pRK5/Myc-mTOR, pRK5/Myc-mTOR-KD, pRK5/HA-raptor were obtained from D. Sabatini via Addgene (nos. 1861, 8482, and 8513 respectively); the pcDNA3/Flag-TBK1-WT, pcDNA3/Flag-TBK1-KD (K38A), pcDNA3/Flag-IKKε-WT, and pcDNA3/Flag-IKKε-KD (K38A) plasmids were from A. Saltiel (Life Sciences Institute, Ann Arbor, MI); the HA-rictor plasmid was from E. Jacinto (Rutgers University, Piscataway, NJ); and the pRK5/Myc-Sin1 plasmid was from K. Inoki (Life Sciences Institute, Ann Arbor, MI)

Generation of recombinant, GST-tagged mTOR in *E. coli*

A fragment of mTOR encoding amino acids 2144-2175 (wild type and a S2159A/T2164A mutant) was subcloned via PCR into vector pGEX-20T for production of GST fusion proteins in the bacterial strain BL21(DE3)LysS. The following primers were used to PCR amplify the mTOR fragment: Primer 1, 5'-gactggatcctatgaccccaaccagccaatc-3'; primer 2, 5'-gactgaattcgcccatcagggtcagcttccg-3'. GST-mTOR was affinity purified on glutathione-sepharose beads via a standard protocol and dialyzed against 10mM Tris pH 7.4, 100 mM NaCl, 1 mM EDTA, 154 mg/L DTT, and 5% glycerol.

***In vitro* kinome screen**

The *in vitro* kinome screen was performed in collaboration with Steve Riddle at Invitrogen/Life Sciences. It involved testing ~300 recombinant kinases on 384-well plate. The kinase reaction contained 25 nM recombinant kinase, 0.125 mg/ml GST-mTOR and 1 mM ATP in kinase buffer containing 50 mM HEPES,

pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, and 0.01% Brij-35. Reactions were incubated at room temperature for 1 hr. The dot blot of the kinase reactions were imaged after incubation with P-mTOR-S2159 primary antibody and Alexa Fluor 488 anti-rabbit secondary antibody.^{32}P incorporation experiment was performed similarly, except the reactions contained 20 nM recombinant kinase, 0.11 mg/ml GST-mTOR (WT or AA), 0.1 mM cold ATP, and trace [γ^{32}P]-ATP. After 1 hr incubation at room temperature, reactions were spotted on nitrocellulose, washed with phosphoric acid and water, and then imaged.

In vitro kinase (IVK) assays

In vitro kinase assays were performed by incubating recombinant GST-mTOR [200 ng] or immunoprecipitated Myc-mTOR substrate with ATP [250 μM] and recombinant [25 nM] or immunoprecipitated TBK1/IKKε in kinase buffer containing 50 mM Tris pH7.5, 12 mM $MgCl_2$, and 1 mM β- glycerophosphate. Reactions were incubated at 30°C for 30 min and stopped by adding sample buffer to a 1x final concentration and boiling for 5 min. Samples resolved on SDS-PAGE, transferred to PVDF membrane, and immunoblotted with P-S2159 antibodies. For drug pretreatments, recombinant kinases were incubated with 10 μM of BX795, CYT, MRT67307, or KBS drugs in kinase buffer on ice for 30 min. BX795, CYT, MRT67307, and KBS drugs were a kind gift from David Barbie (Dana-Farber Cancer Institute, Boston, MA)

Cell culture, drug treatment, and transfection

HEK293-TLR3, IKKε$^{+/+}$and IKKε$^{-/-}$ MEFs were obtained from K. Fitzgerald (University of Massachusetts Medical School, Worcester, MA). TBK1$^{+/+}$ and TBK1$^{-/-}$ MEFs were from Kun-Liang Guan (University of California San Diego, La Jolla, CA). RAW264.7 murine macrophages were from A. Saltiel (Life Sciences Institute, Ann Arbor, MI). HEK293 and HEK293-TLR3 cells, TBK1$^{+/+}$, TBK1$^{-/-}$, IKKε$^{+/+}$, IKKε$^{-/-}$ MEFs, and RAW264.7 murine macrophages were cultured in DMEM that contained high glucose [4.5 g/liter], glutamine [584 mg/liter], and sodium pyruvate [110 mg/liter] (Gibco/Invitrogen) supplemented with 10% fetal

bovine serum (FBS) (Gibco/Invitrogen) (heat inactivated-FBS was used for RAW264.7 murine macrophages) and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were serum deprived via incubation in DMEM supplemented with 0.5% FBS for ~20 hr and stimulated with or without poly(I:C) (Sigma no. 1530) and LPS (Invivogen code: tlrl-eblps). For drug treatment, cells were treated with BX795 [1 μM] or [10 μM] for 1 hr. Unless indicated in the figure legends, HEK293 cells on 60-mm/10-cm plates were transfected according to manufacturer's directions using TransIT-LT1 (Mirus) and a total of 5 μg/10 μg of DNA per plate. The specific amounts of experimental plasmid transfected are stated in the figure legends. Cells were lysed ~24 to 48 hr post-transfection.

Cell lysis, immunoprecipitation, and immunoblotting

Unless indicated otherwise, cells were washed twice with ice-cold PBS and scraped into ice-cold lysis buffer B containing NP-40 [0.5%] and Brij35 [0.1%] as originally described in (2, 84, 168). In Fig. 3.6, cells were lysed in buffer A containing CHAPS [0.3%] (2). Lysates were spun at 13,200 rpm for 5 min at 4°C, and the post-nuclear supernatants were collected. Bradford assay was used to normalize protein levels for immunoprecipitation and immunoblot analysis. For immunoprecipitation, WCLs were incubated with antibodies for 2 hr at 4°C, incubated with protein G- or A-Sepharose beads for 1 hr, washed three times in lysis buffer, and resuspended in 1x sample buffer, as described (2). Samples were resolved on SDS-PAGE and transferred to PVDF membranes by using Towbin transfer buffer, as described (2). Immunoblotting was performed by blocking PVDF membranes in Tris-buffer saline with Tween-20 containing 3% nonfat milk and incubating the membranes in TBST with 2% bovine serum albumin (BSA) containing primary antibodies or secondary HRP-conjugated antibodies, as described (2). Blots were developed by ECL and detected digitally with a Chemi-Doc-It System (UVP).

