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The regulation of mTORC2 kinase activity and complex integrity

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A

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Dedication

This Ph.D. thesis work is dedicated to

My dearest wife

Szu-Wei Lee

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The regulation of mTORC2 kinase activity and

complex integrity

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Growth factor signaling promotes anabolic processes via activation of the PI3K-Akt kinase cascade. Deregulation of the growth factor-dependent PI3K-Akt pathway was implicated in tumorigenesis. Akt is an essential serine/threonine protein kinase that controls multiple physiological functions such as cell growth, proliferation, and survival to maintain cellular homeostasis. Recently, the mammalian Target of Rapamycin Complex 2 (mTORC2) was identified as the main Akt Ser-473 kinase, and Ser-473 phosphorylation is required for Akt hyperactivation. However, the detailed mechanism of mTORC2 regulation in response to growth factor stimulation or cellular stresses is not well understood.

In the first project, we studied the regulation of the mTORC2-Akt signaling under ER stress. We identified the inactivation of mTORC2 by glycogen synthase kinase-3β (GSK-3β). Under ER stress, the essential mTORC2 component, rictor, is phosphorylated by GSK-3β at Ser-1235. This phosphorylation event results in the inhibition of mTORC2 kinase activity by interrupting Akt binding to mTORC2. Blocking rictor Ser-1235 phosphorylation can attenuate the negative impacts of GSK-3β on mTORC2/Akt signaling and tumor growth. Thus, our work demonstrated that GSK-3β-mediated rictor Ser-1235 phosphorylation in response to ER stress interferes with Akt signaling by inhibiting mTORC2 kinase activity.

In the second project, I investigated the regulation of the mTORC2 integrity. We found that basal mTOR kinase activity depends on ATP level, which is tightly regulated by cell metabolism. The ATP-sensitive mTOR kinase is required for SIN1 protein phosphorylation and stabilization. SIN1 is an indispensable subunit of mTORC2 and is required for the complex assembly and mTORC2 kinase activity. Our findings reveal that mTOR-mediated phosphorylation of SIN1 is critical for maintaining complex integrity by preventing SIN1 from lysosomal degradation.

In sum, our findings verify two distinct mTORC2 regulatory mechanisms via its components rictor and SIN1. First, GSK-3β-mediated rictor Ser-1235 phosphorylation results in mTORC2 inactivation by interfering its substrate binding ability. Second, mTOR-mediated Ser-260 phosphorylation of SIN1 preserves its complex integrity. Thus, these two projects provide novel insights into the regulation of mTORC2.

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LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
BSA	Bovine serum albumin
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1- propanesulfonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
elF4E	Eukaryotic translation initiation factor 4E
4EBPs	Eukaryotic initiation factor 4E binding proteins
ER	endoplasmic reticulum
FBS	Fetal bovine serum
FRB	FKBP12-rapamycin binding
GSK3	Glycogen synthase kinase
GST	Glutathione S-transferase
HEAT	Huntington-elongation factor 1A-protein phosphatase 2A (PP2A)Asubunit-TOR
HEK-293T	Human embryonic kidney 293T
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HM	hydrophobic motif
IGF	insulin-like growth factor
IP	Immunoprecipitation
KCl	Potassium chloride

MEF	Mouse Embryonic Fibroblast
$MgCl_2$	Magnesium chloride
mLST8	Mammalian lethal with SEC13 protein 8
mTOR	Mammalian target of rapamycin
NaCl	Sodium chloride
NaF	Sodium fluoride
PCR	Polymerase chain reaction
PDK	phosphoinositide-dependent kinase
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog
raptor	Regulatory associated protein of mTOR
Rheb	Ras homolog enriched in brain
rictor	Rapamycin-insensitive companion of mTOR
S6Ks	Ribosomal protein S6 kinases
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIN1	stress-associated protein kinase (SAPK)-interacting protein 1

Chapter 1

Introduction

1.1 Growth factor-dependent PI3K-Akt signaling pathway

Growth factors have been identified as the autocrine peptide ligands secreted by cells to regulate cell survival, proliferation, and differentiation (Cantley, 2002; Engelman et al., 2006; Fruman et al., 1998). When growth factors bind to their specific receptors, it results in activation of the receptor tyrosine kinase that further initiates phosphorylation of the regulatory docking proteins on tyrosine residues. These tyrosine-phosphorylated sites play an important role to recruit regulatory proteins through Src Homology 2 (SH2) domains and proline-rich binding SH3 domains (Schlessinger and Lemmon, 2003). These growth factor signaling-mediated protein-protein interactions promote phosphatidylinositol-3 kinase (PI3K) activity (Cantley, 2002; Shaw and Cantley, 2006). The well-defined downstream effector of the PI3K pathway is the Akt, also known as protein kinase B (PKB) (Cantley, 2002; Testa and Bellacosa, 2001). Akt is a highly conserved serine/threonine protein kinase, and controls various biological functions such as cell proliferation, growth, survival, and metabolism. Perturbations of the PI3K-Akt signaling are associated with human diseases, such as cancers and metabolic syndromes.

1.2 Downstream effectors and cellular functions of Akt

Akt kinase is a member of the AGC (protein kinase A, G, and C) kinase family that act on a wide spectrum of substrates (Bellacosa et al., 2005). For example, Akt regulates cell cycle through phosphorylation of the cell cycle inhibitors p27 and p21 by causing their cytoplasmic retention (Viglietto et al.,

2002; Zhou et al., 2001a), and also through phosphorylation of the human oncogene product MDM2, which degrades p53 tumor suppressor gene (Zhou et al., 2001b). Akt regulates cell survival through inactivation of the pro-apoptotic protein BAD (Datta et al., 1997; del Peso et al., 1997) and inhibition of the highly conserved substrates identified as the FOXO transcription factors that drive the expression of pro-apoptotic genes (Brunet et al., 1999). Akt controls metabolism by suppressing glycogen synthase kinase-3 (GSK-3) and by stimulating glucose transport and glycolysis (Cross et al., 1995; Kohn et al., 1996). The role of Akt signaling in regulation of cell growth and cell size is linked to its up-regulation of the mTOR pathway by the phosphorylation of tuberous sclerosis complex 2 (TSC2) (Inoki et al., 2002; Manning et al., 2002) and the proline-rich Akt substrate of 40 kDa (PRAS40) (Sancak et al., 2007; Vander Haar et al., 2007).

1.3 Activation of Akt

In the process of the growth factor signaling, binding of the growth factors to the receptors leads to activation and translocation of PI3K to the plasma membrane. Following activation of PI3K and accumulation of phosphatidylinositols-3,4,5-triphosphates (PIP_3), Akt switches its cellular localization to the plasma membrane by binding to PIP_3 via its pleckstrin homology (PH) domain at the N-terminal region (Figure 1-1). In addition to PH domain, it has been recently reported that

Figure 1-1

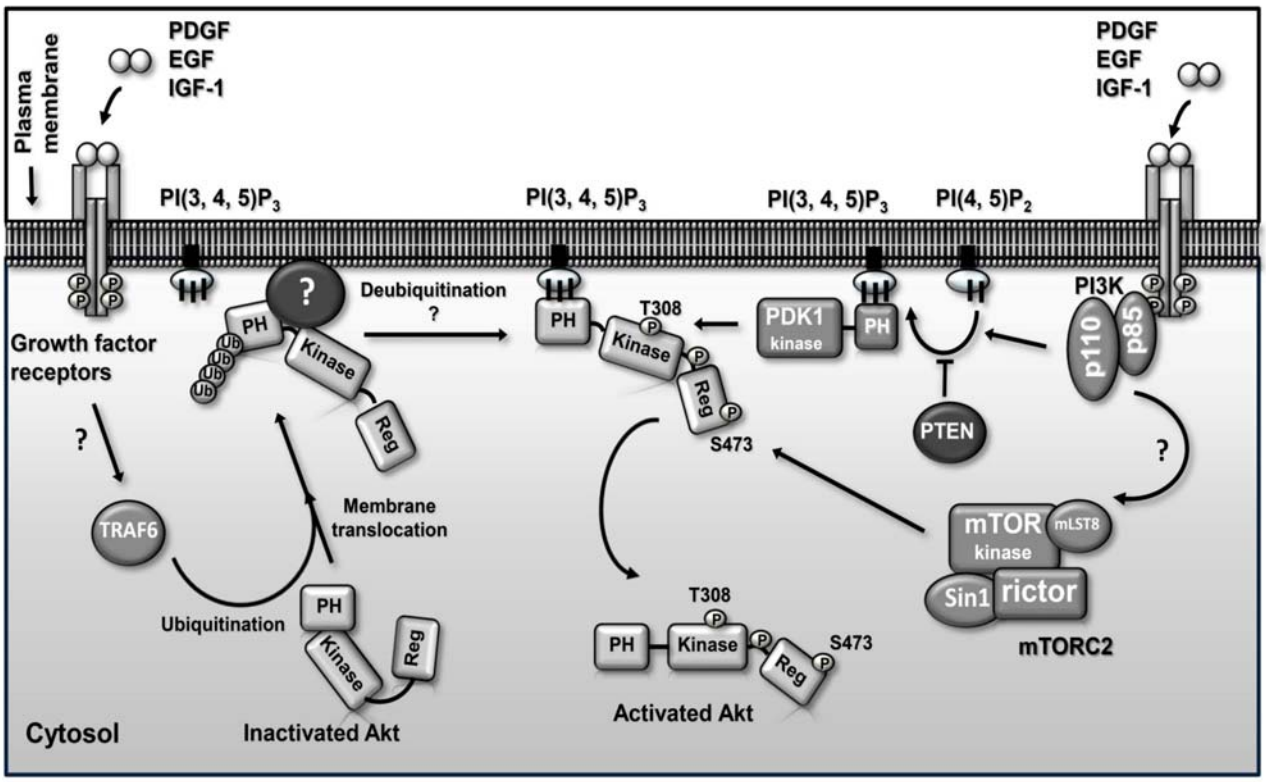

Figure 1-1. Growth factor signaling pathway

Receptors regulated by growth factors activate PI3 Kinase, which recruits inactive Akt to the plasma membrane. Recent report demonstrates that ubiquitination of Akt by TRAF6 is required for its membrane translocation. Moreover, both phosphorylation events by PDK1 and mTORC2 are required for full activation of Akt.

TRAF6 ubiquitinates Akt and assists its membrane translocation (Yang et al., 2009). In the meantime, two residues of Akt are phosphorylated for its full activation. The Thr-308 residue of Akt that locates on its activation loop is phosphorylated by the phosphoinositide-dependent kinase (PDK1), and this phosphorylation is required for kinase activity of Akt (Alessi et al., 1997; Stephens et al., 1998). The second Ser-473 residue resides at the C-terminal region of Akt, and has been defined as the hydrophobic regulatory site. Lately, mTORC2 has been identified as the major Akt Ser-473 kinase (Sarbassov et al., 2005).

Whether Thr-308 phosphorylation of Akt is dependent on Ser-473 phosphorylation is still controversial. In human cancer cells (HT-29 colon, A549 lung, HEK-293T kidney, HeLa cervical, and PC3 prostate), knockdown of mTOR or rictor expression decreases Akt phosphorylation on both Ser-473 and Thr-308 sites (Sarbassov et al., 2005). In this setting, inhibition of mTORC2-mediated Ser-473 phosphorylation results in the decreased Thr-308 phosphorylation of Akt, indicating the importance of Ser-473 phosphorylation for Thr-308 phosphorylation. Moreover, prior Ser-473 phosphorylation is required for PDK1 to phosphorylate Thr-308 in the immortalized cells (Scheid et al., 2002). However, phosphorylation of Akt Thr-308 is not affected by loss of Ser-473 phosphorylation in mTORC2-deficient embryos and MEFs (Guertin et al., 2006). This difference reflects the highly active state of the Akt signaling in cancer cells because Thr-308 phosphorylation of Akt can be boosted by its Ser-473 phosphorylation. In this regard, mTORC2 signaling might be amplified to hyper-activate Akt signaling that

is engaged in tumor growth and progression. Thus, inhibition of mTORC2 in cancer cells should give stronger effects on Akt signaling pathway than in normal tissues.

1.4 Mammalian target of rapamycin (mTOR)

mTOR was initially identified as the target of rapamycin, which is applied as a potential anti-cancer drug and a potent immunosuppressant. The effects of rapamycin on inhibiting proliferation stem from the inactivation of mTOR, an essential regulator of the highly-conserved growth pathway (Harris and Lawrence, 2003). mTOR contains 2,549 amino acids with the predicted molecular weight of 290 kDa. Structurally, mTOR possesses N-terminal tandem HEAT repeats, followed by a FRB domain and a C-terminal kinase domain (Figure 1-2). HEAT repeat is derived from four proteins **H**untington, **E**longation factor 3, PR65/**A**, TO**R**, and is composed of repeated protein motifs (about 47 amino acid residues that form two anti-parallel α-helices and two turns). HEAT repeats compose a rod-like helical structure for regulation of the protein-protein interaction. The FRB domain is the C-terminal stretch of 100 amino acids, and is the docking site for FKBP12/rapamycin complex. Recent reports indicated that the lipid second messenger phosphatidic acid (PA) binds FRB domain and enhances mTOR activity. However, the molecular mechanism of mTOR activation by PA remains unclear. The mTOR kinase domain structurally resembles the kinase domain of PI3K (a lipid kinase), but functions as a serine/threonine protein kinase.

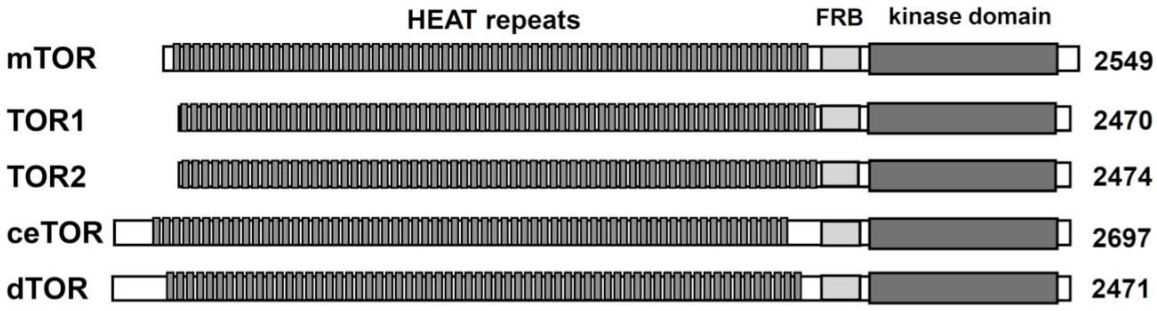

Figure 1-2. The domain structures of the TOR protein

In 1990s, yeast genetic screens first identified two rapamycin target genes, called *TOR 1* and *2*. The high degree of conservation among species, from mammals to yeast, strongly suggests that TOR is an essential cell growth regulator. mTOR is a Serine/Threonine protein kinase with a predicted molecular weight of 290 kDa. Structurally, mTOR contains multiple tandem HEAT repeats in the N-terminal region followed by a FRB domain and a C-terminal kinase domain.

1.5 mTOR complexes

mTOR and its binding partner, mammalian lethal with SEC13 protein 8 (mLST8), exist at least in two distinct complexes (Figure 1-3). mLST8 is a small subunit that contains seven WD40 repeats and physically associates with the mTOR kinase domain (Jacinto et al., 2004; Sarbassov et al., 2004). Interaction between mTOR and mLST8 is required for the kinase activity of mTOR (Kim et al., 2003). The interaction between raptor and mTOR/mLST8 defines the mTOR Complex 1 that regulates protein translation by phosphorylation of its substrates 4EBP1 (eIF4E binding protein 1) and S6K1 (p70 ribosomal protein S6 kinase 1) (Fingar and Blenis, 2004). 4EBP1 binds translational initiator eIF4E and inhibits cap-dependent mRNA translation under basal condition (Gingras et al., 2001). Upon growth factor stimulation, mTORC1 phosphorylates 4EBP1 and relieves its suppressive impact on eIF4E to initiate protein translation. S6K1 binds to eIF3 (eukaryotic initiation factor 3) and remains inactive (Holz et al., 2005). The activated mTORC1 upon growth factor induction results in S6K1 phosphorylation and activation. The active S6K1 phosphorylates ribosomal protein S6 to stimulate protein translation and cell growth (Jeno et al., 1988; Thomas, 2002).

The second mTOR complex (mTORC2) is assembled by binding of mTOR/mLST8 to rapamycin-insensitive companion of mTOR (rictor) and mammalian SAPK interacting protein 1 (SIN1) (Jacinto et al., 2006; Jacinto et al., 2004; Sarbassov et al., 2004). These two unique components SIN1 and rictor most likely carry the regulatory functions of this kinase complex. Sin1 contains a

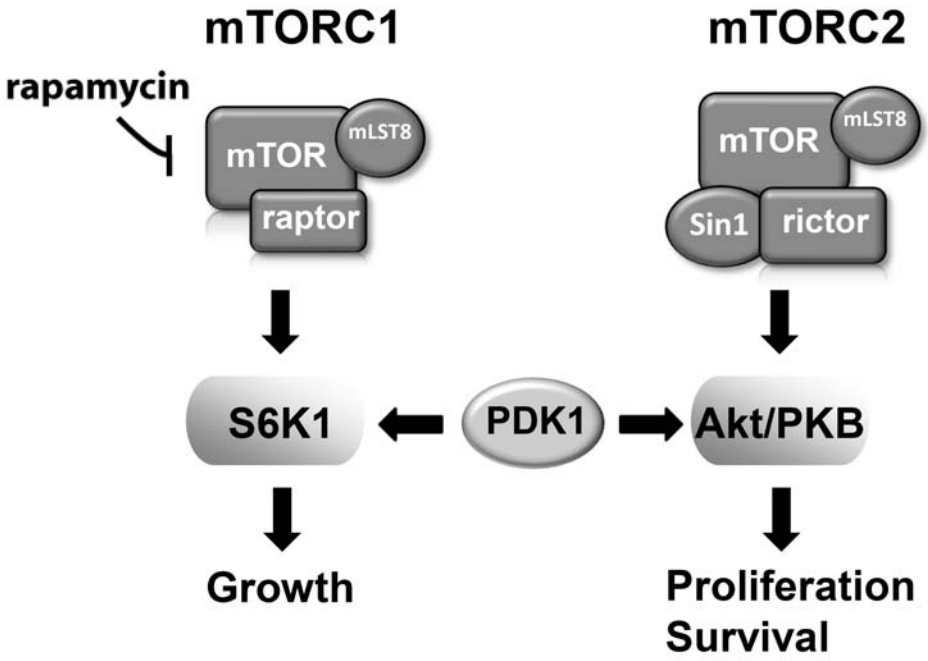

Figure 1-3. Current models of the mTOR complexes

mTOR forms two structurally and functionally distinct molecular complexes: mTORC1 and mTORC2. mTORC1 is composed of mTOR, raptor and mLST8 (also called GβL), which is highly sensitive to rapamycin inhibition. The rapamycin insensitive complex mTORC2 is composed of mTOR, mLST8, rictor and SIN1. Both complexes were shown to regulate downstream targets through phosphorylation on the hydrophobic motif.

N-terminal **C**onserved **R**egion **I**n the **M**iddle (CRIM) domain, the Raf-like Ras binding domain (RBD) and a C-terminal PH domain (Schroder et al., 2007). CRIM domain is a highly conserved region in the SIN1 family. SIN1 RBD domain suggests that Ras is a potential regulator of mTORC2. SIN1 PH domain implies the mTORC2 localization at the plasma membrane. However, the functional roles of these domains are yet to be characterized.

Rictor is a more conserved and larger protein than SIN1. The human rictor protein contains 1,708 amino acids and its sequence analysis did not reveal any homology to known functional domains or proteins (Sarbassov et al., 2004). In spite of this, rictor is a phospho-protein and the functional characterization of this post-translational modification might provide insights in regulation of mTORC2. In recent reports, some phosphorylation sites of rictor were identified, and the Thr-1135 phosphorylation is the growth factor dependent. The functional characterization determined that S6K1 is the kinase of rictor Thr-1135, and this site has been shown to carry the negative impact on mTORC2 by providing the binding site for the 14-3-3 adaptor proteins (Dibble et al., 2009; Julien et al., 2010; Treins et al., 2010). However, our functional studies indicated that this phosphorylation occur independent of mTORC2. Importantly, rictor Thr-1135 site was not essential for regulating mTORC2 kinase activity (Boulbes et al., 2010).

1.6 The mechanism of mTOR inhibition by rapamycin

Rapamycin binds proline isomerase FKBP12 to form a complex, which specifically interacts with the FRB domain of mTOR and inhibits its function

(Figure 1-4) (Kim et al., 2002). In contrast, the FRB domain of mTOR within mTORC2 is not accessible so that mTORC2 cannot be inhibited by rapamycin binding. However, prolonged rapamycin treatment interferes with the assembly of mTORC2. For examples, the prolonged rapamycin treatment results in less abundance of mTORC2 and inhibition of Akt in several cell types, mostly lymphoma cells (Sarbassov et al., 2006).

1.7 Downstream effectors of mTORC2

mTORC2 has been proposed to control phosphorylation of the AGC kinases members including SGK1 (serum and glucocorticoid induced protein kinase 1) (Garcia-Martinez and Alessi, 2008) and PKCα (protein kinase Cα) (Facchinetti et al., 2008; Ikenoue et al., 2008). mTORC2 has been identified as the Ser-422 kinase of SGK1, which functions to stabilize the expression of the ENaC (epithelial sodium channel) to induce sodium transport into epithelial cells and maintain the sodium homeostasis (Loffing et al., 2006). Besides, mTORC2 controls the actin-cytoskeleton probably through phosphorylation of PKCα on Ser-657, but the detailed mechanism remains to be addressed.

The most critical finding about mTORC2 function is the confirmation of this kinase complex as the major Akt kinase at the hydrophobic motif (HM) site Ser-473 (Sarbassov et al., 2005). The functional role of mTORC2

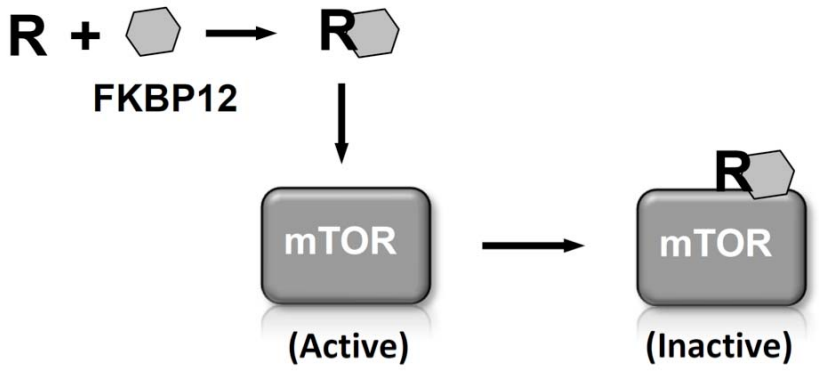

Figure 1-4. Rapamycin/FKBP12 binds and inhibits mTOR

Upon entering the cells, rapamycin binds a small protein receptor called FKBP12 (FK506-binding protein 12 kDa) to form a complex. This complex specifically binds mTOR and inhibits its function.

as the regulatory kinase of Akt has been supported by the mouse genetic studies (Guertin et al., 2006; Jacinto et al., 2006; Shiota et al., 2006; Yang et al., 2006). In addition to Ser-473, mTORC2 has been shown to phosphorylate Akt on Thr-450 turn motif (TM) site (Facchinetti et al., 2008; Ikenoue et al., 2008). Although both sites are regulated by mTORC2, but their roles are different. Regulation of the Akt HM site Ser-473 site is linked to the growth factor-mediated mTORC2 activation. The neighboring TM site Thr-450 of Akt is constitutively phosphorylated by mTORC2 and is required to control Akt protein stability (Oh et al., 2010). During Akt protein translation, mTORC2 can associate with the actively translating ribosome and mediate the Thr-450 phosphorylation. This phosphorylation event facilitates the proper folding of the Akt nascent polypeptide, which prevents misfolded Akt from targeting for K48-linked ubiquitination and proteasomal degradation.

1.8 Glycogen synthase kinase-3β (GSK-3β)

Glycogen Synthase Kinase-3 (GSK-3) is a multifunctional and evolutionally conserved serine/threonine protein kinase (Cohen and Frame, 2001). GSK-3 was discovered as the kinase of glycogen synthase that controls glycogen metabolism (Cross et al., 1995). Two human genes encode GSK-3 isoforms, GSK-3α (51 kDa) and GSK-3β (46 kDa), which exhibit 84 % overall identity and 98 % identity of their kinase domains. The major dissimilarity between two isoforms is that GSK-3α contains an extra N terminal glycine-rich stretch. Besides, these GSK-3 isoforms are functionally distinct because GSK-3α

cannot rescue the embryonically lethal phenotype of GSK-3β deficient mice (Hoeflich et al., 2000). Several recent reports suggest the potential differences between these two isoforms (Kerkela et al., 2008; MacAulay et al., 2007). For examples, GSK-3α inhibition is more effective in causing NF-κB- and cAMP-responsive element-dependent transactivation (Liang and Chuang, 2006). By contrast, GSK-3β depletion has more robust effects in suppressing neuronal death and improving glucose tolerance in skeletal muscle (Liang and Chuang, 2007; Patel et al., 2008).

GSK-3 function is regulated by its phosphorylation. The phosphorylation of the N-terminal serine residue (GSK-3β on Ser-9; GSK-3α on Ser-21) decreased their kinase activity. Certain kinases such as Akt, p90RSK, protein kinase A, and protein kinase C could phosphorylate these serine residues. The phosphorylated serine residue serves as a pseudo substrate that hinders the access of substrates to the active site of GSK-3. By contrast, GSK-3 activity is enhanced by phosphorylation of the tyrosine residue (GSK-3β on Tyr-216 and GSK-3α on Tyr-279), which might take place by autophosphorylation or by additional tyrosine kinases. The regulatory mechanism of the GSK-3 tyrosine phosphorylation remains ill-defined (Frame and Cohen, 2001; Grimes and Jope, 2001).

Moreover, the priming phosphorylation of the substrate by another kinase is required for GSK-3 function. The priming phosphorylation takes place on a serine residue resided four amino acids C-terminal to the GSK-3 phosphorylation site. For instance, casein kinase II performs the priming phosphorylation of

glycogen synthase, and the priming phosphorylation of β-catenin by casein kinase Iα are required for GSK-3 action (Fiol et al., 1987; Liu et al., 2002).

1.9 Biological functions of GSK-3β

GSK-3β regulates various biological pathways such as Wnt/β-catenin (Seidensticker and Behrens, 2000) and Hedgehog pathways, apoptotic process, and cell cycle. GSK-3β dysfunction results in multiple human diseases including bipolar disorder, Alzheimer's disease, cancer and diabetes. In the development of type II diabetes, GSK-3β activation associates with insulin resistance. The impaired response of peripheral tissues (muscle, fat and liver) to insulin signals causes deficient glucose uptake (Tanabe et al., 2008). GSK-3β has been reported to be activated under ER stress (Huang et al., 2009), and ER stress is the potential cause of insulin resistance and type II diabetes (Ozcan et al., 2004). Specific GSK-3β inhibitors stimulate insulin-dependent glucose uptake (Ring et al., 2003). However, the mechanisms of the GSK-3β-dependent insulin resistance remain to be determined.

Chapter 2

Materials and Methods

2.1 Cell lines and culture

MEFs, HeLa, MDA-MB-435, HEK-293T and COS-7 cells were obtained from the American Type Culture Collection and cultured in DMEM/F12 with 10% FBS and penicillin/streptomycin in 5% CO_2 at 37°C. Cell lines were cultured at a density that allows cell division throughout the course of the experiment. MEFs were transfected by Dreamfect reagent following the manufacturer's protocol.

2.2 Antibodies and reagents

Antibodies to Xpress, myc and V5 tag from Invitrogen; antibodies to phospho-Ser2481 mTOR, phospho-Thr1135 and phospho-Ser1235 rictor, mTOR, rictor, raptor, phospho-Ser260 SIN1, phospho-Ser473, phospho-Thr450 and phospho-Thr308 Akt, Akt, phospho-Ser641 GS, GS, phospho-Ser9 and phospho-Tyr216 GSK-3β, GSK-3β, GSK-3α, phospho-Thr980 PERK, PERK, p27, S6K1, phospho-Thr389 S6K1 from Cell Signaling Technologies; antibodies to rictor and tubulin and horseradish peroxidase (HRP)-labeled anti-rabbit, anti-mouse, and anti-goat secondary antibodies from Santa Cruz Biotechnology.

Reagents were obtained from the following sources: Dulbecco's modified Eagle's medium (DMEM)/F12 from Life Technologies; fetal bovine serum (FBS) from HyClone. FuGENE 6 transfection reagent and Complete protease inhibitor cocktail from Roche Applied Science; protein G-Sepharose from Pierce; insulin-like growth factor I (IGF-1) from Peprotech; thapsigargin, tunicamycin, BIO and SB-216763 from Sigma; Dreamfect transfection reagent from OZ Biosciences.

2.3 Letiviral shRNAs and plasmid DNAs

Lentiviral shRNAs targeting humanGSK-3α and GSK-3β were obtained from Sigma (MISSION shRNA). The targeting sequences of the oligo-nucleotides are as follows:

GSK-3β sense strand, GCTGAGCTGTTACTAGGACAA (NM_002093.2-974s1c1);

GSK-3β sense strand, CCCAAACTACACAGAATTTAA (NM_002093.2-1087s1c1);

GSK-3α sense strand, CGGACATCAAAGTGATTGGCA (NM_019884.1-473s1c1);

GSK-3α sense strand, CCTCTCTTCAACTTCAGTGCT (NM_019884.1-1375s1c1).

Lentiviral shRNAs targeting human mTOR were generated and applied as described previously (Sarbassov et al., 2005).

The pcDNA3-hemagglutinin (HA)-GSK-3β-wild type and pBabe-puro-GSK-3β (S9A) were obtained from Addgene. Plasmids pRK5-myc-GCP2, -myc-rictor, myc-raptor, myc-mTOR-WT, myc-mTOR-KD, HA-mLST8, and HA-tubulin were kindly provided by David M. Sabatini (Whitehead Institute for Biomedical Research, Cambridge, MA). The pcDNA3.1-Sin1.1-V5 and pcDNA4-Xp-rictor were generated by the TOPO cloning system (Invitrogen).

2.4 Mutagenesis of rictor

The rictor complementary DNA (cDNA) from the pRK5 plasmid was re-sub-cloned to pcDNA4 by means of the Xpress tag with the pcDNA4/HisMax TOPO TA Expression Kit from Invitrogen. The large size of the plasmid (11 kb) is incompatible with the polymerase chain reaction (PCR)-based mutagenesis. To decrease the size of the plasmid to 6 kb, we sub-cloned the fragment of rictor containing the sequences of the phospho-mutants into pBluescript by Bgl II and

Not I digestion. The primers for mutagenesis were designed based on the QuikChange Primer Design Program (http://www.stratagene.com). The pBluescript plasmid containing the rictor fragment was mutagenized with the QuikChange II XL Mutagenesis Kit (Stratagene). After validation of mutations by sequencing, the mutated rictor fragments were reinserted into the Xp-rictor pcDNA4 plasmid by Bgl II and Not I digestion.

2.5 Retroviral production and infection

Retroviral vectors were propagated in and purified from XL-10 Gold bacterial cells and co-transfected together with the ΔVPR and VSVG plasmids into actively growing cells. One day before transfection, HEK 293T cells (1.2×10^6) were placed on 6-cm dishes in 3 ml of DMEM supplemented with 10% FBS. For production of retroviruses, HEK 293T cells were transfected by the calcium phosphate method with 3 mg of transfer vector pMSCV, 0.6 mg of envelope coding plasmid VSVG, and 2.4 mg of Gag-pol-expressing plasmid.

Retroviruses were harvested 48 hours after transfection and centrifuged at 3,000g at 4°C for 15 min to eliminate any remaining HEK 293T cells. One day before infection, cells to be infected were seeded in six-well plates. Viral supernatant was added at a ratio of 1:1 to the culture medium in the presence of polybrene (8 mg/ml), and the cells were centrifuged at 1,800 rpm for 45 min to increase the infection rate. Cells were incubated with retroviruses for 24 hours. A second infection was performed the next day following the same protocol. After

an additional 24 hours of recovery in regular medium, infected cells were passaged and selected with puromycin (2 mg/ml for 2 days).

2.6 Immunoprecipitations and kinase assays

For immunoprecipitation experiments, the lysis buffer contained 0.3% CHAPS instead of 1% Triton to preserve the integrity of the mTOR complexes. Two micrograms of rictor antibody was added to the cleared cellular lysates (1 mg of protein content in 700 ml) and incubated with rotation at 4°C for 90 min. After 1 hour of incubation with 40 ml of 50% slurry of protein G-agarose, immunoprecipitates captured by protein G-agarose were washed four times with CHAPS-containing lysis buffer and once with rictor-mTOR kinase buffer [25 mM Hepes (pH 7.5), 100 mM potassium acetate, and 2 mM $MgCl_2$].

For *in vitro* mTORC2 kinase reactions, immunoprecipitates were incubated in a final volume of 15 ml at 37°C for 20 min in rictor-mTOR kinase buffer containing 500 ng of inactive Akt1-GST and 1 mM ATP. The reaction was stopped by the addition of 235 µl of ice-cold enzyme dilution buffer [20 mM Mops (pH 7.0), 1 mM EDTA, 0.3% CHAPS, 5% glycerol, 0.1% 2-mercaptoethanol, and bovine serum albumin (BSA; 1 mg/ml)]. After a quick spin, the supernatant was removed from the protein G-agarose, and the 15-µl portion was analyzed by immunoblotting for phospho-Ser473 Akt and total Akt. The pelleted protein G-agarose beads were also analyzed by immunoblotting to determine the abundance of rictor, Sin1, and mTOR in the immunoprecipitates.

For *in vitro* GSK-3β kinase reaction, immunoprecipitates were prepared from MDA-MB-435 cell lysates with rictor antibody. GSK-3β was immunoprecipitated from lysates of human embryonic kidney (HEK) 293T cells expressing GSK-3β that were treated with tunicamycin (2 μg/ml) for 3 hours before cell harvest. *In vitro* GSK-3β kinase assays were performed at 37°C for 30 min in the final volume of 25 ml of GSK-3β kinase buffer containing 4 mM Mops (pH 7.2), 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 4 mM $MgCl_2$, 0.05 mM dithiothreitol (DTT), 40 μM BSA, and 1 mM ATP. Immunoprecipitates captured by protein G-agarose were washed with rictor-mTOR kinase buffer for subsequent *in vitro* mTORC2 kinase reactions as indicated above.

2.7 Cell lysis and immunoblotting

Cells were rinsed with ice-cold phosphate-buffered saline (PBS) before lysis in buffer containing 40 mM Hepes (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM sodium glycerophosphate, 50 mM NaF, 1% Triton X-100, and protease inhibitor cocktail (Roche Applied Science). The scraped lysates were incubated for 20 min at 4°C to complete lysis. The soluble fractions of cell lysates were isolated by centrifugation at 13,000 rpm at 4°C for 12 min. Samples of the cellular lysates containing an equal amount of proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. Proteins were then visualized by immunoblotting and detected with enhanced chemiluminescence (ECL) with the Immobilon Western kit (Millipore).

2.8 Preparation of liver tissue extracts for immunoblotting

Powdered livers were homogenized on ice with a Teflon glass homogenizer in 1 ml of ice-cold buffer [20 mM tris-HCl (pH 7.4), 20 mM NaCl, 1 mM EDTA, 20 mM β-glycerophosphate, 5 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] containing 0.1% Tween 20 and were centrifuged at 1,000g for 20 min at 4°C to pellet insoluble material. The protein concentration of the supernatant was determined. Liver extract samples (containing 50 to 100 mg of total protein) were subjected to SDS-PAGE in 7.5% polyacrylamide gels followed by Western blot analysis with indicated antibodies.

2.9 Tumor xenografts

MEFs constitutively expressing wild-type, S1235A, or S1235D-rictor were transformed by H-Ras overexpression. Protein extracts from wild-type-, S1235A-, or S1235D-rictor-expressing MEFs indicated equal protein amounts of rictor and H-Ras by Western blotting analysis. The MEFs (5×10^6 cells per mouse) were injected subcutaneously into the upper flank region of 6-week-old immunodeficient nude mice (n = 5 for each group).

Tumor size was measured after 15 days and the tumor volume was determined with the standard formula L × W2 × 0.5, where L is the longest length and W is the shortest length in millimeters. The differences in the tumor volume from mice injected by wild-type-, S1235A-, or S1235Drictor-expressing MEFs

were compared by one-way analysis of variance (ANOVA). Nude mice were killed and the tumors were excised.

2.10 In vitro substrate binding assay

For the in vitro substrate binding assay, rictor immunoprecipitates were incubated in a final volume of 15 ml at 37°C for 20 min in rictor-mTOR kinase buffer containing 100 ng of Akt1-GST with or without ATP. The immunoprecipitates were then washed four times with CHAPS-containing lysis buffer. The pelleted protein G-agarose beads were analyzed by immunoblotting to determine the protein abundance of Akt1-GST, rictor, Sin1, and mTOR in the immunoprecipitates.

2.11 Drosophila RNAi and analysis

RNAi against *Drosophila* GSK-3 was performed as previously described (Sarbassov et al., 2005). dsRNA targeting Drosophila GSK-3 was synthesized by *in vitro* transcription (IVT) in 20-ml reactions with a MEGAscript T7 kit (Ambion). DNA templates for IVT were generated by PCR from total Drosophila S2 cellular genomic DNA. The primers (which incorporated a 5' and 3' T7 promoter) for dGSK-3 dsRNA synthesis were as follows:

The dGSK-3 forward primer:

5'-TAATACGACTCACTATAGGCCGTTGACGAGTTTGTGTGT

The dGSK-3 reverse primer:

5'-TAATACGACTCACTATAGGAAACTCGGCGACTGTTTGTT

The underlined region indicates the T7 promoter sequence. The dsRNA products were purified with an Invitrogen PureLink PCR Purification Kit. Final dsRNA concentrations were measured on a Nano-Drop spectrophotometer.

Drosophila S2 cells were prepared for dsRNA addition by seeding 1×10^6 cells in 2 ml of *Drosophila* Schneider's medium to each well in six-well culture plates. dsRNAs were transfected with FuGENE 6 (Roche). Briefly, 6 µl of FuGENE was added to 94 µl of Drosophila SFM (Invitrogen) followed by the addition of 4 mg of dGSK-3 dsRNA. Four micrograms of green fluorescent protein (GFP) dsRNA was used as control. Tubes were gently mixed and incubated for 20 min at room temperature. FuGENE-dsRNA complexes were then administered to cells by adding the entire mixture drop-wise around wells and then swirling to ensure even dispersal. After 6 hours of dsRNA addition, the medium was changed to avoid potential negative effects of FuGENE on cell viability. Additional FuGENE-dsRNA complexes were added to wells on each of the following 3 days. After 4 days of incubation to allow turnover of the target mRNAs, cell lysates were subjected to immunoblotting for the amounts of phospho- and total dAkt, GSK-3, and tubulin.

2.12 Purification of the soluble FLAG-mLST8/myc-mTOR heterodimer

FLAG-mLST8 was co-transfected with myc-mTOR cDNA in HEK-293T cells. After a 48 h transfection, cells were washed with cold PBS, lysed with 0.3% CHAPS buffer, and incubated with mild agitation for 20 min at 4 °C. The lysate was transferred to the spinning columns for centrifugation for 15 min at 10,000

rpm. After centrifugation, the supernatant was used for the FLAG affinity purification as described previously (Yip et al., 2010). FLAG M2 affinity resins were washed three times in 0.3% CHAPS lysis buffer and packed into a 10 ml Bio-Rad column. The cellular lysates were applied through the column seven times. After running the lysates, the column was washed with 20 ml of lysis buffer and 10 ml of elution buffer (40 mM HEPES, pH 7.4, 500 mM NaCl, 0.1% CHAPS). The FLAG-mLST8/myc-mTOR heterodimer was eluted by the elution buffer with 0.5 mg/ml of the FLAG peptide. The eluted fractions from number 2 to 6 were combined, dialyzed with mTORC2 kinase buffer, and concentrated with Millipore Amicon Ultra-15 centrifugal filter units. The purified FLAG-mLST8/myc-mTOR heterodimer was analyzed by immunoblotting and applied for the *in vitro* mTORC2 assembly.