Generation of Flp-In stable cell lines

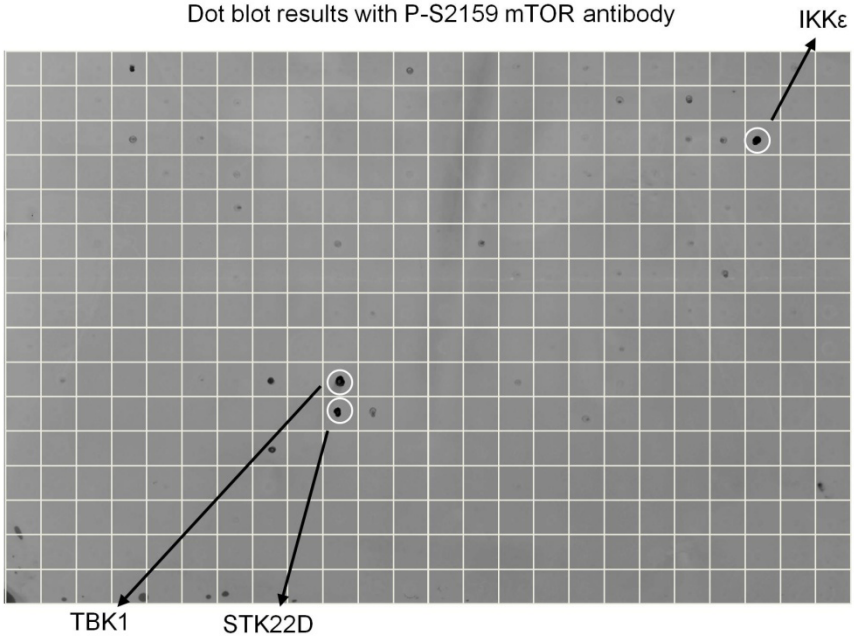
The Invitrogen Flp-In system was used to generate stable HEK293 cell lines that express AU1-mTOR-WT allele, as described (84). Flp-In-HEK293-AU1-mTOR-WT cells were maintained in high glucose DMEM supplemented with 10% FBS and hygromycin [100 µg/mL].

Image editing

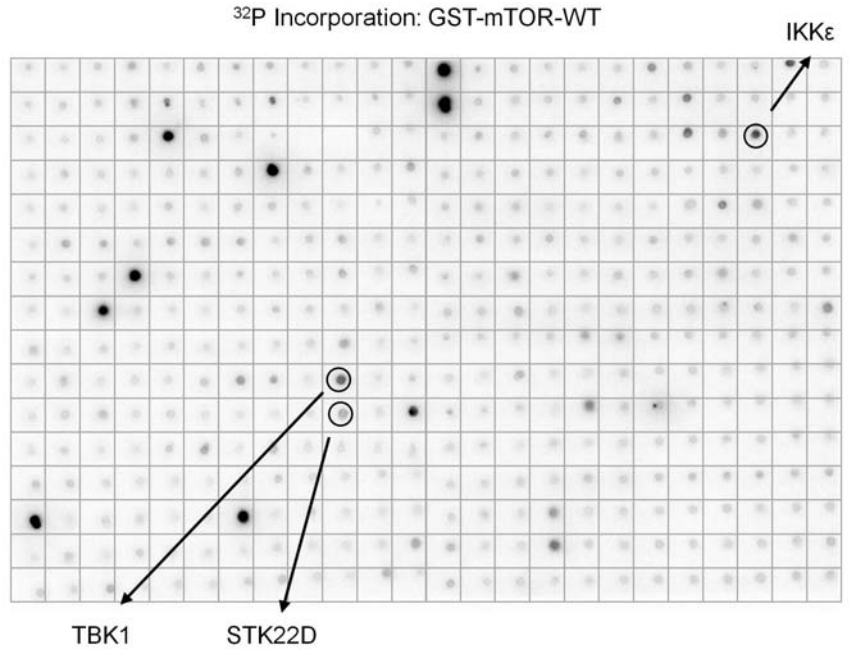
For some figures, irrelevant lanes were removed from a scanned autoradiograph and flanking lanes juxtaposed using Adobe Photoshop. The presence of a thin, vertical black line indicates such a modification.

Figure 3.1 *In vitro* kinome screen to identify potental mTOR S2159 kinases.
A. Dot blot with P-S2159 mTOR antibody. An *in vitro* kinome screen was performed using recombinant GST-mTOR-WT as substrate and ~300 recombinant kinases. Dot blot with the P-S2159 antibody was performed to detect mTOR phosphorylation. TBK1, IKKε, and STK22D kinases gave the strongest P-S2159 mTOR signals.
B. Site-specific ^{32}P incorporation. Similar to A, except that GST-mTOR WT (upper panel) or GST-mTOR-AA (lower panel) was used as substrate and [$\gamma^{32}P$]-ATP was added to the reactions. ^{32}P incorporation was detected by autoradiography. TBK1, IKKε, and STK22D phosphorylated GST-mTOR-WT but not GST-mTOR-AA.

A.



B.