2.13 The *in vitro* mTORC2 assembly and kinase reaction

Myc-rictor and SIN1-V5 cDNAs were co-transfected in HEK-293T cells. After 48-h transfection, cells were washed with cold PBS, lysed with 0.3% CHAPS buffer, and subjected for centrifugation for 15 min at 10,000 rpm. The supernatant was applied for immunoprecipitation with anti-V5 antibody. The myc-rictor/SIN1-V5 immunoprecipitates were incubated with or without the purified soluble FLAG-mLST/myc-mTOR heterodimer at room temperature for 2 h in mTORC2 kinase buffer with 1 mM ATP. After washing three times with 0.3% CHAPS lysis buffer, the immunoprecipitates were used for the *in vitro* mTORC2 kinase reaction with wild-type Akt as the substrate.

Chapter 3

ER stress inhibits mTORC2-Akt signaling through GSK-3β-mediated phosphorylation of rictor

Most of this work has been published in:

C.-H. Chen, T. Shaiken, T. R. Peterson, R. Aimbetov, A. K. Bissenbaev, S.-W. Lee, J. Wu, H.-K. Lin, D. D. Sarbassov, ER stress inhibits mTORC2 and Akt signaling through GSK-3β–mediated phosphorylation of rictor. *Sci. Signal*. 4, ra10 (2011).

Rationale

Although the functional role of mTORC2 as the major regulatory Ser-473 kinase of Akt is well established, the regulatory mechanisms of mTORC2 remains poorly characterized. Within mTORC2, the essential components rictor and SIN1 most likely carries the regulatory functions of this kinase complex. Particularly, rictor is a phospho-protein and the functional characterization of this post-translational modification might provide insights in regulation of mTORC2. In this study, I aimed to identify rictor phosphorylation site(s) that is essential for mTORC2 regulation.

Growth factor signaling is sensitive to certain stress conditions, such as endoplasmic reticulum (ER) stress. ER stress is a common pathophysiological state associated with metabolic and neuronal disorders as well as human cancers. Here, we found that inhibition of the mTORC2 and Akt signaling by ER stress is associated with the increased rictor phosphorylation on Ser-1235. This phosphorylation event occurs in mouse tissues under ER stress condition, suggesting that rictor Ser-1235 has a biological function and could potentially affect the mTORC2 kinase activity. Therefore, I initiated this research project to further investigate whether mTORC2 kinase activity is suppressed under ER stress. I also aimed to identify the kinase for rictor Ser-1235 phosphorylation, and to determine the biological functions of rictor Ser-1235 phosphorylation.

Results

3.1. ER stress-mediated inhibition of Akt signaling associates with rictor phosphorylation at Ser-1235

To address if the mTORC2 activity is sensitive to stress condition, we analyzed its activity and rictor phosphorylation in response to osmotic stress. Acute osmotic stress induced by treatment of cells with 0.5 M sorbitol caused inhibition of the mTORC2-Akt signaling, which was shown by the substantial decrease of the mTORC2 kinase activity and Akt Ser-473 phosphorylation (Figure 3-1A). Notably, the osmotic stress induced by sorbitol led to increased rictor phosphorylation at Ser-1235 as shown in cell lysate and rictor immunoprecipitate (Figure 3-1A). Osmosis of water from cells is linked to ER stress induction (Lee and Linstedt, 1999). Our initial experiment in tissue culture of cancer cells indicates a stress-inducible phosphorylation of rictor on Ser-1235. We hypothesized that if this site has a biological function, this phosphorylation event would also occur in mouse tissues under ER stress.

Thapsigargin and tunicamycin are two well-characterized compounds known to induce ER stress. Thapsigargin is an inhibitor of SERCA (sarco/endoplasmic reticulum calcium ATPase). Thapsigargin inhibits the cell to transport calcium to the endoplasmic and sarcoplasmic reticula and results in the elevated cytosolic calcium concentration. The disruption of homeostatic balance of the Ca^{2+} concentration in the ER leads to ER stress induction.

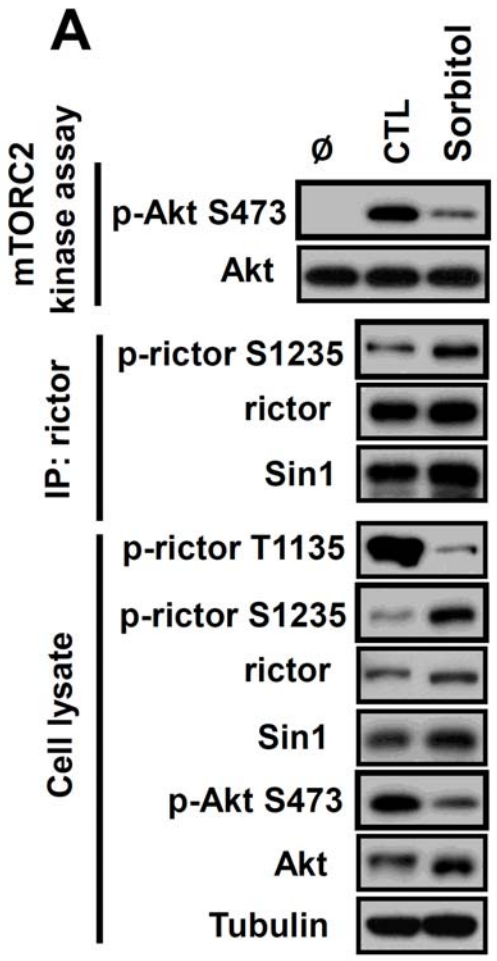

Figure 3-1. Inhibition of mTORC2 signaling by ER stress is linked to rictor phosphorylation.

(A) Inhibition of mTORC2 activity by osmotic stress correlates with rictor Ser-1235 phosphorylation. HEK 293T cells were incubated with 0.5 M sorbitol for 30 min to induce osmotic stress. Rictor immunoprecipitates (IP) were used for *in vitro* kinase assays with full-length wild-type Akt1 as the substrate. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

Tunicamycin blocks the N-linked protein glycosylation and causes incorrect protein folding and membrane targeting. The accumulation of the misfolded proteins triggers ER stress. To extend our cellular data to the *in vivo* study, we injected mice with both compounds to induce ER stress and analyzed the mouse liver extracts. The induction of ER stress was indicated by phosphorylation of the ER stress marker PERK (protein kinase RNA-like endoplasmic reticulum kinase) at Thr-980 (Figure 3-1B). Similarly, ER stress resulted in decreased Akt Ser-473 phosphorylation and augmented rictor Ser-1235 phosphorylation. The data implied that Ser-1235 is a novel ER-stress-inducible rictor phosphorylation site.

3.2. Inhibition of GSK-3 prohibits ER stress-mediated Ser-1235 phosphorylation of rictor and up-regulates mTORC2 kinase activity

Based on the sequence alignment result, the surrounding sequence of rictor Ser-1235 (SS-S^{1235}-PS) resembles the BCL-3 (B cell lymphoma 3-encoded protein) Ser-394 (SS-S^{394}-PS) site known to be phosphorylated by GSK-3. Importantly, the GSK-3 kinase activity is inducible by ER stress (Song et al., 2002). Thus, it is possible that stress-induced rictor phosphorylation at Ser-1235 is regulated by GSK-3. Indeed, treatment of cells with tunicamycin and thapsigargin led to GSK-3 activation, as shown by the increased tyrosine phosphorylation of GSK-3 at Tyr-216 (Hughes et al., 1993; Wang et al., 1994) and the elevated phosphorylation of the GSK-3 substrate, glycogen synthase (GS) at Ser-641 (Figure 3-2A). Moreover, inhibition of GSK-3 in stressed cells by

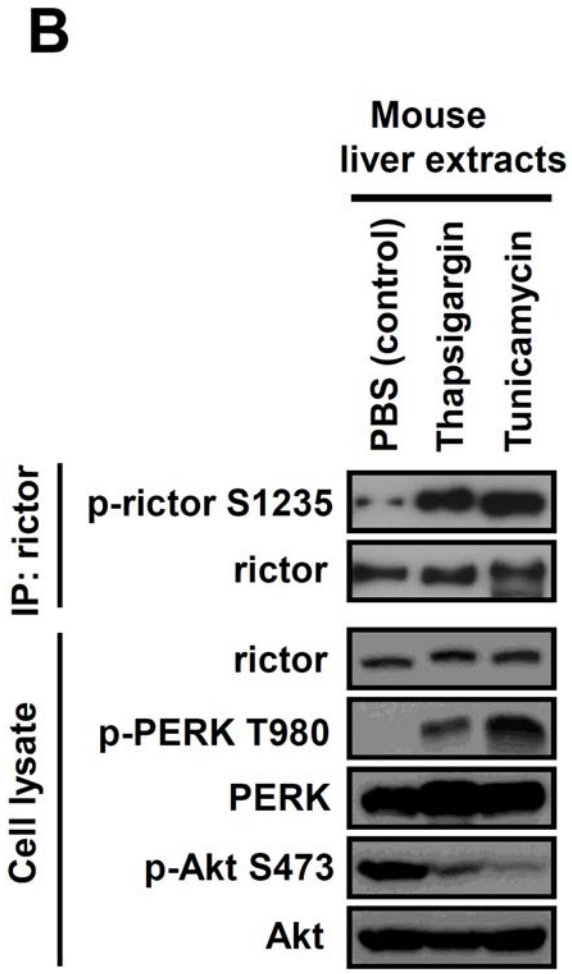

Figure 3-1. Inhibition of mTORC2 signaling by ER stress is linked to rictor phosphorylation.

(B) Regulation of rictor Ser-1235 phosphorylation by ER stress in mice. Thapsigargin (1 μg/g in PBS) or tunicamycin (2 μg/g in 150 mM glucose) was injected intraperitoneally into 4-month-old mice for 24 hours to induce ER stress. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

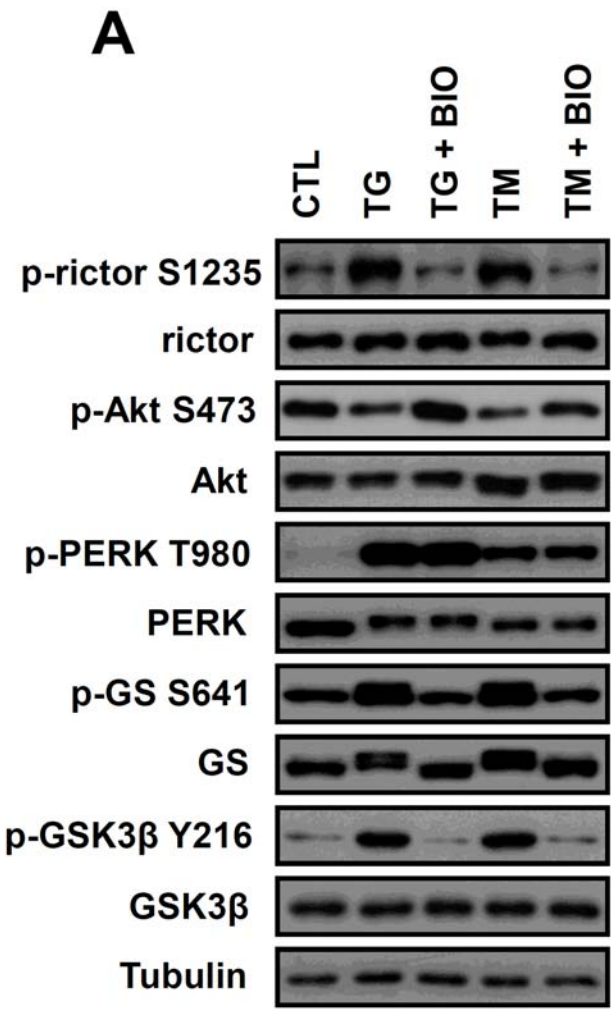

Figure 3-2. ER stress–dependent phosphorylation of rictor at Ser-1235 and mTORC2 signaling requires GSK-3 activity.

(A) GSK-3 mediates ER stress-induced phosphorylation of rictor at Ser-1235. MDA-MB-435 cells were treated with 2 μM thapsigargin (TG) or tunicamycin (TM) (2 μg/ml) for 6 hours, and then with 5 mM of the GSK-3 kinase inhibitor BIO for 3 hours before cell harvest. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

6-bromoindirubin-3′-oxime (BIO) prevented the induction of rictor phosphorylation at Ser-1235, implying that GSK-3 regulates rictor phosphorylation. The ER stress-mediated dephosphorylation of Akt was also dependent on GSK-3 function, demonstrating that Akt Ser-473 phosphorylation is inversely correlated with rictor Ser-1235 phosphorylation.

We further studied if GSK-3 regulates Akt signaling in cell culture mediums containing high (10 %) or low (1 %) serum. Inhibition of GSK-3 by two distinct inhibitors, BIO and SB-216763, promoted Akt Ser-473 and Thr-308 phosphorylation in both high- and low-serum conditions (Figure 3-2B). So, inhibition of GSK-3 suffices to activate Akt signaling in different conditions, which is coherent to recent reports that GSK-3 inhibitors cause Akt-dependent phenotypes including glucose uptake and cell proliferation (Mussmann et al., 2007; Ring et al., 2003).

Because inhibition of GSK-3 also reduced rictor phosphorylation at Ser-1235, we reasoned that GSK-3 inhibition-dependent Akt activation may associate with the changes of mTORC2 activity. To examine this idea, we immunopurified mTORC2 from cells treated with or without GSK-3 inhibitors and conducted the *in vitro* mTORC2 kinase assay using full length GST-Akt as the substrate. We detected a higher kinase activity of mTORC2 with GSK-3 inhibition (Figure 3-2C). These results indicated that inhibition of GSK-3 augments mTORC2 kinase activity; moreover, this impact was coherently accompanied with the declined rictor phosphorylation at Ser-1235.

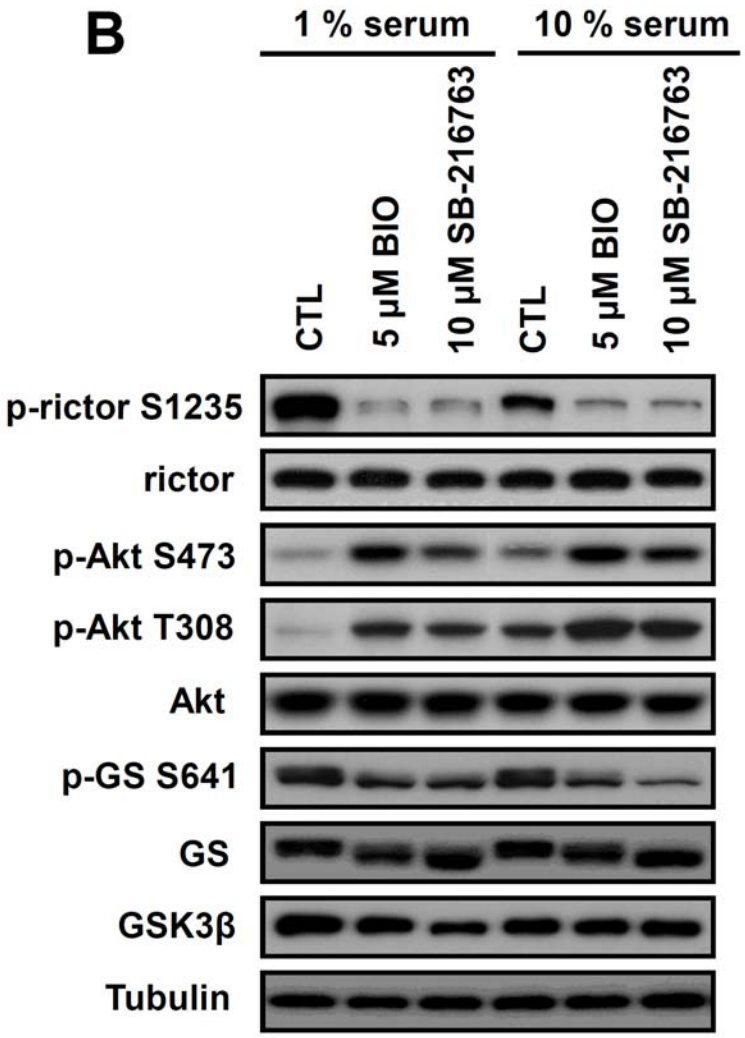

Figure 3-2. ER stress–dependent phosphorylation of rictor at Ser-1235 and mTORC2 signaling requires GSK-3 activity.

(B) GSK-3 inhibitors induce Akt activation. MDA-MB-435 cells were incubated with 5 μM BIO or 10 μM SB-216763 for 5 hours under low- or high-serum (1 or 10% FBS, respectively) conditions. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

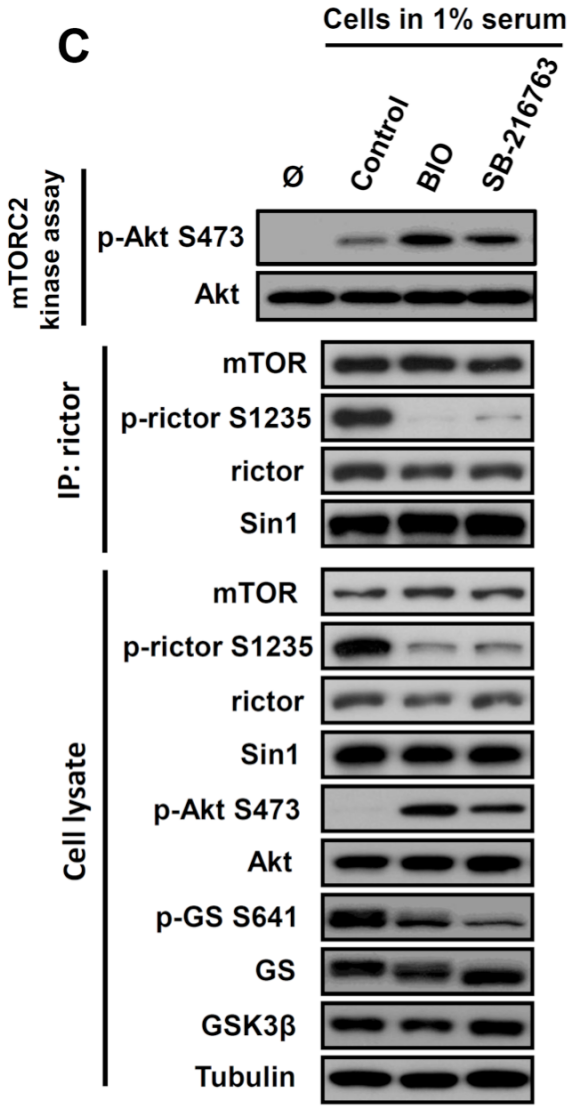

Figure 3-2. (C) Inhibition of GSK-3 induces mTORC2 kinase activity. MDA-MB-435 cells were incubated with BIO or SB-216763 as in Figure 3-2B under low-serum conditions. Rictor immunoprecipitates were used for *in vitro* kinase assays with full-length WT Akt1 as the substrate. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

3.3. The inhibitory regulation of mTORC2 and Akt by GSK-3 are conserved in *Drosophila*

Alignment of rictor orthologs indicates that the first 1,000 amino acids of human rictor protein (total 1,708 amino acids) are highly conserved among eukaryotes (Sarbassov et al., 2004), and Ser-1235 residue is conserved in vertebrates (Figure 3-3A). Although the first 1,000 amino acids conserved region of human rictor sequence poorly aligns with the invertebrate *Drosophila melanogaster* rictor sequence, a small region within the adjacent sequence of human rictor Ser-1235 (from amino acids 1209 to 1238) does align with its *Drosophila* ortholog (from amino acids 1209 to 1237) with a potential phosphorylation site at Ser-1233 (Figure 3-3B). Thus, we reasoned that TORC2 and Akt regulation by GSK-3 is possibly conserved in the *Drosophila*. To address this possibility, we performed GSK-3 inhibition in *Drosophila* S2 cells. Under low-serum (1% FBS) condition, *Drosophila* Akt (dAkt) phosphorylation at the TORC2-dependent Ser-505 residue (Sarbassov et al., 2005) increased after BIO treatment (Figure 3-3C). There is solely single gene in *Drosophila* encoding GSK-3. We knocked down this gene with double-stranded RNA (dsRNA)-mediated RNA interference (RNAi) (Sarbassov et al., 2005). Knockdown of GSK-3 promoted dAkt phosphorylation at Ser-505 (Figure 3-3D), implying that Akt activity was inhibited by GSK-3 in *Drosophila* cells. In addition, GSK-3 was required for declined dAkt Ser-505 phosphorylation under ER stress (Figure 3-3E).

Figure 3-3. The conservation of the rictor S-1235 site

(A) The Ser-1235 site in rictor is conserved in vertebrates. Rictor vertebrate ortholog protein sequences were aligned with HomoloGene website (http://www.ncbi.nlm.nih.gov/homologene).

(B) The alignment of human and *Drosophila* sequences within the area of the Ser-1235 site in human rictor.

(These figures were adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

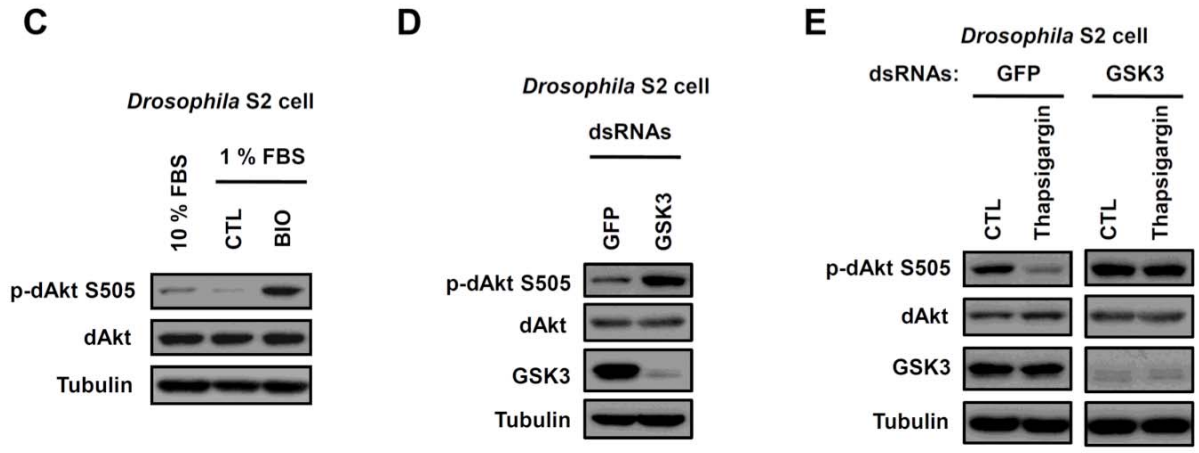

Figure 3-3. GSK-3-dependent regulation of Akt is conserved in the invertebrate fly *Drosophila melanogaster*

(C) Similar to mammalian cells, Akt phosphorylation is increased by inhibition of GSK3 in *Drosophila* S2 cells. *Drosophila* S2 cells were incubated in 1% FBS containing *Drosophila* Schneider's medium for 16 hours and then treated with 20 μM BIO for 3 hours.

(D) Knockdown of GSK3 in *Drosophila* S2 cells induces phosphorylation of Akt. GSK3 and GFP dsRNAs were transfected with Fugene 6 into S2 cells. After four days, S2 cells were collected and analyzed by Immunoblotting.

(E) Phosphorylation of Akt in *Drosophila* S2 cells is regulated by ER stress. *Drosophila* S2 cells with or without GSK-3 knockdown were treated with thapsigargin (10 μM) for 24 hours.

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3.4. GSK-3β is required for rictor phosphorylation at Ser-1235

Our data demonstrated that ER stress-dependent rictor phosphorylation and mTORC2 inactivation is dependent on GSK-3 activity. In human, there are two structurally similar but functionally distinct GSK-3 isoforms: GSK-3α and GSK-3β. The selective GSK-3 inhibitors SB-216763 and BIO recognize the ATP-binding pocket of GSK-3, which shares structural similarity in GSK-3β and GSK-3α. Therefore, it is unlikely that GSK-3 inhibitors could discriminate between the two isoforms. To examine if both isoforms are required for rictor Ser-1235 phosphorylation, we knocked down each isoform employing two different shRNAs specifically targeting GSK-3β or GSK-3α. Depletion of GSK-3β rather than GSK-3α prevented rictor Ser-1235 phosphorylation (Figure 3-4A).

We further determined the role of GSK-3β in regulation of rictor phosphorylation and Akt signaling by using GSK-3β-deficient MEFs. Switching GSK-3β wild-type MEFs from high- to low-serum conditions caused raised rictor Ser-1235 phosphorylation, and this elevated phosphorylation was linked to reduced Akt Ser-473 phosphorylation (Figure 3-4B). By contrast, we did not observe the same effects in GSK-3β-null MEFs that were switched from high- to low-serum conditions (Figure 3-4B). Moreover, we analyzed the impact of ER stress to wild-type and GSK-3β-deficient MEFs. Wild-type MEFs displayed similar ER stress response as that of human cancer cells (Figure 3-2A); however, ER stress induction did not promote rictor

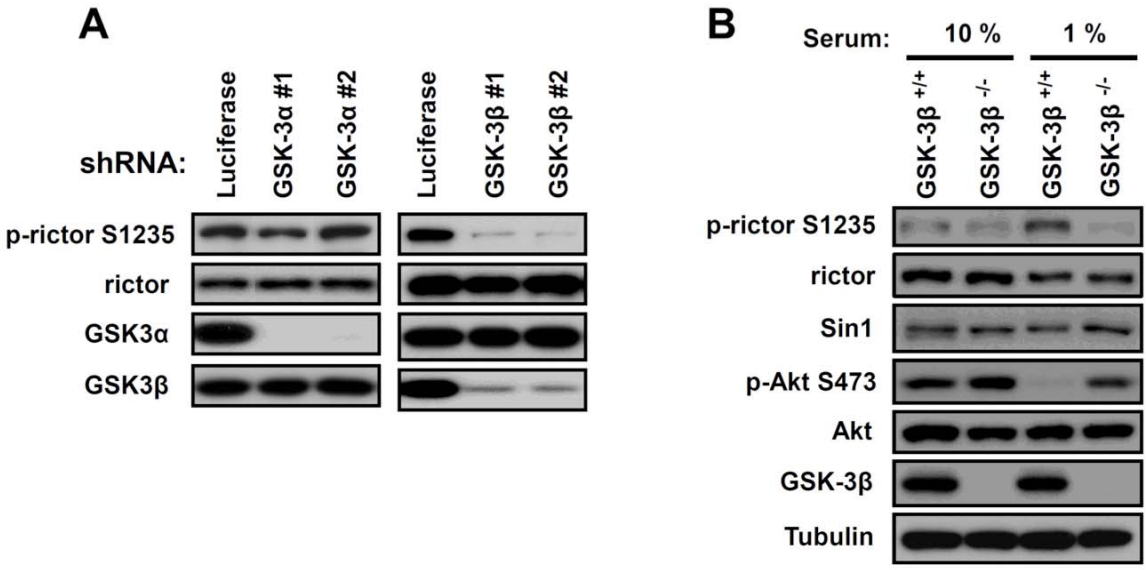

Figure 3-4. GSK-3β is required for ER stress-induced phosphorylation of rictor at Ser-1235 and inhibition of Akt signaling.

(A) Phosphorylation of rictor at Ser-1235 in cells requires GSK-3β. shRNAs targeting luciferase, GSK-3α, or GSK-3β were lentivirally transduced into MDA-MB-435 cells.

(B) Phosphorylation of rictor at Ser-1235 was not detected and phosphorylation of Akt at Ser-473 phosphorylation was increased in GSK-3β-null MEFs. GSK-$3\beta^{+/+}$ and GSK-$3\beta^{-/-}$ MEFs were incubated with 10 or 1% FBS-containing medium for 16 hours.

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phosphorylation at Ser-1235 or suppress Akt Ser-473 phosphorylation in GSK-3β-deficient MEFs (Figure 3-4C). Previous findings showed that these two GSK-3 isoforms have distinct functions (Hoeflich et al., 2000; Lee et al., 2007; Ruel et al., 1993). Our results expanded the notion that GSK-3β, instead of GSK-3α, controls rictor Ser-1235 phosphorylation and inhibits Akt signaling in response to ER stress.

3.5. GSK-3β phosphorylates Ser-1235 of rictor and inhibits mTORC2 kinase activity

Since GSK-3β-mediated rictor phosphorylation caused declining of the mTORC2 activity, we inferred that augment of GSK-3β protein level might also affect mTORC2 function. Certainly, overexpression of GSK-3β is effective to inhibit the kinase activity of mTORC2, which was linked to increased rictor phosphorylation at Ser-1235 (Figure 3-5A). Although rictor phosphorylation at Ser-1235 is dependent on the presence of GSK-3β and its activity, we could not make a conclusion that GSK-3β is the direct kinase of rictor. Thus, we further tested if GSK-3β could directly phosphorylate rictor by performing the *in vitro* GSK-3β kinase assay. Incubation of the immunopurified mTORC2 with or without active GSK-3β showed that GSK-3β directly phosphorylated Ser-1235 of rictor (Figure 3-5B). Following the *in vitro* GSK-3β kinase reaction, we applied the immunopurified mTORC2 for the kinase assay and found that its kinase activity decreased when rictor was phosphorylated by GSK-3β at Ser-1235 (Figure 3-5B). Furthermore,

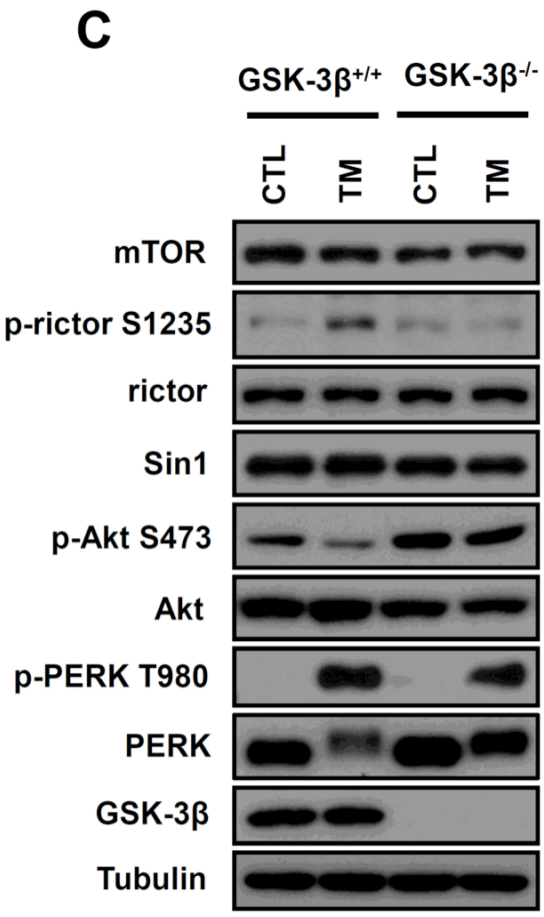

Figure 3-4. GSK-3β is required for ER stress-induced phosphorylation of rictor at Ser-1235 and inhibition of Akt signaling.

(C) GSK-3β-null MEFs show resistance to ER stress–dependent Akt inactivation. GSK-3β$^{+/+}$ and GSK-3β$^{-/-}$ MEFs were treated with tunicamycin (2 μg/ml) for 6 hours. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

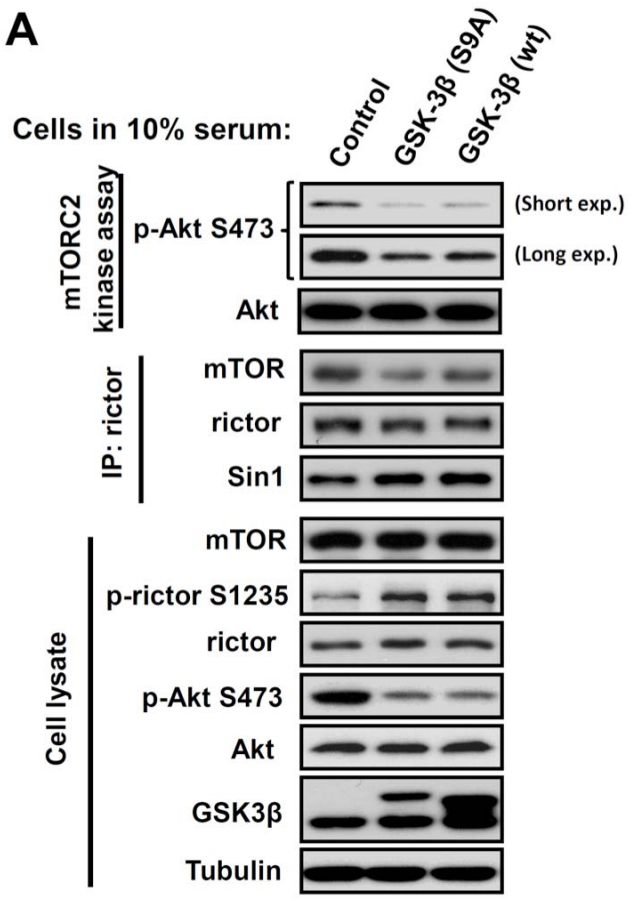

Figure 3-5. GSK-3β phosphorylates rictor at Ser-1235 and inhibits phosphorylation of Akt by mTORC2.

(A) GSK-3β overexpression enhances phosphorylation of rictor at Ser-1235 and inhibits phosphorylation of Akt by mTORC2. Rictor immunoprecipitates were prepared from lysates of COS-7 cells transfected with WT or mutant GSK-3β and used for *in vitro* kinase assays with full-length WT Akt1 as the substrate. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

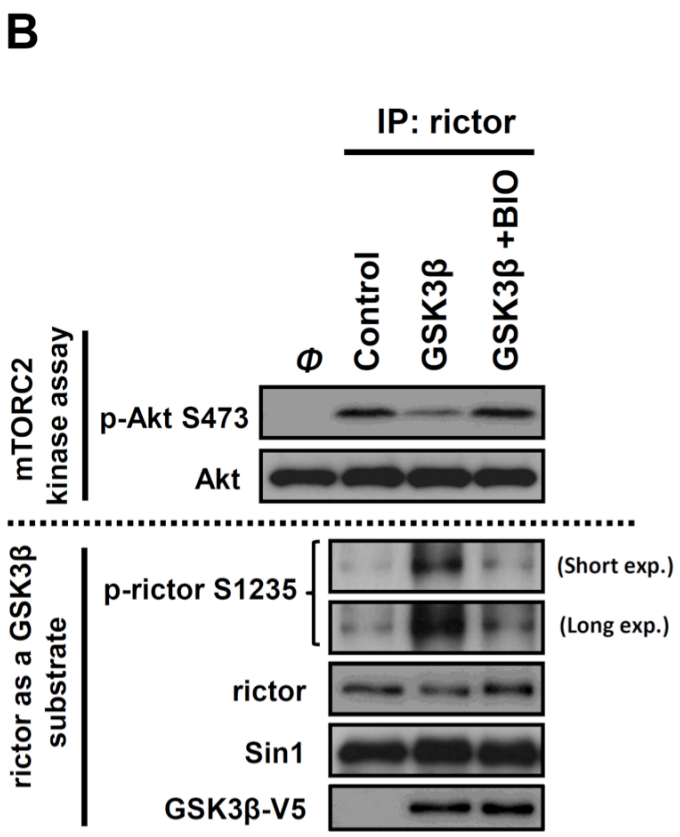

Figure 3-5. GSK-3β phosphorylates rictor at Ser-1235 and inhibits phosphorylation of Akt by mTORC2.

(B) GSK-3β phosphorylates rictor at Ser-1235 and inhibits phosphorylation of Akt by mTORC2. GSK-3β was immunopurified from HEK 293T cells transfected with GSK-3β; cells were treated with tunicamycin (2 μg/ml) for 3 hours before harvest. Rictor immunoprecipitates were prepared from MDA-MB-435 cell lysates. The *in vitro* GSK-3β kinase assays were performed on rictor immunoprecipitates. As a control, GSK-3β was preincubated with 10 μM BIO before the kinase reaction. The *in vitro* mTORC2 kinase assays were subsequently performed on rictor immunoprecipitates. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

rictor phosphorylation was eliminated by inhibition of GSK-3, demonstrating that GSK-3β, but not a contaminating kinase, directly phosphorylated rictor at Ser-1235. Therefore, GSK-3β inhibits mTORC2 kinase activity by directly phosphorylating rictor on Ser-1235.

3.6. Blocking Ser-1235 phosphorylation of rictor prevents mTORC2 inactivation by GSK-3β

We reasoned that if rictor Ser-1235 were the main regulatory site of GSK-3β, mutation on this site would alter GSK-3β-dependent regulation of mTORC2 function. We over-expressed either wild-type rictor or its Ser-1235 to Ala (S1235A) phospho-mutant that prevents phosphorylation in rictor-deficient MEFs. We detected higher Akt Ser-473 phosphorylation in the rictor S1235A mutant-expressing MEFs in comparison with those expressing wild-type rictor (Figure 3-6A). In addition, overexpression of GSK-3β led to decreased Akt Ser-473 phosphorylation in the presence of wild-type rictor. On the contrary, mTORC2-mediated Akt phosphorylation was not changed by overexpression of GSK-3β in the context of rictor S1235A mutant (Figure 3-6A). Thus, mutation of the rictor Ser-1235 is sufficient to prevent the mTORC2 inactivation by GSK-3β.

Likewise, we observed more intense Akt Ser-473 phosphorylation in MEFs stably expressing the rictor S1235A in comparison with those expressing wild-type rictor or the Ser-1235 to Aspartic acid (S1235D) mutant that mimics phosphorylation (Figure 3-6B). We further investigated ER stress responses in

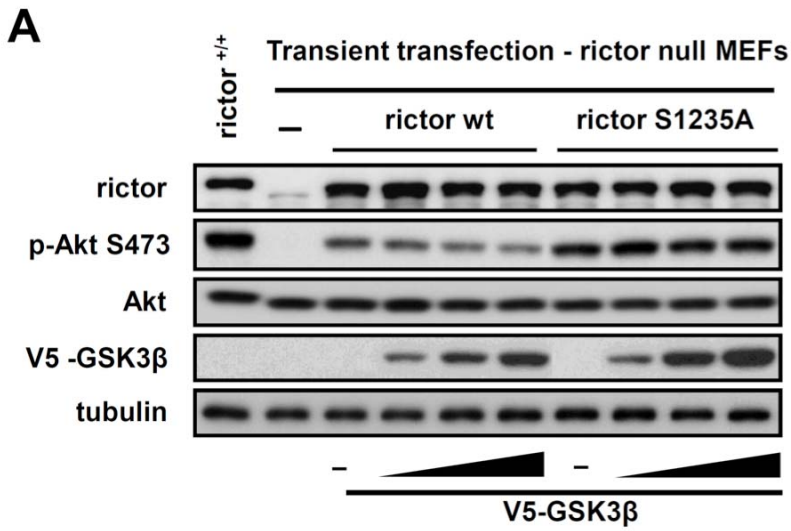

Figure 3-6. Blocking Ser-1235 phosphorylation of rictor prevents mTORC2 inactivation by GSK-3β.