32P Incorporation: GST-mTOR-WT
IKKε
TBK1
STK22D

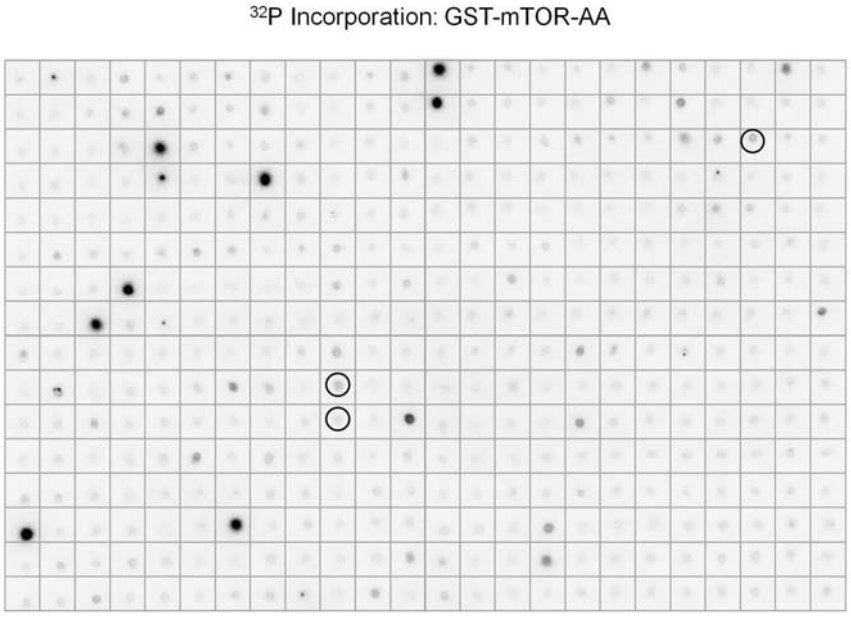
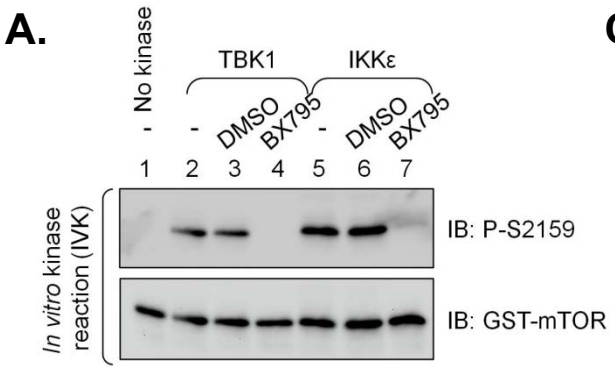
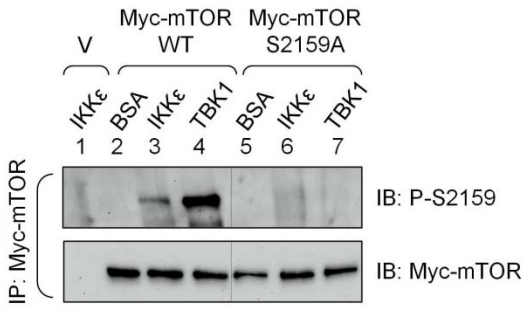
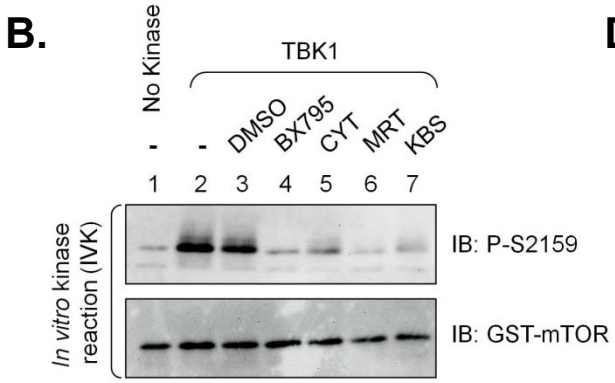
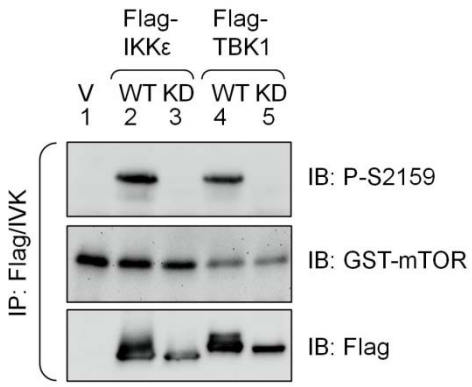

32P Incorporation: GST-mTOR-AA

Figure 3.2 Recombinant TBK1 and IKKε phosphorylate mTOR S2159 *in vitro.*
A. *In vitro* kinase (IVK) assays with recombinant GST-mTOR substrate. Recombinant TBK1 or IKKε [25 nM] was pretreated with DMSO vehicle control or the TBK1/IKKε inhibitor BX795 [10 μM] for 30 minutes on ice in kinase buffer. Start cocktail containing 200 ng of GST-mTOR and 250 μM ATP was then added. The reactions were incubated at 30°C for 30 minutes. SDS-PAGE sample buffer was added to a 1x final. Samples were boiled for 5 min, resolved on SDS-PAGE, and immunoblotted with the indicated antibodies.
B. *In vitro* kinase assays in the presence of several TBK1 inhibitors. Similar to A except that recombinant TBK1 was pretreated with DMSO vehicle control or various TBK1 inhibitors: BX795 [10 μM], CYT [10 μM], MRT67307 [10 μM], or KBS [10 μM] for 30 minutes on ice in kinase buffer.
C. Recombinant IKKε and TBK1 phosphorylate full-length mTOR on S2159. HEK293 cells on 10-cm plates were transiently transfected with 10 μg vector control, Myc-mTOR-WT or -S2159A plasmids. WCLs were immunoprecipitated with Myc antibodies. Immunoprecipitated Myc-mTOR was subjected to *in vitro* kinase assay in the presence of BSA control or recombinant kinases IKKε/TBK1, similar to A. Samples were immunoblotted with the indicated antibodies.
D. Immunoprecipitated IKKε and TBK1 phosphorylate mTOR S2159 *in vitro*. HEK293 cells on 60-mm plates were transiently transfected with 5 μg vector control, Flag-IKKε-wild type (WT), Flag-IKKε-kinase dead (KD), Flag-TBK1-WT, or Flag-TBK1-KD. WCLs were immunoprecipitated with Flag antibodies. Immunoprecipitated Flag-IKKε-WT, Flag-IKKε-KD, Flag-TBK1-WT, or Flag-TBK1-KD was subjected to *in vitro* kinase (IVK) assay using recombinant GST-mTOR similar to that shown in A, B, and C. Samples were immunoblotted with the indicated antibodies.
E.The sequence surrounding the mTOR S2159 phosphorylation site fits well with TBK1 and IKKε consensus target phospho motifs.
Note: WT, wild type; KD, kinase dead









E.

mTOR P-S2159 : S-I-A-P-pS-L-Q-V-I

TBK1 target motif : S-x-x-x-pS

IKKε target motif : x-x-x-**Y**-x-p**S**-**L**-x-**Y**-x

FP I WF

M MF

Figure 3.3 TBK1/IKKε and mTOR coimmunoprecipitate.
Flp-In-HEK293 cells on 10-cm plates stably expressing vector control (-) or AU1-mTOR (+) were transiently transfected with10 μg vector, Flag-TBK1-WT, Flag-TBK1-KD, Flag-IKKε-WT, or Flag-IKKε-KD. WCLs were immunoprecipitated with AU1 antibodies and immunoblotted as indicated. WCLs were also immunoblotted directly.
Note: WT, wild type; KD, kinase dead.