(A) GSK-3β inhibits Akt phosphorylation by phosphorylation of rictor at Ser-1235. Rictor-null MEFs grown in 10% serum were transfected with cDNAs encoding WT rictor or the S1235A mutant and with increasing amounts of GSK-3β cDNA. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

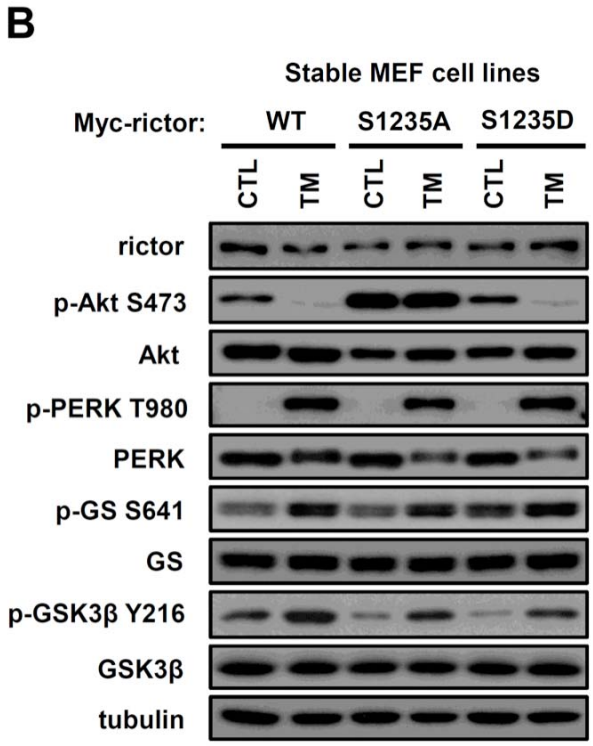

Figure 3-6. Blocking Ser-1235 phosphorylation of rictor maintains higher mTORC2 activity.

(B) Stable expression of the rictor S1235A phospho-mutant shows high basal phosphorylation of Akt that is insensitive to ER stress. Rictor-null MEFs constitutively expressing WT rictor or the phospho-mutants were treated with tunicamycin (0.3 μg/ml) for 8 hours. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

the MEFs restored with wild-type rictor or its phospho-mutants. Treatment of tunicamycin equally induced ER stress and GSK-3β activation in all cell lines (Figure 3-6B). However, Akt phosphorylation at Ser-473 did not decline under ER stress in the rictor S1235A-expressing MEFs (Figure 3-6B). This suggested that Ser-1235 of rictor is important for the GSK-3β impacts on mTORC2 and Akt signaling. Surprisingly, the S1235D mutant-expressing MEFs (which mimics constitutive rictor Ser-1235 phosphorylation) remained sensitive to ER stress at the level similar to that detected in the wild-type rictor-reconstituted MEFs. This inferred that ER stress suppresses Akt signaling not only by interfering with mTORC2 activity, but also by inhibiting upstream regulator of mTORC2 in the growth factor/PI3K signaling.

It is interesting that single rictor phosphorylation event can alter the activity of a huge protein complex (about 550 kD). Several possible mechanisms might be accountable for the impact of rictor Ser-1235 phosphorylation on mTORC2 activity, such as destruction of complex integrity or interference of the substrate binding. To test which notion is correct, we first analyzed the integrity and activity of the mTORC2 reconstituting either wild-type rictor or its phospho-mutants. We observed similar amount of intact mTORC2 from MEFs expressing rictor wild type, S1235A, or S1235D-mutant (Figure 3-6C), suggesting that neither of the phospho-mutants changes the complex integrity of mTORC2. Consistently, kinase activity of the rictor S1235A-containing mTORC2 was higher than that of mTORC2 complexes restoring either wild-type rictor or S1235D mutant (Figure 3-6C, upper panel). This scenario occurred in the complexes from the

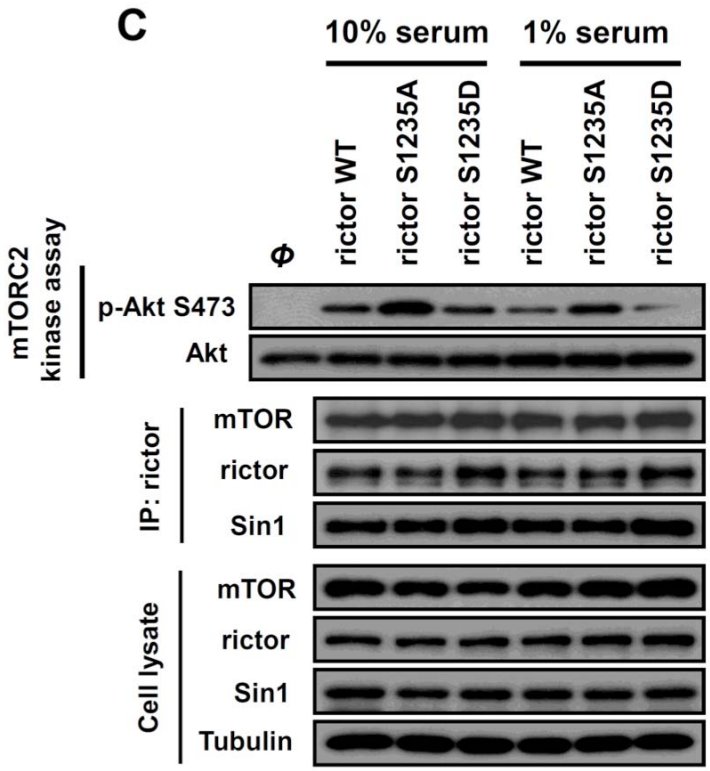

Figure 3-6. Blocking Ser-1235 phosphorylation of rictor maintains higher mTORC2 kinase activity.

(C) Increased phosphorylation of Akt is observed with mTORC2 complexes containing the rictor S1235A mutant. Rictor immunoprecipitates from rictor-null MEFs stably expressing WT rictor or the phospho-mutants and grown in 10 or 1% serum for 16 hours were used for *in vitro* kinase assays. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

high- or low-serum cell culture conditions. The data demonstrated that rictor Ser-1235 phosphorylation regulates mTORC2 kinase activity, but not complex integrity.

3.7. GSK-3β-mediated rictor phosphorylation at Ser-1235 hinders the mTORC2 substrate binding

Since rictor in conjunction with SIN1 regulates substrate specificity of mTORC2, the phosphorylation of rictor at Ser-1235 might affect the binding of Akt to mTORC2. To test this hypothesis, we developed the mTORC2 substrate-binding assay in which the immunopurified mTORC2 was incubated with full length GST-Akt1 protein in the absence of ATP. Following incubation, the unbound proteins were thoroughly washed, and the protein level of GST-Akt bound to mTORC2 was analyzed by immunoblotting. First, we examined the interaction between mTORC2 and Akt in cells treated with tunicamycin or sorbitol (ER-stressed). We detected less GST-Akt protein bound to mTORC2 that was purified from ER stressed cells in comparison with control cells (Figure 3-7A). Therefore, ER stress causes deficient substrate binding ability of mTORC2, leading to mTORC2 inactivation. Given that ER stress-dependent mTORC2 inactivation requires GSK-3β, GSK-3β-mediated rictor phosphorylation at Ser-1235 may influence mTORC2 substrate binding. Accordingly, we further analyzed the impacts of the rictor phospho-mutantion on the Akt binding ability of mTORC2. The equal amount of mTORC2 purified from rictor wild

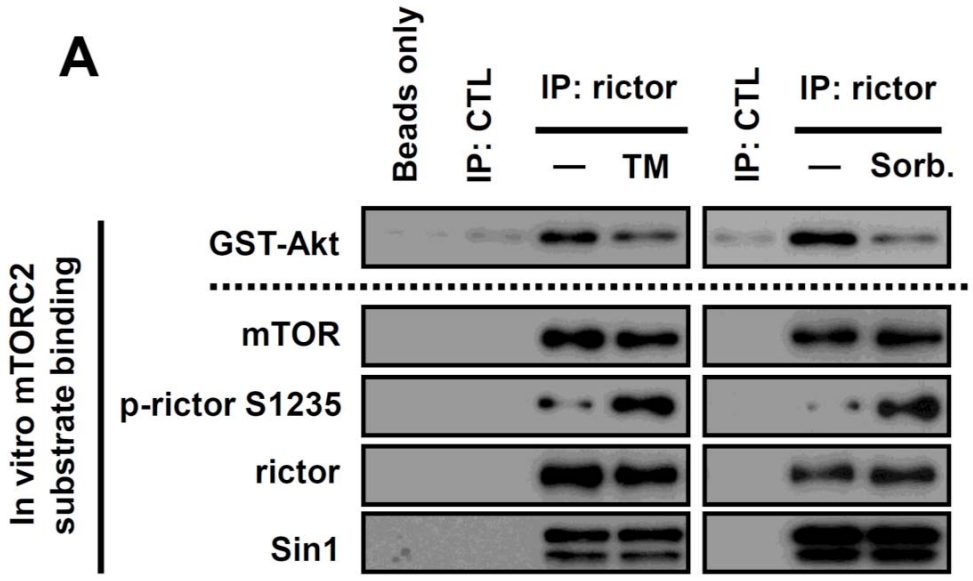

Figure 3-7. Phosphorylation of rictor at Ser-1235 inhibits mTORC2 kinase activity by interfering with mTORC2 binding to Akt.

(A) ER stress inhibits mTORC2 substrate binding. MDA-MB-435 cells were treated with tunicamycin or sorbitol (Sorb.) to induce stress. Rictor immunoprecipitates were used for the *in vitro* substrate binding assays with WTGST-Akt as the substrate and analyzed by immunoblotting. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

type, S1235A, or S1235D-mutant restored MEFs was employed for GST-Akt binding assay. The mTORC2 complex carrying wild-type rictor interacted with more GST-Akt compared to control (Figure 3-7B, left panel). In comparison with mTORC2 carrying wild-type rictor, the rictor S1235A phospho-mutant containing complex exhibited stronger GST-Akt binding ability; in contrast, the complex containing rictor S1235D phospho-mutant bound less amount of the substrate. The interaction of mTORC2 with its substrate was transient and dynamic since the binding was undetectable if ATP exists in the reaction buffer (Figure 3-7B, right panel). By contrast, when ATP is absent, the GST-Akt is inefficiently released from mTORC2. Under this setting, the bound GST-Akt protein amount represents the Akt-binding capacity of mTORC2. Therefore, these results demonstrated that the molecular mechanism of the mTORC2 inactivation caused by rictor Ser-1235 phosphorylation was due to interference of Akt binding to mTORC2.

3.8. GSK-3β-mediated rictor phosphorylation at Ser-1235 controls cell proliferation and tumor growth

Our findings showed that GSK-3β-dependent rictor phosphorylation at Ser-1235 declines the mTORC2-Akt signaling. Since Akt controls cell proliferation, we inferred that rictor S1235A mutation may more strongly induce cell proliferation in comparison with wild-type rictor or the S1235D mutation. To test this possibility, we assessed the proliferation rate of these stable MEFs reconstituted with equal amount of the wild-type rictor and its phospho-mutants

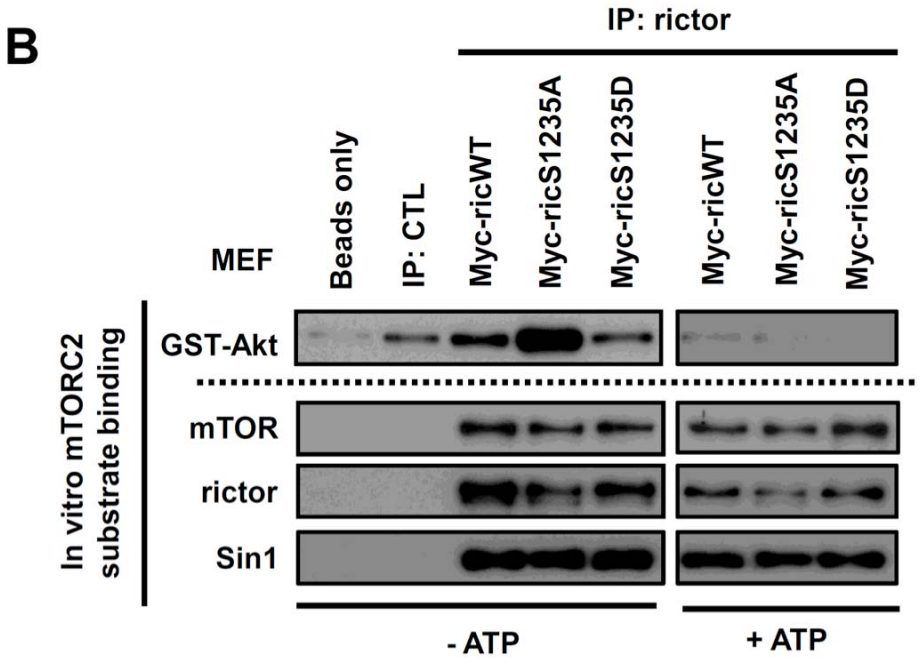

Figure 3-7. Phosphorylation of rictor at Ser-1235 inhibits mTORC2 kinase activity by interfering with mTORC2 binding to Akt.

(B) Phosphorylation of rictor at Ser-1235 regulates mTORC2 substrate binding. Rictor immunoprecipitates from rictor-null MEFs stably expressing WT rictor or the phospho-mutants were used for the *in vitro* substrate binding assays with WT GST-Akt as the substrate. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

(Figure 3-8A, left panel). Restoration of the wild-type rictor into rictor-deficient MEFs augmented cell proliferation rate by 2.27 times. Restoration of the rictor S1235A mutant promoted cell proliferation more potently than the wild-type rictor (2.67 times in comparison with non-restored rictor-deficient MEFs and 30 % higher than rictor-WT restored MEFs). In contrast, MEFs restored with the rictor S1235D mutant displayed lower cell proliferation capacity (1.83 times related to non-restored rictor-deficient MEFs; 50 % less than rictor-WT restored MEFs) (Figure 3-8A, right panel). Notably, the cell proliferation rate of the rictor S1235A-restored MEFs was twice higher than that of the rictor S1235D-restored MEFs. This finding indicates that GSK-3β-mediated rictor Ser-1235 phosphorylation is critical for controlling cell proliferation.

To expand our findings to the *in vivo* tumor xenograft system, we analyzed the impacts of rictor Ser-1235 phosphorylation on subcutaneous tumor growth. First, MEFs restored with rictor-WT or its phospho-mutants were transformed by overexpressing H-Ras (Figure 3-8B, left panel). Mice injected with the rictor S1235A-restored MEFs developed larger tumors in comparison with those injected with rictor-WT-restored MEFs (Figure 3-8B, right panel; Figure 3-8C). On the contrary, mice injected with MEFs containing the rictor S1235D mutation generated smaller tumors than those injected with either WT or S1235A rictor-expressing MEFs (Figure 3-8B, right panel; Figure 3-8C). The data demonstrated that rictor Ser-1235 phosphorylation is significant for controlling cell proliferation and tumor growth.

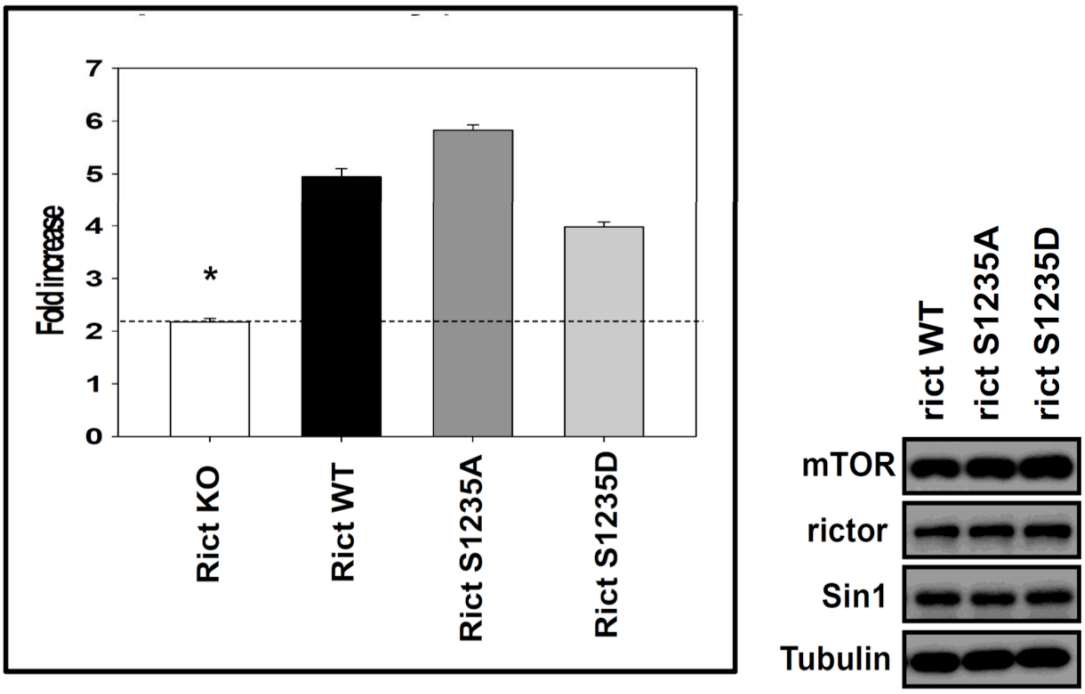

Figure 3-8. Phosphorylation of rictor at Ser-1235 inhibits cell proliferation. (A) WT rictor or the phospho-mutants were reintroduced into rictor-null MEFs with the lentiviral expression system. Cell proliferation measurements were performed by counting cells after 48 hours. The ratio of proliferation rate was graphed with GraphPad Prism 5 software. *P < 0.001 for all pair wise comparisons; one-way ANOVA, post hoc intergroup comparisons with Holm-Sidat test. (This figure was adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

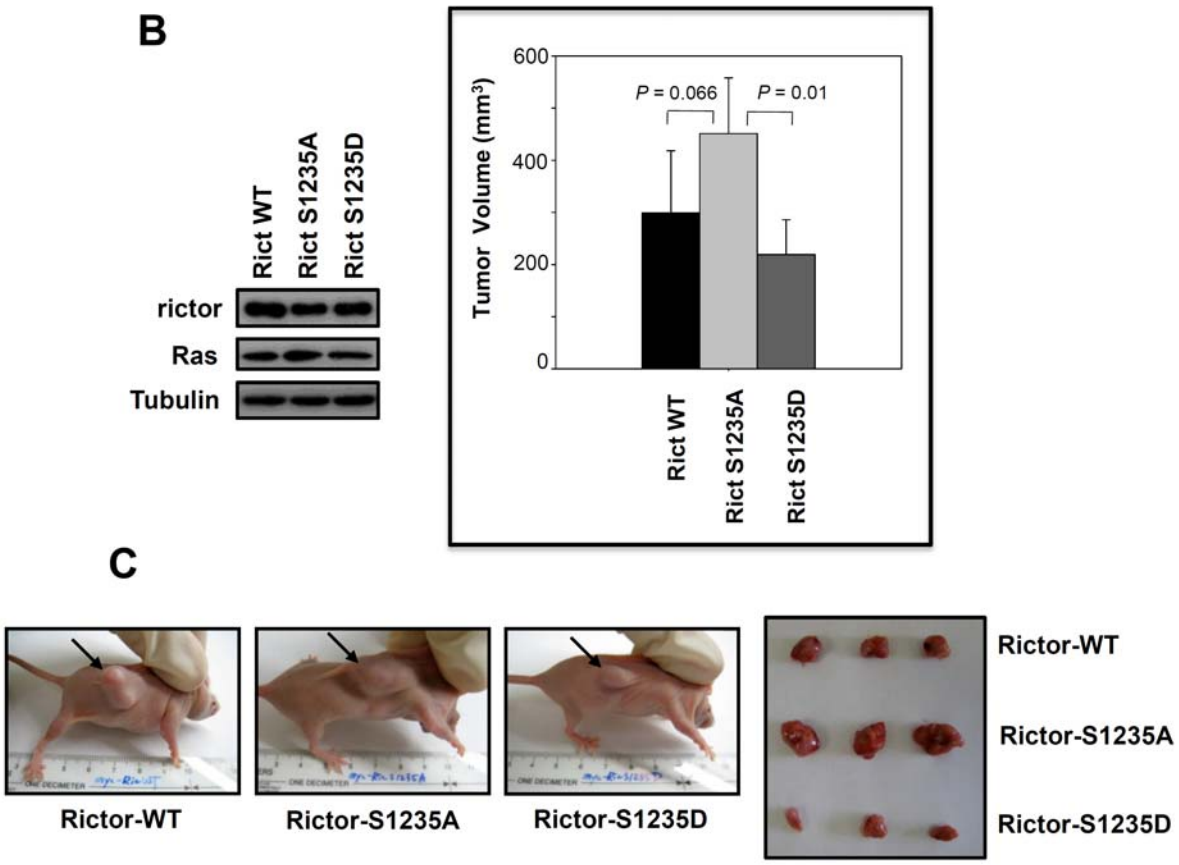

Figure 3-8. Phosphorylation of rictor at Ser-1235 inhibits tumor growth.

(B) MEFs described in Figure 3-7A were transformed by overexpression of the oncogenic form of H-Ras and injected into 6-week-old immunodeficient nude mice (n = 5 for each group; 5×10^6 cells per mouse). Tumor size was measured after 15 days, the tumor sizes were calculated, and the volumes were presented by histogram. *One-way ANOVA, post hoc intergroup comparisons with Holm-Sidat test; the P values are indicated. (C) Mice injected with MEFs expressing each form of rictor were killed and representative images of the excised tumors are shown. (These figures were adopted from our previously published work in *Sci. Signal*. 4, ra10, 2011; the permission was received from the journal.)

3.9. Discussion

Although ER stress is linked to inhibition of the Akt signaling in the pathogenesis of human diseases, the molecular mechanisms of how ER stress inhibits Akt signaling remain to be defined. In our study, we observed that ER stress induces GSK-3β-mediated rictor phosphorylation at Ser-1235, which suppresses mTORC2 kinase activity by impeding its substrate binding (Figure 3-9). Our study provides a novel insight to fill the gaps of knowledge and clarifies the overlooked balance between two kinases, Akt and GSK-3β. Initially, GSK-3β has been identified as the substrate of Akt. Now, our study outlines the importance and mechanism that GSK-3β, the highly conserved and essential kinase in all eukaryotes, controls the anabolic growth factor signaling. This finding transforms GSK-3β from Akt substrate to the dominant stress-dependent regulator of Akt. It explains how ER stress inhibits growth factor signaling and how GSK-3β carries the tumor suppressive effect.

In terms of GSK-3β-mediated phosphorylation, most GSK-3β substrates need priming phosphorylation at adjacent sites to promote the phosphorylation by GSK-3β. Nevertheless, several GSK-3β substrates do not require priming step, such as cyclin D1 and BCL3 (Diehl et al., 1998; Viatour et al., 2004). Besides, there is no serine residue at the adjacent priming site of rictor for prior phosphorylation. The resemblance of phosphorylation sites between rictor and BCL3 implies that priming phosphorylation is not required for GSK-3β-mediated rictor phosphorylation at Ser-1235. Our study also indicates that GSK-3β-

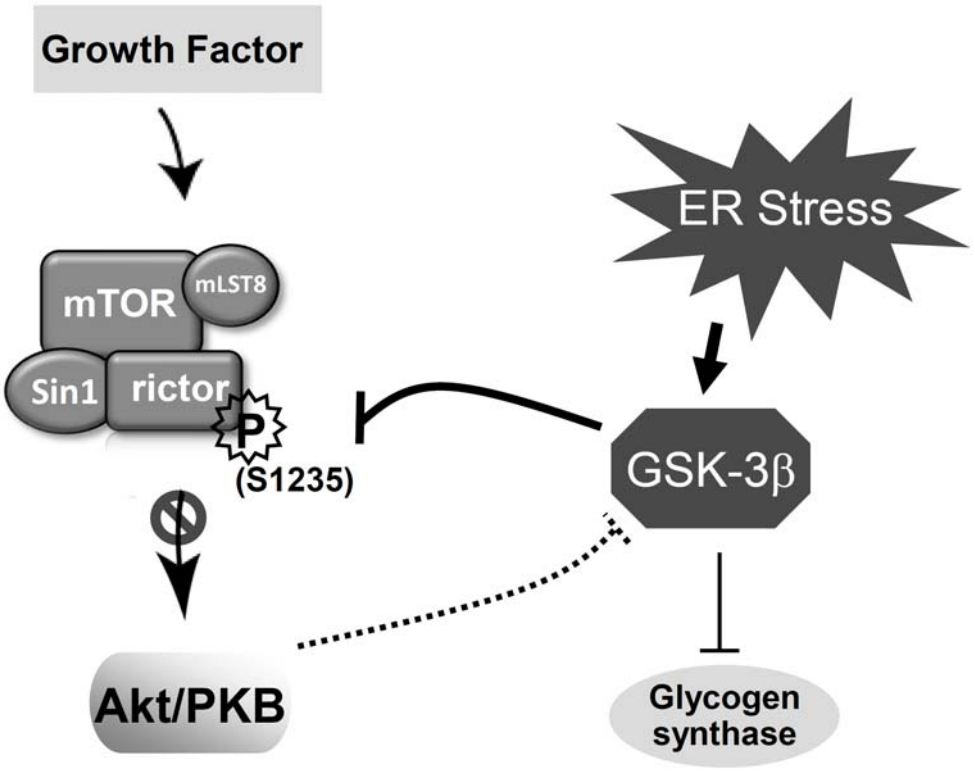

Figure 3-9. The proposed working model for the first project.

Initially, GSK-3β has been identified as the substrate of Akt. In my first project, we showed that under ER stress rictor is phosphorylated by GSK-3β at Ser-1235. This phosphorylation event causes inhibition of mTORC2 kinase activity by interfering with substrate binding.

mediated mTORC2 regulation is evolutionally conserved in vertebrates and invertebrate fly *Drosophila melanogaster*.

In addition, our finding indicates that one specific Ser-1235 phosphorylation on rictor inhibits Akt binding ability of the multi-protein complex (approximately 550 kDa). Other phosphorylation sites of rictor in proximity to Ser-1235 including the Thr-1135 and Ser-1177 sites do not affect mTORC2 activity (Boulbes et al., 2010). Accordingly, we inferred that rictor Ser-1235 site locates in proximity to the Akt-binding pocket of mTORC2 and this phosphorylation event interrupts with Akt binding to mTORC2. In addition, rictor Ser-1235 phosphorylation did not disrupt the complex integrity of mTORC2, indicating that Ser-1235 residue does not locate near the binding site of rictor to its interacting partners.

Furthermore, previous mouse genetic models are consistent with our study in which GSK-3β obstructs the insulin-dependent Akt signaling, providing a link between GSK-3β and insulin resistance (Srinivasan et al., 2005; Tanabe et al., 2008). This explains why GSK-3 inhibitors act as the insulin-mimetic agents by stimulating glucose uptake and cell proliferation. GSK-3β becomes a promising target for treating multiple human diseases such as metabolic disorders (Cohen and Goedert, 2004; Rayasam et al., 2009). Hence, our finding connects clinical applications of GSK-3 inhibitors with activation of mTORC2/Akt signaling, and is particularly significant in development of these inhibitors for effective treatment of metabolic disorders. So far, most GSK-3 inhibitors could not distinguish between GSK-3α and GSK-3β because they are ATP competitive

inhibitors and the ATP binding sites of these two isoforms are very similar. To overcome the low specificity of this type of GSK-3 inhibitors, the development of the non-ATP competitive inhibitors (e.g. substrate competitive inhibitors) is very important. However, the substrate competitive inhibitors have a disadvantage that they usually bind weakly to the kinase (just like the dynamic substrate-enzyme interaction). So, chemical modifications are required to improve the efficacy of the substrate competitive inhibitors by enhancing their binding affinity to GSK-3β.

In our study, GSK-3β is highly activated under ER stress due to the observation that the phosphorylation of its substrate glycogen synthase (Ser-641) and auto-phosphorylation site (Tyr-216) (Chen et al., 2011). However, how ER stress controls GSK-3β activity remains scarcely characterized. It is important to address this question in the future. Our preliminary study also indicates that GSK3β-dependent rictor Ser-1235 phosphorylation is linked to a biphasic effect on mTORC2. As an immediate effect we observe inhibition of the mTORC2 kinase activity, and as a long-term effect we detect an accelerated degradation of rictor. It is common that most of the known substrates of GSK3β are rapidly degraded by proteosomal pathway where GSK3β-mediated phosphorylation serves as a recognition motif for the β-Trcp E3 ubiquitin ligase (Xu et al., 2009). It will be interesting to identify the mechanism for the Ser-1235 phosphorylation-dependent rictor degradation.

Chapter 4

The homeostatic ATP sensor mTOR maintains the mTORC2 complex integrity

The first part of this work has been published in:
C.-H. Chen, D. D. Sarbassov, The mTOR (Mammalian Target of Rapamycin) Kinase Maintains Integrity of mTOR Complex 2. *The Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394 (2011)

The second part of this work has been submitted to *Molecular Cell* Journal

Rationale

The central component of the growth signaling mTOR assembles into two structurally and functionally different complexes (mTORC1 and mTORC2). The second complex, mTORC2, is a multi-protein kinase composed of mTOR, mLST8, SIN1 and rictor. In my last project (Chapter 3), we demonstrated that one specific rictor phosphorylation at Ser-1235 inhibits Akt binding of mTORC2. However, the regulatory mechanism of the mTORC2 complex integrity is not well characterized.

In this study, I developed a reconstitution system of mTORC2 by co-expression of its essential components (mTOR, mLST8, rictor, and SIN1), and observed that the mTOR-dependent SIN1 phosphorylation is critical for maintaining complex integrity by preventing SIN1 degradation. SIN1 is a critical component for mTORC2 complex formation and its kinase activity. Thus, I aimed to investigate the mechanism how mTOR kinase regulates integrity of mTORC2.

Results

4.1. The mTORC2 reconstitution by co-expression of its four components

According to previous studies, mTORC2 components display distinct binding affinity (Frias et al., 2006; Sarbassov et al., 2004). A mild cell lysis buffer containing the CHAPS detergent could maintain the assembly of this kinase complex, whereas mTORC2 dissociates to two heterodimers (rictor/SIN1 and mTOR/mLST8) under stringent lysis condition with the Triton X-100 detergent. The biochemical studies suggest that the initial stage of the mTORC2 formation

is the dimerization of the mTOR/mLST8 and rictor/SIN1, respectively. Then, these two heterodimers assemble a relatively low affinity hetero-tetramer that is sensitive to the stringent lysis buffer containing Triton X-100.

First, we tested the formation of the rictor/SIN1 heterodimer by co-expressing rictor and SIN1. The V5-tagged SIN1 was co-transfected with the myc-tagged rictor, GCP2, or raptor cDNAs (Figure 4-1A, lower panel). Immunopurification via the myc antibody showed that SIN1 only bound to rictor rather than the control proteins GCP2 and raptor (Figure 4-1A, upper panel). Moreover, the interaction between rictor and SIN1 is required to stabilize mutual expression since SIN1 is highly unstable without its binding partner rictor (Frias et al., 2006). In order to reach the equivalent SIN1 expression, the five-fold amount of SIN1 cDNA was co-transfected with the control proteins GCP2 or raptor. Similarly, the co-expression of mTOR and mLST8 showed the formation of the mTOR/mLST8 heterodimer (Figure 4-1B). We observed that mTOR specifically interacted with mLST8 but not tubulin by pulling down myc-mTOR. Thus, the initial step of our reconstitution studies indicated the specific assembly of the rictor/SIN1 and mTOR/mLST8 heterodimers.

Subsequently, we generated the reconstituted mTORC2 by co-expression of its four recombinant components (Figure 4-1C). After immunopurification of myc-mTOR, we analyzed its binding proteins and observed that SIN1 or rictor itself does not interact with mTOR/mLST8 heterodimer. We also detected low

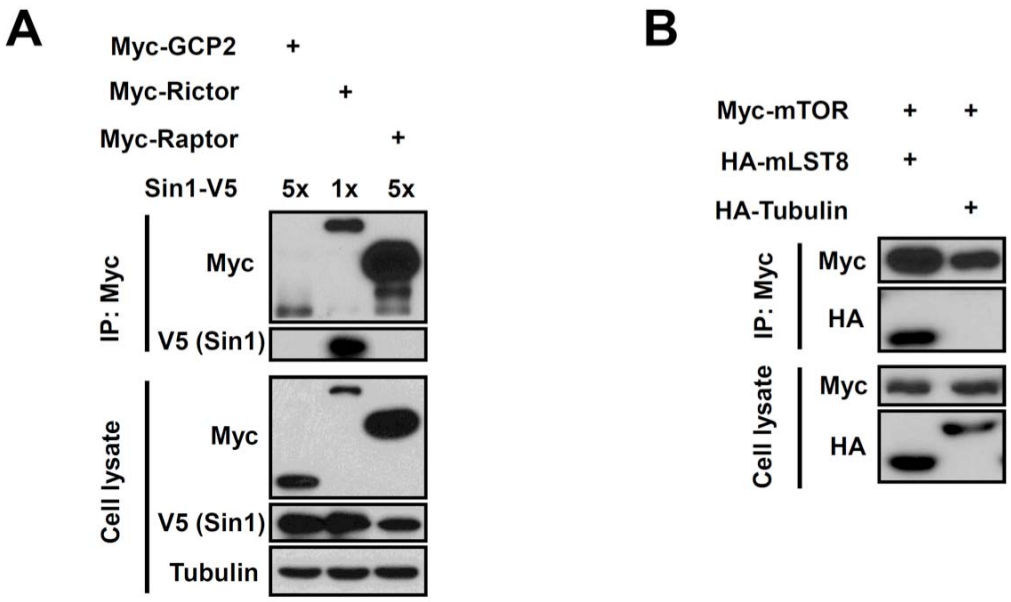

Figure 4-1. Reconstitution of the functional mTORC2 complex by expressing its recombinant components.

(A) Rictor interacts with SIN1. SIN1-V5 plasmid DNA was co-transfected with myc-rictor into HEK-293T cells by Lipofectamine 2000, and cells were analyzed 48 h following transfection. Cell lysates were applied for immunoprecipitation (IP) with anti-myc antibody. The immunoprecipitates and cell lysates were analyzed by immunoblotting with anti-myc and anti-V5 antibodies.

(B) mTOR interacts with mLST8. Myc-mTOR construct was co-transfected with HA-mLST8 in HEK-293T cells. The negative control is presented by expressing HA-α-tubulin with myc-mTOR. (These figures were adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

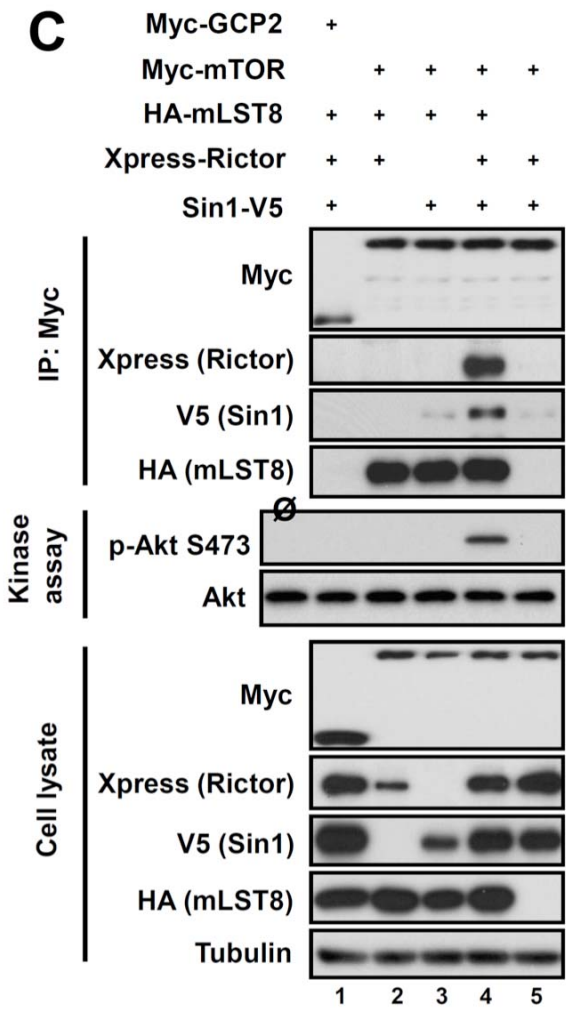

Figure 4-1. Reconstitution of the functional mTORC2 complex by expressing its recombinant components.

(C) Interaction of mTOR with other mTORC2 components. The differently tagged recombinant components of mTORC2 were transiently expressed in HEK-293T cells, and the assembled complexes were purified by the immunoprecipitation of myc-mTOR. (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

protein abundance of SIN1 and rictor alone because rictor/SIN1 heterodimer formation is required to stabilize their expression. Similarly, mTOR itself did not bind to rictor/SIN1 heterodimer, suggesting that the heterotrimer does not form during the mTORC2 assembly. When all four components of mTORC2 were co-transfected, we observed the functional mTORC2 formation as shown by the *in vitro* mTORC2 kinase assay (Figure 4-1C). Our study of the mTORC2 reconstitution system is consistent to the previous findings that SIN1, rictor and mLST8 are indispensable components for the assembly and function of mTORC2 (Frias et al., 2006; Guertin et al., 2006; Jacinto et al., 2006). Importantly, the endogenous mTORC2 components do not interfere with the recombinant mTORC2 reconstituted by transiently expressed mTOR, mLST8, rictor, and SIN1.

We further examined if the reconstituted mTORC2 is regulated by growth factor signaling as the endogenous complex by analysis of its activity in growth factor responsive COS-7 cells (Roudabush et al., 2000). After co-transfection of the mTORC2 components, we stimulated the serum-starved COS-7 cells by IGF-1 and immunopurified the reconstituted mTORC2 followed by the *in vitro* kinase reaction (Figure 4-1D). The mTORC2 purified from cells grown in 10% serum showed the low basal kinase activity and the complex from serum-starved cells did not exhibit kinase activity. In contrast, we observed the robust kinase activity of mTORC2 in IGF-stimulated cells. Thus, the reconstituted mTORC2 resembles its native form that is also controlled by growth factor signaling.

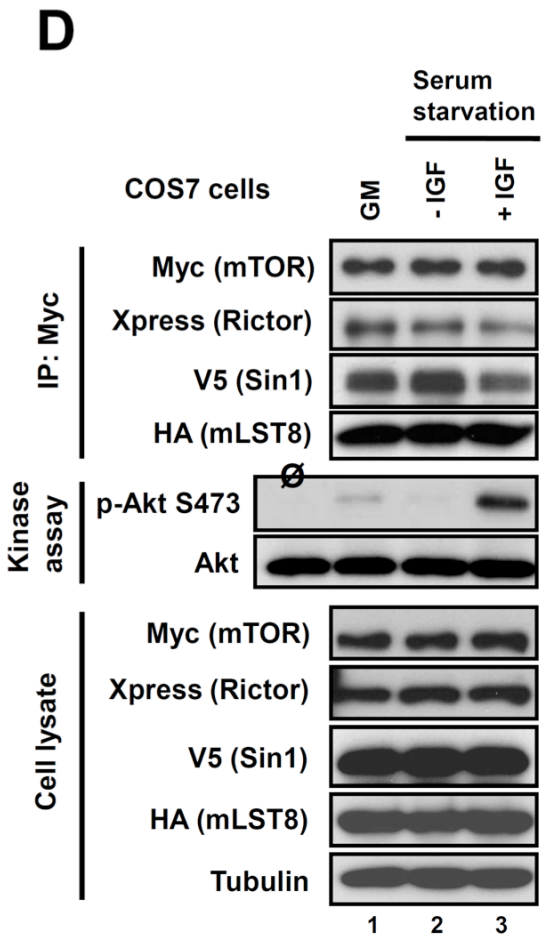

Figure 4-1. Reconstituted mTORC2 kinase activity was stimulated by IGF-1. (D) The differently tagged recombinant components of mTORC2 were transiently expressed in COS-7 cells, and the assembled complexes were purified by the immunoprecipitation of myc-mTOR. Lane 1, COS-7 cells expressing recombinant mTORC2 was cultured in growth medium containing 10% serum. Lanes 2 and 3, COS-7 cells expressing recombinant mTORC2 were cultured in serum-free medium for 16 h and then treated without or with IGF-I (100 ng/ml) for 30 min, respectively. (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

4.2. Reconstitution of mTORC2: The kinase activity of mTOR is required for Akt Ser-473 phosphorylation

In the process of the growth factor-dependent Akt activation, the Ser-473 phosphorylation is required to stabilize the active conformation of Akt (Pearce et al., 2010). Characterization of the Ser-473 kinase (PDK2) will provide a novel insight for the Akt regulation. Recent biochemical and mouse genetic studies clarified mTORC2 as the major Ser-473 kinase of Akt (Guertin et al., 2006; Jacinto et al., 2006; Sarbassov et al., 2005; Shiota et al., 2006; Yang et al., 2006). Although mTOR serves as the kinase within mTORC2, there was no direct evidence indicating mTOR as the Ser-473 kinase of Akt. In order to characterize the role of the mTOR kinase within mTORC2, we employed the reconstitution of mTORC2 carrying the kinase-dead form of mTOR. We co-transfected the cDNAs of the wild-type or kinase-dead mTOR with other essential mTORC2 components and analyzed the mTORC2 activity in the duplicate experiments. The mTORC2 reconstituted with the kinase-dead mTOR did not exhibit kinase activity (Figure 4-2A), indicating the essential role of the mTOR kinase domain for the kinase activity of mTORC2. Previous reports suggested that Akt auto-phosphorylation at Ser-473 occurs in regulation of Akt (Toker and Newton, 2000). To characterize the potential impact of Akt auto-phosphorylation, we performed the kinase reaction by using the kinase-inactive form of GST-Akt. The reconstituted wild-type mTORC2 exhibited the robust kinase activity toward the kinase-inactive form of GST-Akt, demonstrating that Akt auto-phosphorylation does not take place during the mTORC2-mediated Akt phosphorylation (Figure 4-2A).