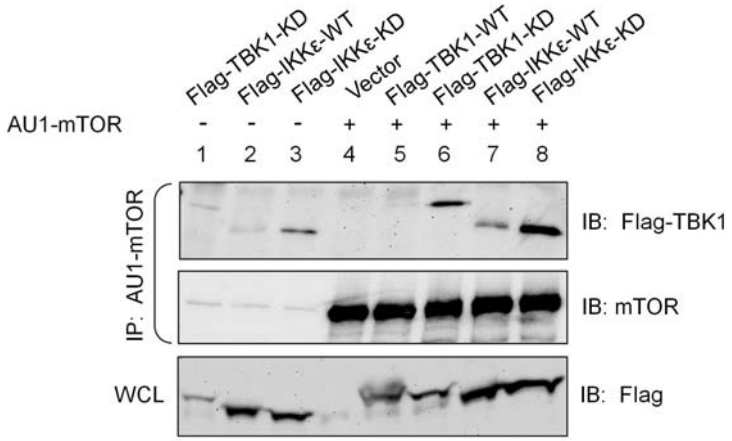


Figure 3.4 TBK1$^{-/-}$ and IKKε$^{-/-}$ MEFs show reduced mTOR S2159 phosphorylation.
A. TBK1$^{+/+}$ vs. TBK1$^{-/-}$ MEFs. Endogenous mTOR was immunoprecipitated from TBK1$^{+/+}$ and TBK1$^{-/-}$ MEFs with mTOR antibodies and immunoblotted with the indicated antibodies. WCLs were also immunoblotted directly.
B. IKKε$^{+/+}$ vs. IKKε$^{-/-}$ MEFs. Similar to A, except that IKKε$^{+/+}$ and IKKε$^{-/-}$ MEFs were used.
Note: NS, non-specific band.

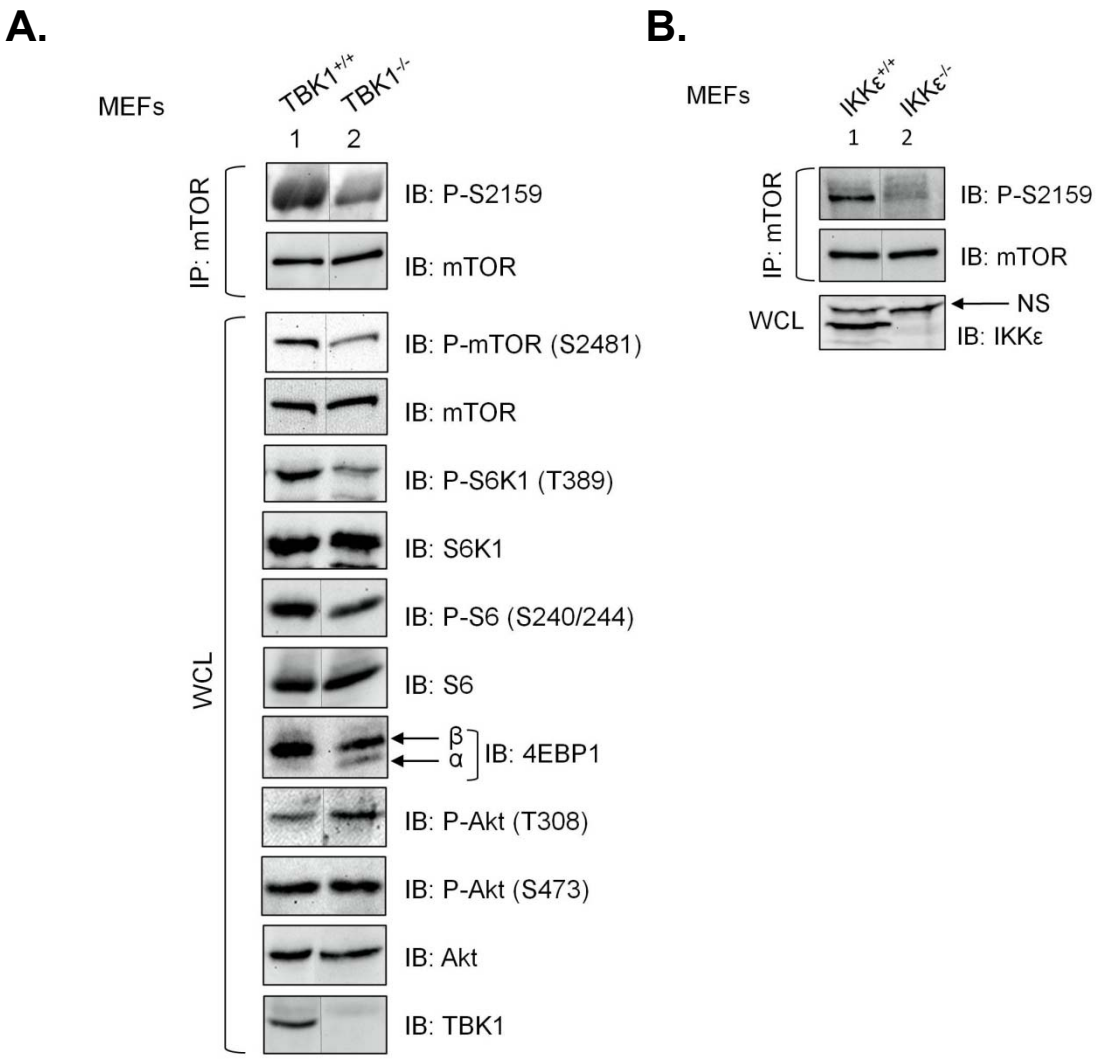


Figure 3.5 Overexpression of TBK1 and IKKε increases mTOR S2159 phosphorylation and S2481 autophosphorylation in intact cells.