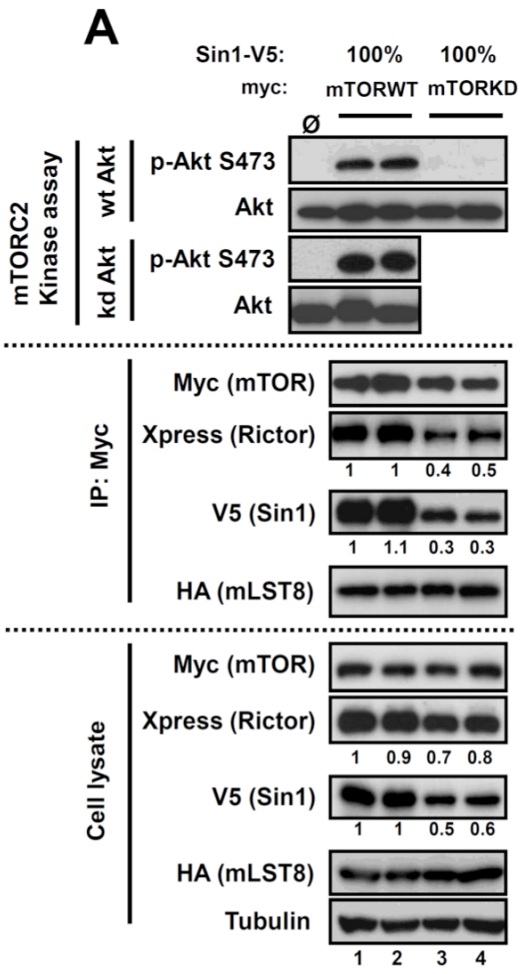

Figure 4-2. Critical role of mTOR kinase activity in mTORC2 function.

(A) The kinase-dead mTOR forms inactive mTORC2 and causes deficiency in assembly of the complex. The study has been performed in duplicate: Xpress-rictor, SIN1-V5, and HA-mLST8 constructs were co-transfected with myc-mTOR wild type (WT) (lanes 1 and 2) or myc-mTOR kinase-dead (KD) (lanes 3 and 4) in HEK-293T cells. Immunoprecipitates (IP) prepared with anti-myc antibody were used for the *in vitro* kinase assay with wild-type or kinase-dead Akt as the substrate. (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

In the subsequent analysis, we found that the kinase-dead mTOR did not interrupt expression and interaction with its binding partner mLST8; however, it resulted in the declined expression of rictor and SIN1, which led to ineffective formation of the mTORC2 (Figure 4-2A). In addition, SIN1 protein displayed the slower migratory form in the complex with wild-type mTOR rather than kinase-dead mTOR (Figure 4-2A). This indicated that mTOR kinase activity might be required for SIN1 protein modification, which is linked to regulation of SIN1 protein stability. The experiment in Figure 4-2A showed that the kinase-dead mTOR caused a profound impact on the rictor and SIN1 expression that resulted in low abundance of mTORC2. To overcome the uneven mTORC2 assembly, we optimized the formation of the complex with wild-type mTOR by co-expressing only 30 % of the SIN1 cDNA (Figure 4-2B). The comparable amounts of the immunopurified mTORC2 complexes assembled with mTOR-WT or mTOR-KD were applied for *in vitro* kinase assay. Contrary to mTOR-WT, the mTORC2 reconstituted with kinase-dead mTOR again did not exhibit the kinase activity. Similarly, the SIN1 co-expressed with mTOR-WT showed the slower mobility in the gel, indicating that SIN1 protein modification is dependent on mTOR kinase activity. Collectively, our analysis showed that mTOR kinase within mTORC2 is required for mTORC2 function as the Akt Ser-473 kinase, and also contributes to the regulation of SIN1 through protein modification.

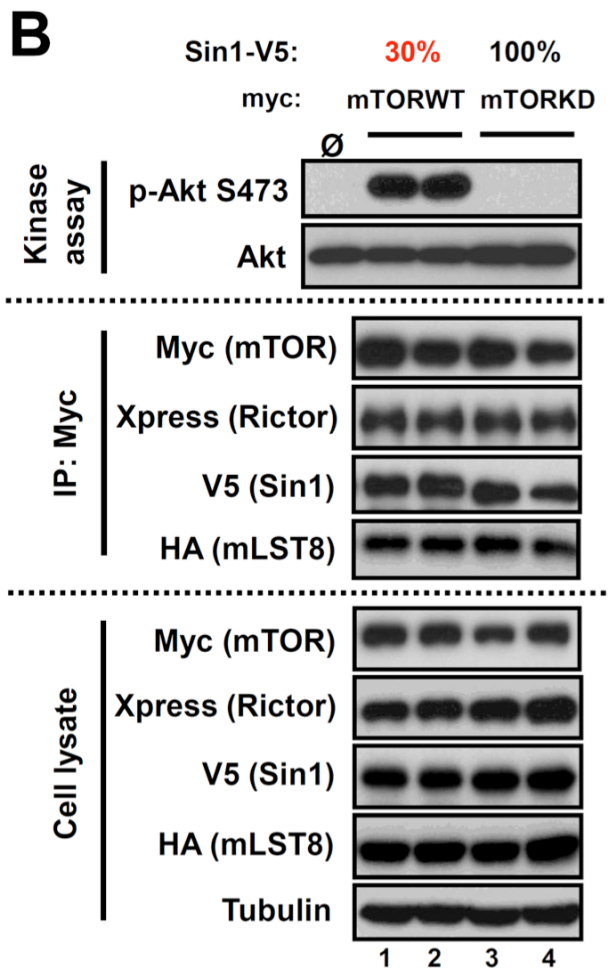

Figure 4-2. Critical role of mTOR kinase activity in mTORC2 function.

(B) The study similar to that in Figure 4-2A was performed by optimizing expression and assembly of the mTORC2 assembled with the wild-type or the kinase-dead mutant of mTOR. To optimize expression, the myc-mTOR-WT was co-transfected with Xpress-rictor, 30% of SIN1-V5, and HA-mLST8 constructs in HEK-293T cells (lanes 1 and 2). (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

4.3. Phosphorylation of SIN1 occurs upon assembling into mTORC2 complex *in vitro*

Our data shown in Figure 4-2 indicate that SIN1 protein abundance and its modification depend on the mTOR kinase activity. Most likely, the observed SIN1 protein modification is caused by phosphorylation. To test this possibility, we reconstituted and purified mTORC2 followed by phosphatase treatment (Figure 4-3A). Only the active form of phosphatase changed the SIN1 mobility from the slower to faster migratory form, suggesting that SIN1 is phosphorylated within mTORC2 assembled with wild-type mTOR.

To further investigate if SIN1 is the mTOR substrate, we examined the mTOR-mediated SIN1 phosphorylation by the *in vitro* mTORC2 assembly. First, we purified the co-expressed FLAG-mLST8/myc-mTOR heterodimer by FLAG peptide elution. Then, the immunopurified myc-rictor/V5-SIN1 heterodimer bound to protein G-agarose beads was incubated with soluble FLAG-mLST8/myc-mTOR heterodimer. After co-incubation, the protein G-agarose beads were thoroughly washed and analyzed for the complex formation and kinase activity of mTORC2. Indeed, these two heterodimers have integrated to mTORC2 as shown by its four components (Figure 4-3B). The *in vitro* assembled mTORC2 exhibited kinase activity toward Akt Ser-473. In addition, SIN1 protein changed mobility to the slower migratory form following *in vitro* mTORC2 reconstitution (Figure 4-3B), resembling its phosphorylated form before phosphatase treatment (Figure 4-3A). These results indicated that SIN1 is the mTOR substrate.

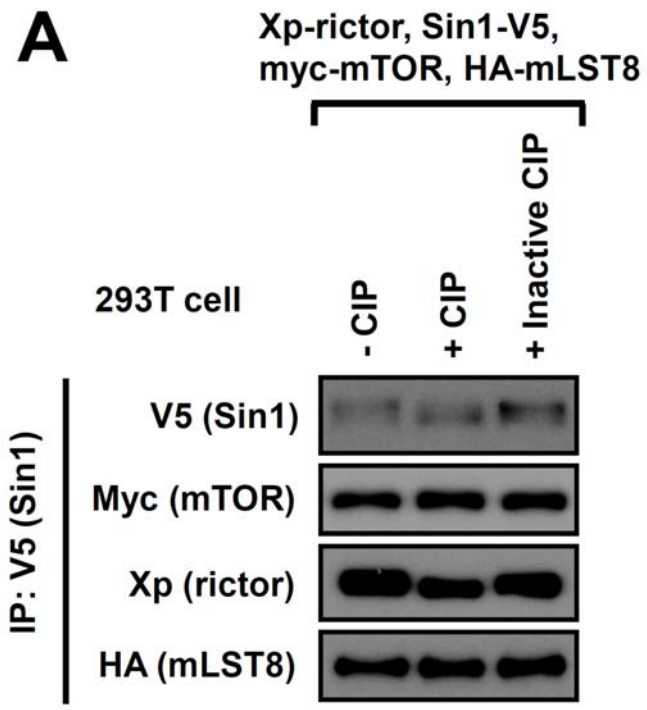

Figure 4-3. SIN1 is a substrate of mTOR.

(A) SIN1 as a component of mTORC2 is a phospho-protein. Myc-mTOR-WT cDNA was co-transfected with Xpress-rictor, Sin1-V5, and HA-mLST8 cDNAs in HEK-293T cells. Cell lysates were applied for immunoprecipitation with anti-V5 antibody. The immunoprecipitates were incubated with or without calf intestinal phosphatase (*CIP*) or inactive CIP (pretreated with 50 mM EDTA). CIP treatment resulted in a faster migratory form of SIN1, suggesting that the mobility shift of SIN1 band is indeed due to phosphorylation/dephosphorylation. (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

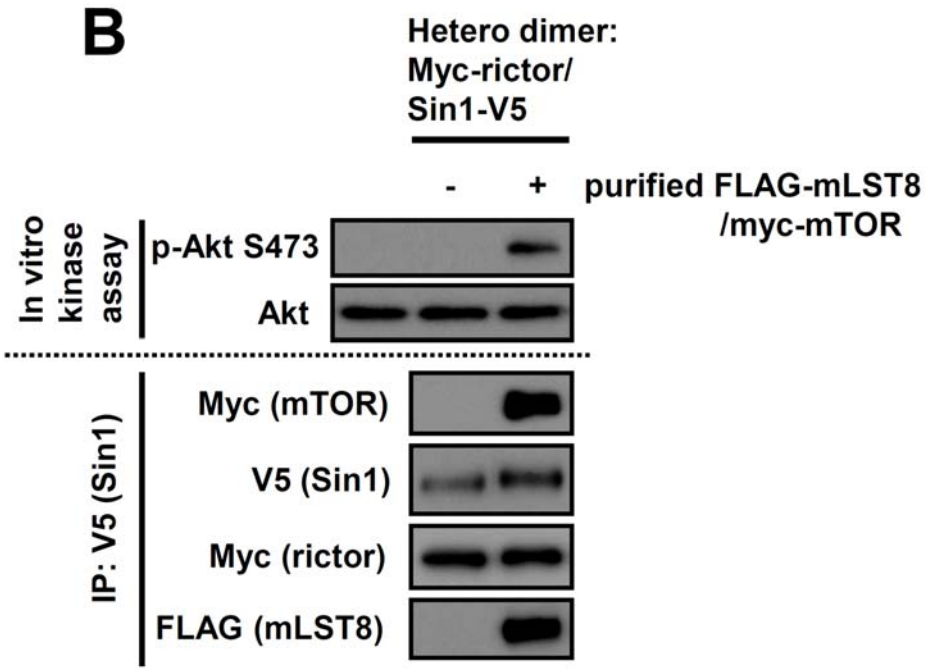

Figure 4-3. SIN1 is a substrate of mTOR.

(B) SIN1 as a component of mTORC2 is a substrate of mTOR. *In vitro* mTORC2 kinase assay was performed by incubation of two heterodimers, rictor/SIN1 and mTOR/mLST8. First, FLAG purification was conducted from HEK-293T cells with FLAG-mLST/myc-mTOR co-transfection. Then, the purified soluble FLAG-mLST/myc-mTOR heterodimer was incubated with immunopurified myc rictor/SIN1-V5 by the V5 antibody at room temperature for 2 h in mTORC2 kinase buffer with 1 mM ATP. After washing three times with 0.3% CHAPS lysis buffer, the immunoprecipitates were used for the *in vitro* mTORC2 kinase reaction by using wild-type Akt as the substrate. (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

4.4. The lysosomal degradation of SIN1 protein is caused by the inactive mTOR kinase

Our data suggested that SIN1 protein expression is dependent on the mTOR-mediated SIN1 phosphorylation. To further inspect the regulation of SIN1 by mTOR kinase, we studied SIN1 protein half-life by the inhibition of protein synthesis. As described in Figure 4-2B, we normalized the reconstituted mTORC2 by reducing SIN1 expression with wild-type mTOR. Following cycloheximide treatment, we detected the shorter SIN1 protein half-life in the mTOR-KD co-expression (Figure 4-4A), suggesting that declined SIN1 protein level results from accelerated protein degradation. The quantification of the immunoblotting image of SIN1 displayed the greater effect of mTOR-KD on diminishing the SIN1 half-life approximately eight times in comparison with mTOR-WT (Figure 4-4B).

The ubiquitin-proteasomal and lysosomal degradation are two major representatives of protein degradation pathways. To verify which pathway regulates SIN1 protein degradation, we reconstituted mTORC2 with the mTOR-KD form in cells and studied effects of MG132 (proteasome inhibitor) or chloroquine (lysosome inhibitor). The Skp2-mediated proteasomal pathway is required for the degradation of the cell cycle regulator p27 protein (Carrano et al., 1999). We observed the considerable increase of the p27 protein after MG132 treatment (Figure 4-4C) revealing that the drug was effective in inhibition of the proteosomal pathway. However, the suppression of the proteosomal pathway did not block SIN1 degradation as shown by the low SIN1 protein amount in the

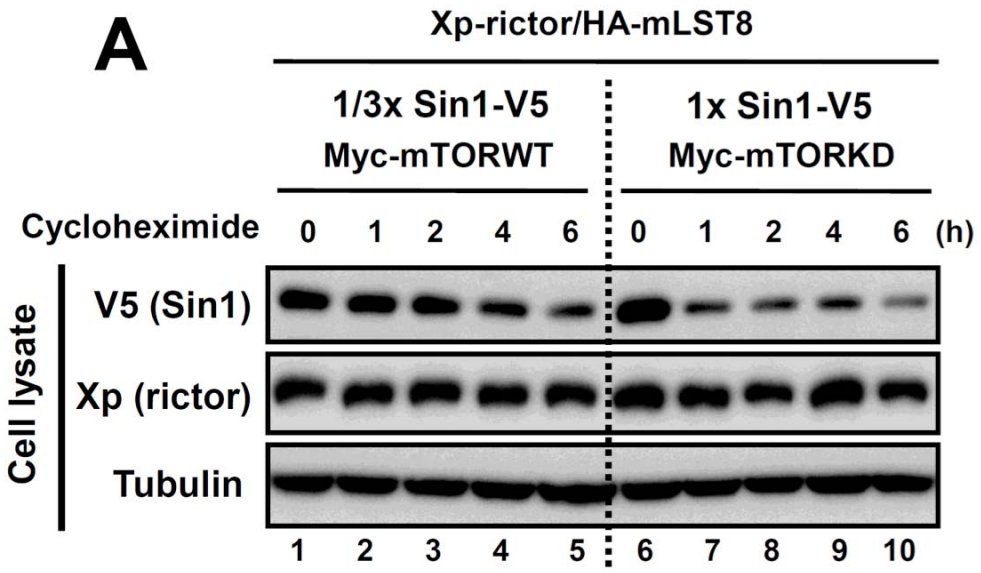

Figure 4-4. mTOR kinase activity stabilizes SIN1 by preventing its lysosomal degradation.

(A) Optimized reconstitution of mTORC2 by co-expression of its components was performed as in Figure 4-2B. 48 h following transfection, cells were treated with 20 μg/ml cycloheximide and lysed at the indicated time points. (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

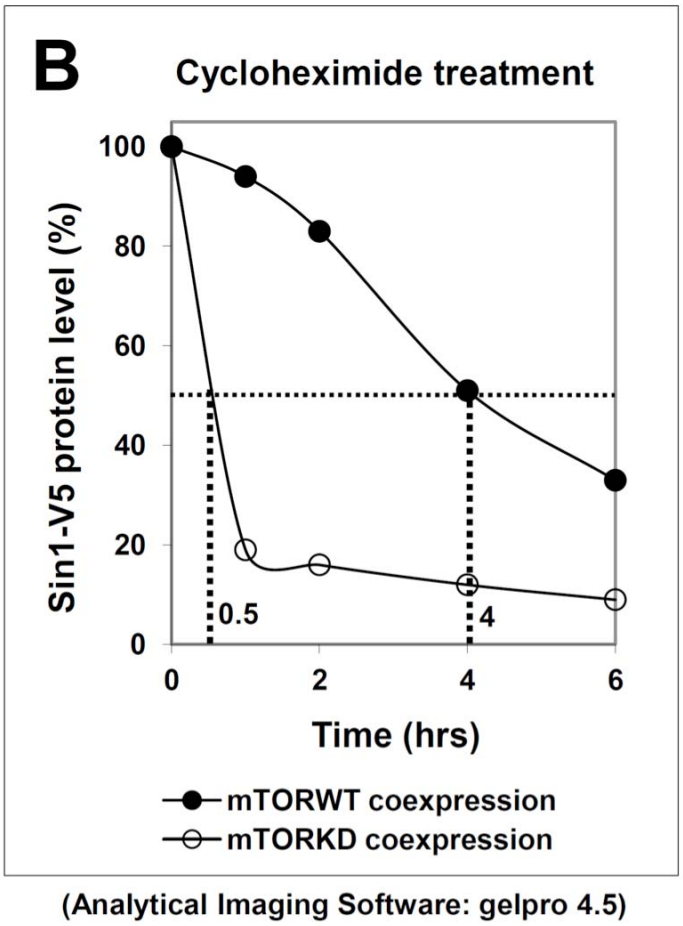

Figure 4-4. mTOR kinase activity stabilizes SIN1 by preventing its lysosomal degradation.