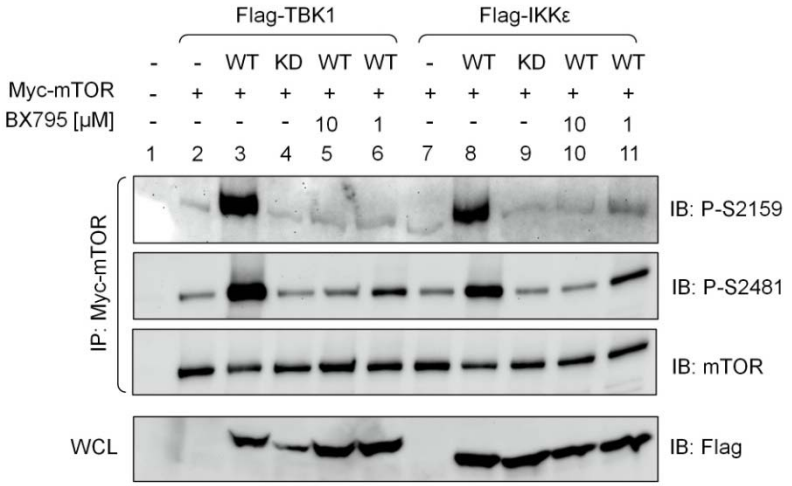
A. Analysis of mTOR S2159 phosphorylation and S2481 autophosphorylation upon TBK1/IKKε overexpression. HEK293-TLR3 cells on 10-cm plates were transfected with vector control or Myc-mTOR [8 μg] together with Flag-TBK1-WT, Flag-TBK1-KD, Flag-IKKε-WT, or Flag-IKKε-KD [2 μg]. Before lysis, cells were pretreated with or without BX795 for 2 hr. WCLs were immunoprecipitated with Myc antibodies and immunoblotted, as indicated. WCLs were also immunoblotted directly.

B. TBK1 and IKKε overexpression increases mTOR S2159 phosphorylation within mTORC1, promotes S2481 autophosphorylation within mTORC1 and mTORC2, and induces raptor and rictor phosphorylation. HEK293-TLR3 cells on 10-cm plates were transiently transfected with vector control, Myc-mTOR [7 μg], Flag-TBK1 [2 μg] or Flag-IKKε [2 μg], and HA-Raptor [1.5 μg] (left panel) or HA-rictor [1.5 μg] and Myc-Sin1 [1.5 μg] (right panel). WCLs were immunoprecipitated with HA antibodies and immunoblotted, as indicated. WCLs were also immunoblotted directly. Note: Lanes 2 and 3; 4 and 5 are duplicates.
LE: Light exposure; DE: Dark exposure

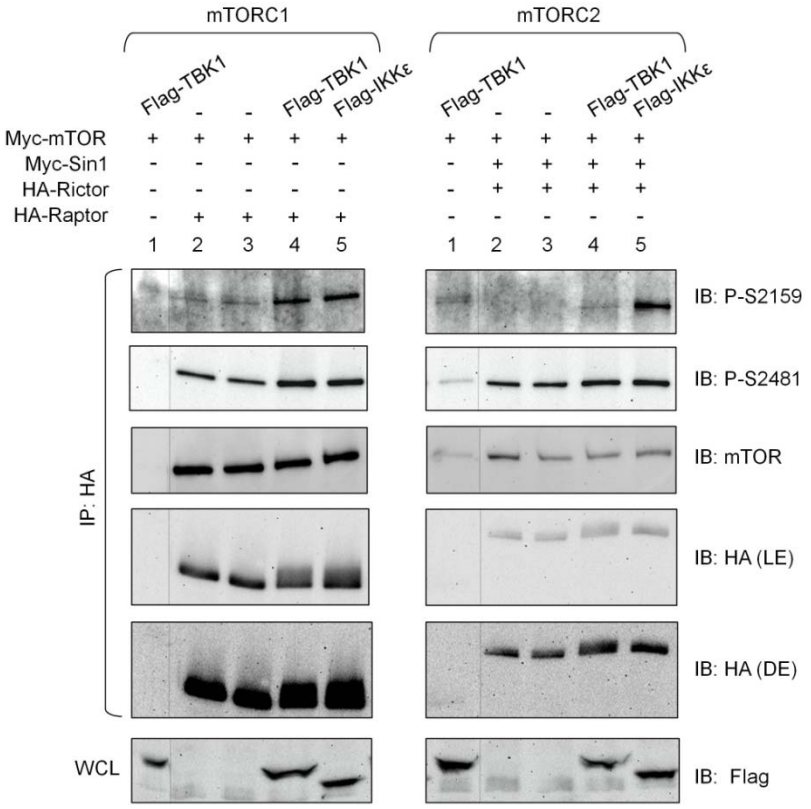
C. TBK1 overexpression promotes raptor S863 phosphorylation. HEK293-TLR3 cells on 10-cm plates were transiently co-transfected with vector control or HA-raptor [1 μg] together with or without Flag-TBK1 [2 μg] and Myc-mTOR [7μg]. Before lysis, cells were pre-treated with or without BX795 for 2 hr. WCLs were immunoprecipitated with HA antibodies and immunoblotted, as indicated.

D. Poly(I:C) stimulation promotes mTOR S2159 phosphorylation in HEK293-TLR3 cells. HEK293-TLR3 cells on 10-cm plates were transiently transfected with vector control or Myc-mTOR [8 μg] together with or without Flag-TBK1 [2 μg]. Cells were serum deprived in DMEM-FBS [0.5%] (~20 hr) and stimulated with or without poly (I:C) [50 μg/ml] for 2 hr. WCLs were immunoprecipitated with Myc antibodies and immunoblotted, as indicated. WCLs were also immunoblotted directly.

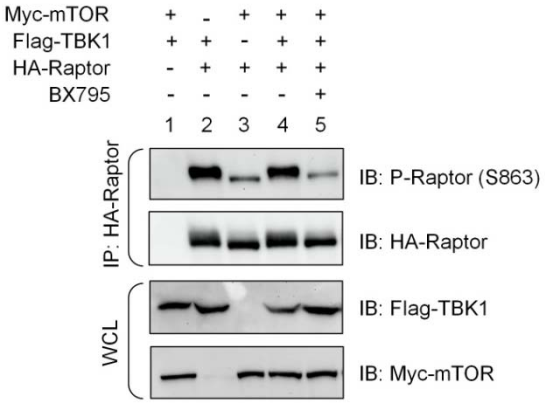
A.



B.


mTORC1
mTORC2
Flag-TBK1
Flag-TBK1
Flag-IKKε
Myc-mTOR
Myc-Sin1
HA-Rictor
HA-Raptor
1 2 3 4 5
IP: HA
WCL
IB: P-S2159
IB: P-S2481
IB: mTOR
IB: HA (LE)
IB: HA (DE)
IB: Flag

C.


Myc-mTOR
Flag-TBK1
HA-Raptor
BX795
1 2 3 4 5
IP: HA-Raptor
WCL
IB: P-Raptor (S863)
IB: HA-Raptor
IB: Flag-TBK1
IB: Myc-mTOR

D.

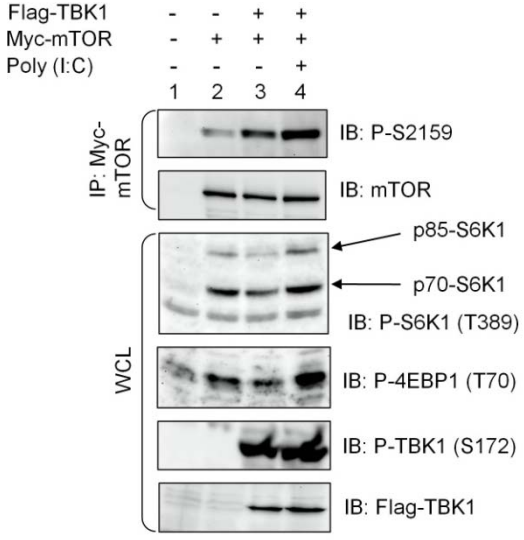
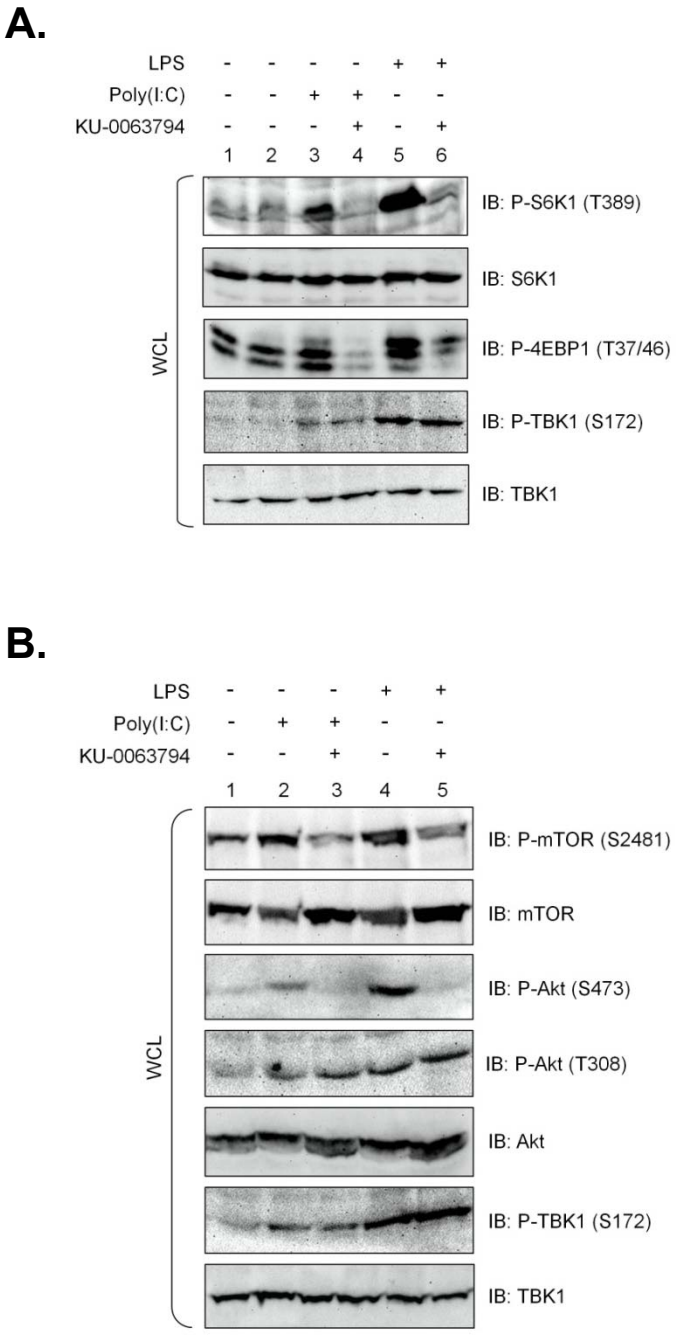

Flag-TBK1
Myc-mTOR
Poly (I:C)
1 2 3 4
IP: Myc-mTOR
WCL
IB: P-S2159
IB: mTOR
p85-S6K1
p70-S6K1
IB: P-S6K1 (T389)
IB: P-4EBP1 (T70)
IB: P-TBK1 (S172)
IB: Flag-TBK1

Figure 3.6 Activation of TLR3 and TLR4 promotes mTOR signaling in RAW264.7 murine macrophages.
A. Activation of TLR3 and TLR4 promotes mTORC1 signaling. RAW264.7 cells on 10-cm plates were pretreated with or without KU-0063794 [100 nM] for 1 hr and stimulated with or without poly (I:C) [30 μg/ml] or LPS [100 ng/ml] for 2 hr and lysed. WCLs were directly immunoblotted with the indicated antibodies. Note: Lanes 1 and 2 are duplicates.
B. Activation of TLR3 and TLR4 promotes mTOR intrinsic kinase activity and mTORC2 signaling in RAW264.7 cells. Same as A.



Chapter 4

Conclusion

Aberrant regulation of mTORC1 signaling contributes to a host of diseases including cancer, benign tumor syndromes, type II diabetes, and cardiovascular diseases. mTOR is an evolutionarily conserved serine/threonine protein kinase that functions as an environmental sensor to regulate protein synthesis, cell growth (increase in cell size/mass), and cell proliferation. Although the intra- and extra-cellular signals that regulate mTORC1 signaling (i.e. growth factors; nutrients; cellular stress) are well established, the molecular mechanisms underlying mTORC1 regulation remain poorly defined. Accumulating evidence in the literature indicates that reversible phosphorylation regulates mTOR and its partner proteins. Upon insulin stimulation, Akt and mTOR, for instance, phosphorylate PRAS40 to blunt its inhibitory function on mTORC1 (97, 222, 263, 308, 313). Insulin/PI3K signaling also promotes mTOR-mediated raptor S863 phosphorylation to promote mTORC1 function (99, 315). Activated mTOR also phosphorylates deptor, leading to its degradation and thus relieving its mTOR inhibitory action (238). Ras activation leads to MAPK and RSK-mediated phosphorylation of Tsc2 (193, 255, 295) and raptor (39, 40) to promote mTORC1 function. In response to energy deprivation, AMPK phosphorylates both Tsc2 and raptor to suppress mTORC1 function (117, 148). Thus, diverse upstream signals regulate mTORC1 signaling by regulating phosphorylation of PRAS40, Tsc2, and raptor on multiple sites. mTOR contains several phosphorylation sites, as well. These include S2481, S2448, T2446, and S1261 (2, 51, 52, 135, 212, 237, 273). S2481 is an mTOR autophosphorylation site (237). Stimulation of cells with amino acids or insulin promotes, whereas inhibition of mTOR by drug inhibitors

or amino acid depletion reduces mTOR S2481 autophosphorylation, suggesting that S2481 autophosphorylation monitors mTOR intrinsic catalytic activity (284). Near S2481 lies S2448, a site phosphorylated by the mTORC1 substrate S6K1. Mitogenic signals, amino acids, and insulin stimulation increase, whereas rapamycin and wortmannin treatment decreases mTOR S2448 phosphorylation (51, 52, 135, 273). mTOR phosphorylation on T2446, on the other hand, increases during energy depletion and decreases upon insulin and nutrient stimulation (51, 273), suggesting that phosphorylation of mTOR T2446 negatively regulates mTORC1 function, although no experimental data supports this notion (51, 273). Although the regulation of mTOR S2481, S2448, and T2446 phosphorylation by upstream signals is well established, the functional significance of these phosphorylation sites in mTORC1 or mTORC2 function remains poorly understood. S1261 is a more recently identified mTOR site, phosphorylation of which increases in both mTORC1 and mTORC2 upon insulin stimulation via the PI3K/Akt pathway in 3T3-L1 adipocytes (2). mTOR S1261 phosphorylation promotes mTORC1 intrinsic catalytic activity, downstream signaling to S6K1 and 4E-BP1, and cell growth, providing the first evidence that site-specific mTOR phosphorylation regulates mTORC1 function (2).

To elucidate the molecular mechanisms underlying mTORC1 regulation, we further investigated the phosphorylation of mTOR itself. We identified S2159 and T2164 as novel sites of mTOR phosphorylation that lie at the N-terminus of mTOR kinase domain. Both S2159 and T2164 residues are evolutionarily conserved (Fig. 2.1D). mTOR S2159 and T2164 phosphorylation acts in a cooperative manner to promote mTORC1 signaling towards its well characterized substrates S6K1 and 4E-BP1 under steady-state conditions as well as upon insulin or amino acid stimulation (Chapter 2) (84).

Raptor has a dual function in regulating mTORC1 signaling. While a tighter, more detergent resistant conformation of the mTOR-raptor interaction restricts downstream mTORC1 signaling, upon insulin or amino acid stimulation the mTOR-raptor interaction becomes more detergent sensitive, which likely reflects an allosteric conformational change in mTORC1 that enables substrate

recruitment and phosphorylation (84, 168). Insulin stimulation also weakens the inhibitory raptor-PRAS40 interaction via both Akt- and mTORC1-mediated phosphorylation of PRAS40 (97, 222, 263, 308, 313). In this study, we show that mTOR S2159 and T2164 phosphorylation weakens the inhibitory mTOR-raptor and raptor-PRAS40 interactions. mTOR S2159 and T2164 phosphorylation also increases mTORC1 intrinsic catalytic activity, as monitored by mTOR S2481 autophosphorylation. Thus, our work reveals that mTOR S2159 and T2164 phosphorylation employs several mechanisms to increase mTORC1 signaling capacity: mTOR S2159 and T2164 phosphorylation weakens inhibitory mTORC1 component interactions and promotes mTORC1 intrinsic catalytic activity, which leads to increased S6K1 and 4EBP1 phosphorylation (84) (Chapter 2). Importantly, mTOR S2159 and T2164 phosphorylation not only promotes biochemical mTORC1 signaling to S6K1 and 4E-BP1, but also contributes to cell growth and G1-phase cell cycle progression (84) (Chapter 2).

In addition to promoting cell growth and cell cycle progression, other cell biological functions of mTORC1 signaling include augmenting *de novo* lipid biosynthesis and inhibiting autophagy (158, 181, 241). Thus, in the future it will be interesting to investigate whether mTOR S2159 and T2164 phosphorylation also contributes to these cell biological functions of mTORC1. In addition, it will also be important to test whether mTOR S2159 and T2164 phosphorylation regulates mTORC2 function to its downstream targets Akt, SGK1, and PKCα.

Moving forward, we performed a kinome screen and identified IKK-related kinases TBK1 and IKKε as candidate kinases for mTOR S2159. TBK1 and IKKε share 64% sequence identity and overlapping functions with each other. Upon viral or bacterial infection, TLR3 and TLR4 found on immune cells, respectively, activate TBK1 and IKKε to promote type I IFN synthesis and initiate innate immune responses (96, 130, 276). We confirmed the kinome screen results by demonstrating that both TBK1 and IKKε phosphorylate mTOR on S2159 directly *in vitro* (Chapter 3). In addition, both TBK1 and IKKε physically interact with mTOR, validating our findings further. Interestingly, the kinase dead (KD) mutants of TBK1 and IKKε interact with mTOR more strongly relative to the wild

type TBK1 and IKKε, indicating that the interaction between TBK1/IKKε and mTOR takes place in a hit and run manner, such that lack of phosphorylation on mTOR S2159 slows down the release of kinase dead TBK1/IKKε from mTOR. Previous studies also reported similar dynamics of kinase-substrate interactions. Catalytically inactive MEK, for instance, interacts with its substrate ERK more robustly compared to wild type MEK (251).

In addition to *in vitro* kinase reactions, we demonstrate that TBK1 and IKKε promote mTOR S2159 phosphorylation in intact cells. TBK1$^{-/-}$ and IKKε$^{-/-}$ single knockout MEFs display reduced levels of mTOR S2159 phosphorylation relative to wild type MEFs, indicating that TBK1 and IKKε indeed represent physiological kinases for mTOR S2159. Furthermore, overexpression of TBK1 and IKKε boosts mTOR S2159 phosphorylation, which increases further when TLR3 is stimulated with poly(I:C).

Although we have identified TBK1 and IKKε as kinases for mTOR S2159, the kinase(s) for T2164 site remains unknown. Our initial studies suggested that S2159 and T2164 act in a cooperative manner to promote mTORC1 signaling, however neither TBK1 nor IKKε phosphorylate mTOR on T2164 (84) (data not shown). These data are consistent with our earlier observations, which indicated that a staurosporine-sensitive kinase (i.e. TBK1 and IKKε) mediates mTOR S2159 phosphorylation, while a staurosporine-insensitive kinase mediates mTOR T2164 phosphorylation (84) (Chapter 2). It is entirely possible, however that parallel signaling inputs that employ different kinases regulate mTOR S2159 and T2164 phosphorylation simultaneously to promote mTOR function.

Our studies show that TBK1 is important for mTORC1 function since under steady-state conditions, TBK1$^{-/-}$ MEFs display impaired mTOR intrinsic catalytic activity and reduced mTORC1 signaling to S6K1, S6, and 4E-BP1. This role of TBK1 in promoting mTORC1 function is independent of its recently identified role as an Akt T308 and S473 kinase, since under steady-state conditions, TBK1$^{-/-}$ MEFs do not display impaired Akt phosphorylation on either T308 or S473 (155, 224, 325). Consistently, activation of TLR3 and TLR4 in RAW264.7 murine macrophages promotes mTOR intrinsic catalytic activity and

downstream mTORC1 and mTORC2 signaling towards S6K1, S6, 4E-BP1 and Akt, respectively. Whether TBK1/IKKε-dependent mTOR S2159 phosphorylation mediates the role of TLR3 and TLR4 in promoting mTORC1 and mTORC2 function remains a very important question for future research. It will be also interesting to investigate what molecules other than TBK1 and IKKε function downstream of TLR3 and TLR4 to promote mTORC1 and mTORC2 signaling.

TBK1 and IKKε not only regulate mTOR intrinsic catalytic activity and S2159 phosphorylation but also induce raptor and rictor phosphorylation, likely on multiple sites, as observed by extensive mobility shift on SDS-PAGE gels. Considering the role of raptor and rictor phosphorylation in controlling mTORC1 and mTORC2 function, respectively, it will be very interesting to uncover specifically which phosphorylation sites TBK1 and IKKε regulate on these proteins. It is also entirely possible that TBK1 and IKKε control phosphorylation of other mTORC1 and mTORC2 components such as PRAS40, deptor, and mSin1. Investigating these questions will help our understanding of TBK1- and IKKε-mediated regulation of mTORC1 and mTORC2 function.

Uncovering the direct role of TBK1- and IKKε-dependent mTOR S2159 phosphorylation remains an important area for future research. It is possible that mTOR S2159 phosphorylation contributes to the role of TBK1 and IKKε in initiating innate immune signaling. Several studies, indeed, point out a role for mTORC1 signaling in promoting innate immunity. mTORC1 augments the cap-dependent translation of IFN regulated mRNAs to enhance innate immune responses (61). For instance, Tsc1/2-mTORC1-4EBP axis increases IRF7, ISG15, and CXCL10 mRNA translation upon virus infection to promote innate immunity and establish an antiviral state (61, 163). Additional studies suggest that not only the mTORC1-4E-BP1 axis but also the mTORC1-S6K1 axis contributes to innate immunity by promoting type I IFN synthesis in pDCs (35). Overall, these studies indicate that mTORC1 positively regulates innate immune responses through its well-characterized substrates S6K1 and 4E-BP1 (35, 61, 163). Thus, upon viral or bacterial infection, in addition to phosphorylating and activating IRF3 and IRF7, TBK1 and IKKε may employ an additional mechanism

to promote the translation of IFN regulated mRNAs by augmenting mTORC1 function through mTOR S2159 phosphorylation.

Chronic inflammation induces tumorigenesis through aberrantly regulated NF-κB signaling (162, 233). It is possible that the immune related TBK1 and IKKε kinases also play role in inflammatory carcinogenesis. In agreement with this, emerging data suggest that oncogenic Ras signaling increases TBK1 and IKKε activities, which augments aberrant Akt and NF-κB signaling to promote cellular transformation and cancer cell survival (14, 21, 224, 325). Thus, in the future, we plan to test whether oncogenic Ras increases TBK1- and IKKε-dependent mTOR S2159 phosphorylation to promote tumor cell survival. Additionally, IKKε$^{-/-}$ mice are protected from diet-induced obesity and insulin resistance associated with type II diabetes as well as chronic inflammation in liver and adipose tissue (53). Interestingly, the role of aberrantly regulated mTOR signaling in these disease states is well established (i.e. carcinogenesis, type II diabetes, obesity) (70, 133, 341). Thus, it is tempting to speculate that deregulation of TBK1- and IKKε-dependent mTOR S2159 phosphorylation may contribute to progression of these diseases, which we would like to test in the future. Overall, our findings presented in this study may help the development of novel therapeutics to treat these prevalent human disorders.

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