(B) Graphic represents mTOR kinase-dependent SIN1 half-life shown in (A). SIN1-V5 signals were quantified by GelPro 4.5 software. The half-life of SIN1 co-expressed with mTOR-WT is shown as **filled circles**, and its half-life when co-expressed with mTOR-KD is shown as **open circles**. (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

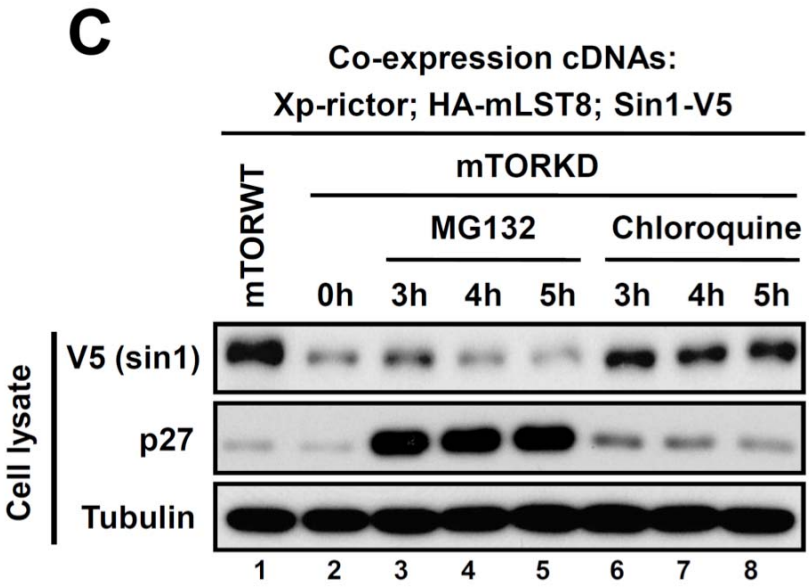

Figure 4-4. mTOR kinase activity stabilizes SIN1 by preventing its lysosomal degradation.

(C) myc-mTOR-KD or its wild-type construct was co-transfected with Xpress-rictor, SIN1-V5, and HA-mLST8 constructs into HEK-293T cells. After 48-h transfection, cells were treated with 30 μM MG132 or 100 μM chloroquine and collected at the indicated time points. (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

presence or absence of MG132. Only the lysosomal inhibitor chloroquine led to elevated SIN1 protein level comparable to that in the co-expression of the wild-type mTOR (Figure 4-4C). Thus, these results demonstrated that SIN1 degradation caused by deficient mTOR kinase is dependent on lysosomal degradation pathway.

4.5. Endogenous SIN1 protein level depends on mTOR expression and its kinase activity

Our findings based on the recombinant mTORC2 reconstitution system indicated that protein stability of SIN1 depends on mTOR kinase activity. To confirm our results, we validated if mTOR regulates the endogenous SIN1 protein. Indeed, the knockdown of mTOR by expressing specific shRNAs led to the declined protein amount of the endogenous SIN1 (Figure 4-5A). This negative impact correlates well with the mTOR knockdown efficiency since the smaller SIN1 protein amount is observed in cells containing the shRNA#2, which causes the stronger suppressive effect on the mTOR protein level.

Next, we addressed if mTOR inhibition is able to mimic the impact on SIN1 protein stability by mTOR-KD. We applied the highly specific mTOR inhibitor TORIN in MDA-MB-435 cells (Liu et al., 2010; Thoreen et al., 2009), which resulted in a potent mTOR inactivation as shown by the de-phosphorylation of the mTORC2 substrate Akt (Ser-473 and Thr-450) and the mTORC1 substrate S6K1 (Thr-389) (Figure 4-5B). TORIN also inhibited the mTOR auto-phosphorylation at Ser-2481 (Peterson et al., 2000). By contrast, the

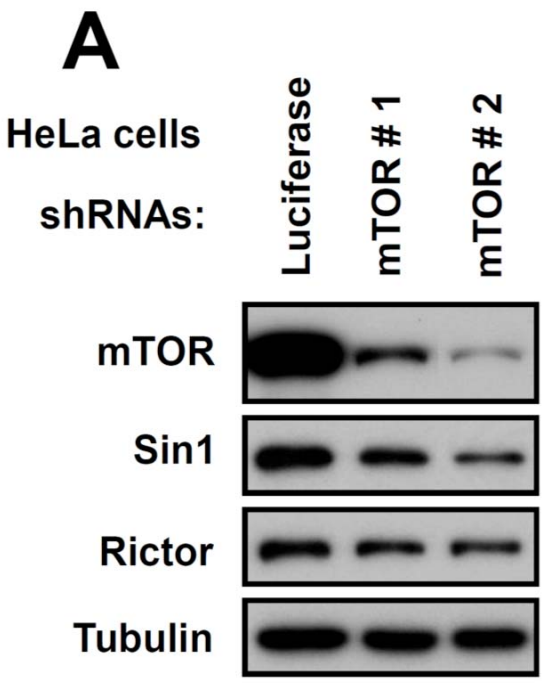

Figure 4-5. Endogenous SIN1 expression is dependent on mTOR kinase activity.

(A) SIN1 protein abundance decreased with a loss of mTOR. The mTOR knockdown has been performed by the lentiviral expression of two shRNAs in HeLa cells. A nonspecific luciferase shRNA was used as a control. (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

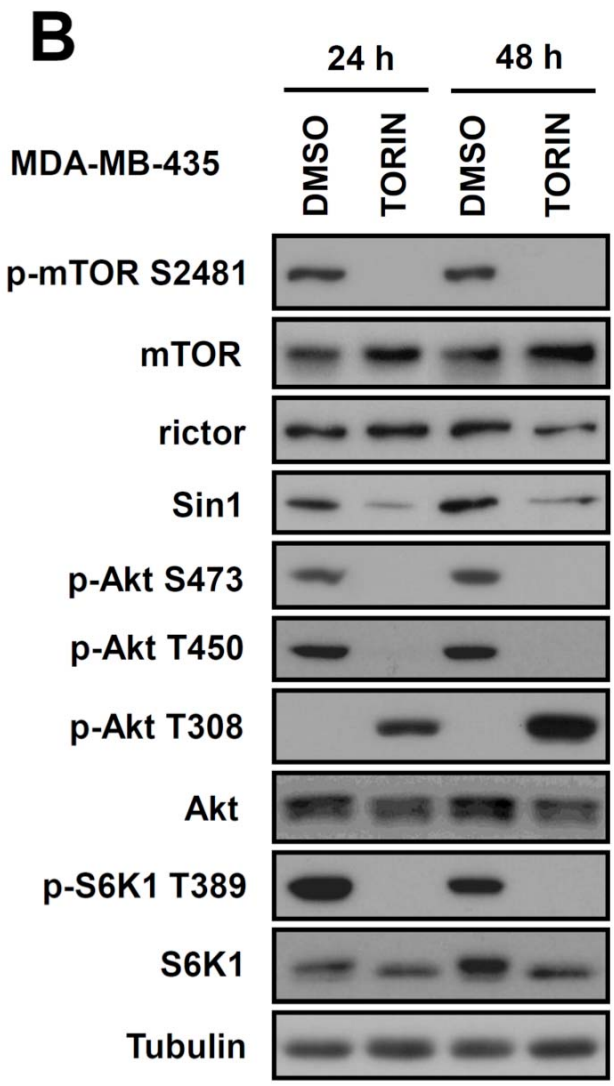

Figure 4-5. Endogenous SIN1 expression is dependent on mTOR kinase activity.

(B) mTOR kinase activity controls abundance of SIN1. MDA-MB-435 cells were incubated with specific mTOR inhibitor (0.5 μM TORIN) for 24 or 48 h. (This figure was adopted from our previously published work in *Journal of Biological Chemistry* Vol. 286, No. 46, 40386-40394, 2011; the permission was received from the journal.)

PDK1-dependent Akt phosphorylation at Thr-308 has been stimulated by the mTOR kinase inactivation, which is consistent to the previous report (Peterson et al., 2009). This outcome is likely due to the compensatory effect caused by the mTORC2-dependent inhibition of Akt. Notably, the prolonged mTOR inhibition by TORIN led to a considerable reduction of the endogenous SIN1 protein level (Figure 4-5B). Therefore, our results demonstrate that mTOR and its kinase activity is required for protein expression of SIN1 within either endogenous or reconstituted mTORC2.

4.6. SIN1 protein abundance and mTORC2 integrity is regulated by the amount of cellular glucose

Previous research suggested that nutrient signals positively regulate mTOR activity. In the absence of the amino acids in cells, it showed strong reduction of the mTORC1 activity. Moreover, AMPK negatively regulates mTORC1/S6K activity under energy deprivation. However, how mTORC2 responds to the nutrient or energy depletion is still unclear. According to our study of the mTORC2 reconstitution, the mTOR kinase activity is required for maintaining SIN1 protein stability and integrity of mTORC2. To identify the physiological conditions that cause mTOR dysfunction and SIN1 degradation, we studied the metabolic stress conditions in cells. Glucose is the essential energy source for cells to produce ATP; amino acids are the key elements for protein synthesis. MDA-MB-435 and HeLa cells were incubated in mediums without glucose or amino acids for 20 hours (Figure 4-6A).

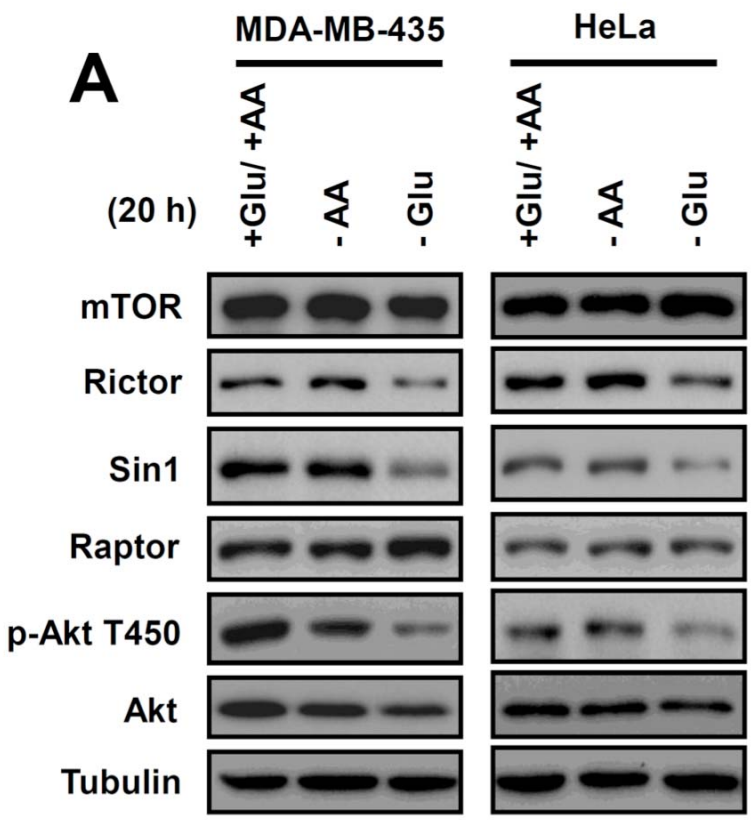

Figure 4-6. Glucose starvation, but not amino acid deprivation, causes rictor and SIN1 protein degradation and mTORC2 dissociation.

(A) Decreased SIN1 and rictor protein abundance under glucose starvation is associated with reduced basal mTORC2 activity toward Akt Thr-450. MDA-MB-435 and HeLa cells were incubated with glucose-free or amino acid-free mediums for 20 hours, respectively. Immunoblotting was used to detect phosphorylation of Akt at Thr-450 and the amount of the indicated proteins in the cell lysates.

Prolonged glucose starvation led to SIN1 degradation and inhibited phosphorylation of Akt at Thr-450, which is a well-characterized constitutive phosphorylation site of mTORC2 (Facchinetti et al., 2008; Ikenoue et al., 2008). Because rictor stability requires its binding to SIN1, we detected rictor degradation due to less SIN1 protein abundance after glucose depletion. In contrast, we did not have the similar observation from cells cultured in amino acid-free medium.

SIN1 is an essential component of mTORC2 and required for complex formation. By pulling down mTOR from cells under glucose starvation at the shorter time point (16 hours), we found that SIN1 and rictor did not bind mTOR efficiently (Figure 4-6B). Under this setting, the dissociation of raptor from mTOR was associated with the elevated AMPK-dependent raptor phosphorylation at Ser-792. It suggests that AMPK might play a role in the regulation of mTORC1 assembly. The above results indicated that integrity of mTORC2 and mTORC1 is sensitive to glucose depletion, but not amino acid starvation.

4.7. The mTOR Kinase functions as the ATP sensing component of mTORC2

Why glucose deprivation causes mTORC2 inactivation, dissociation and SIN1 degradation? Glucose is the essential source for generating ATP. We measured ATP level from cells under glucose starvation and found that cellular ATP ratio gradually decreased in the time-dependent manner (Figure 4-7A). Previous study showed that mTOR has lower ATP binding ability and is a

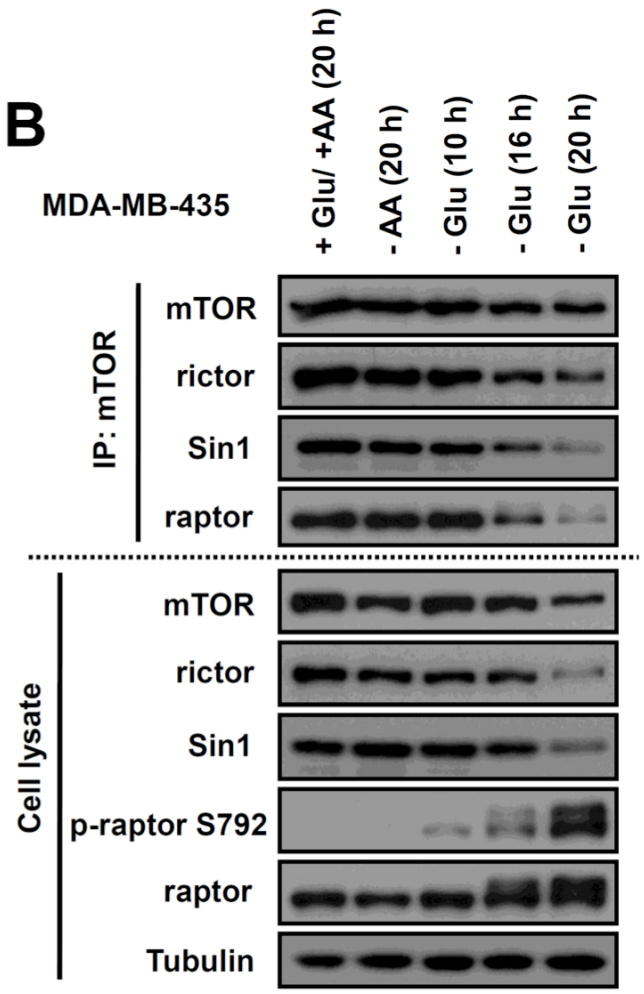

Figure 4-6. Glucose starvation, but not amino acid deprivation, causes rictor and SIN1 protein degradation and mTORC2 dissociation.

(B) Deficient complex integrity of the mTORC1 and mTORC2 is caused by glucose deprivation. MDA-MB-435 cells were incubated with glucose-free medium for 10, 16 and 20 hours or amino acid-free medium for 20 hours, respectively. The mTOR immunoprecipitates were prepared from cell lysates to examine the complex formation of the mTORC1 and mTORC2.

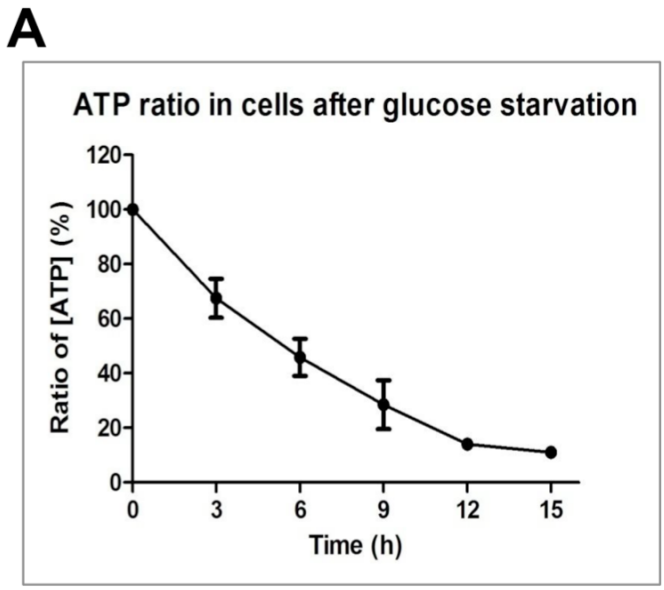

Figure 4-7. Cellular ATP ratio gradually decreases under glucose starvation condition with the time-dependent manner.

(A) MDA-MB-435 cells were incubated with glucose-free medium for 0, 3, 6, 9, 12 and 15 hours, and collected for quantitative detection of ATP by luciferase driven bioluminescence assay (Roche ATP Bioluminescence Assay Kit CLS II). Three independent experiments were shown for each condition.

homeostatic ATP sensor (Dennis et al., 2001). Thus, a cellular ATP level may directly affect mTOR kinase activity. To prove this, we immunopurified mTORC2 and analyzed the *in vitro* mTORC2 kinase activity toward Akt under different ATP concentrations (Figure 4-7B). We detected the highest kinase activity of mTORC2 at the concentration of ATP above the 1.2 mM range, whereas most protein kinases require about 0.1 mM ATP for full activation (Dennis et al., 2001). Using the same ATP titration conditions for mTORC1, we obtained similar results for Thr-389 phosphorylation of S6K1 by mTORC1 purified by raptor immunoprecipitation (Figure 4-7C). These findings support the role of mTOR kinase as the ATP sensor. We also interpret that mTORC2 kinase activity declined under glucose starvation is mediated by ATP depletion. Since SIN1 protein stability depends on its phosphorylation by mTOR, the mTORC2 kinase activity highly sensitive to the ATP depletion under glucose starvation led to SIN1 degradation and complex dissociation.

4.8. Phosphorylation of SIN1 at Ser-260 is dependent on the ATP-sensitive mTORC2 activity

To further characterize the mTOR-dependent regulation of SIN1, we attempted to identify SIN1 phosphorylation site. We applied the mTORC2 reconstitution system by co-expressing wild type mTOR or its kinase-dead form with other three essential components (Xp-rictor, V5-SIN1 and HA-mLST8). By pulling down V5-SIN1, the enriched samples from 10 immunoprecipitates were applied for SDS-PAGE and Commassie Blue staining (Figure 4-8A). Mass

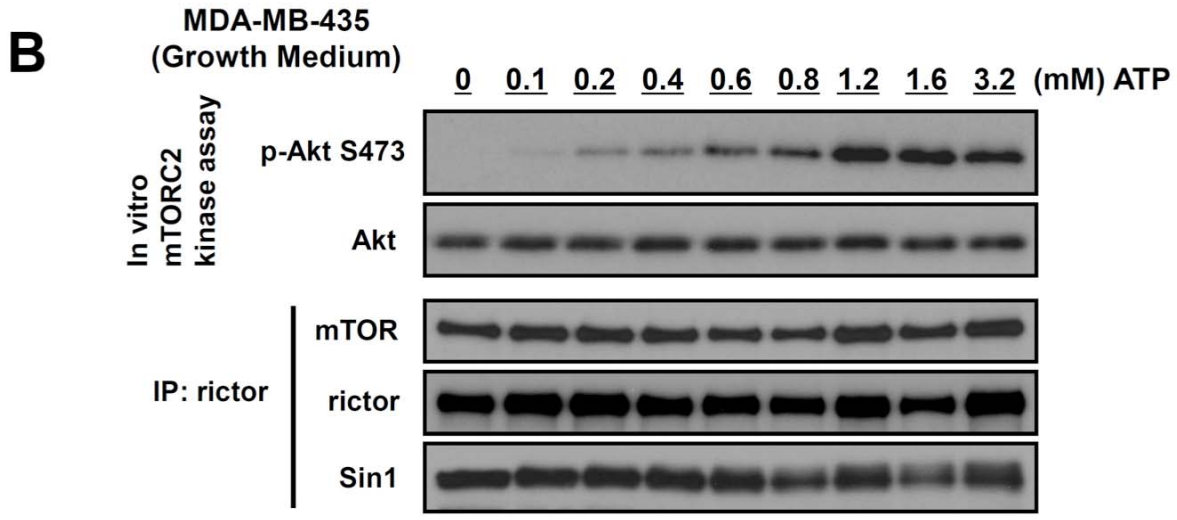

Figure 4-7. The activity of the mTOR kinase complexes is sensitive to ATP concentration.

(B) For mTORC2 kinase reaction, rictor immunoprecipitates prepared from lysates of MDA-MB-435 cells were used for *in vitro* kinase assays with full-length WT-Akt1 as the substrate and different amount of the ATP (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, and 3.2 mM, respectively).

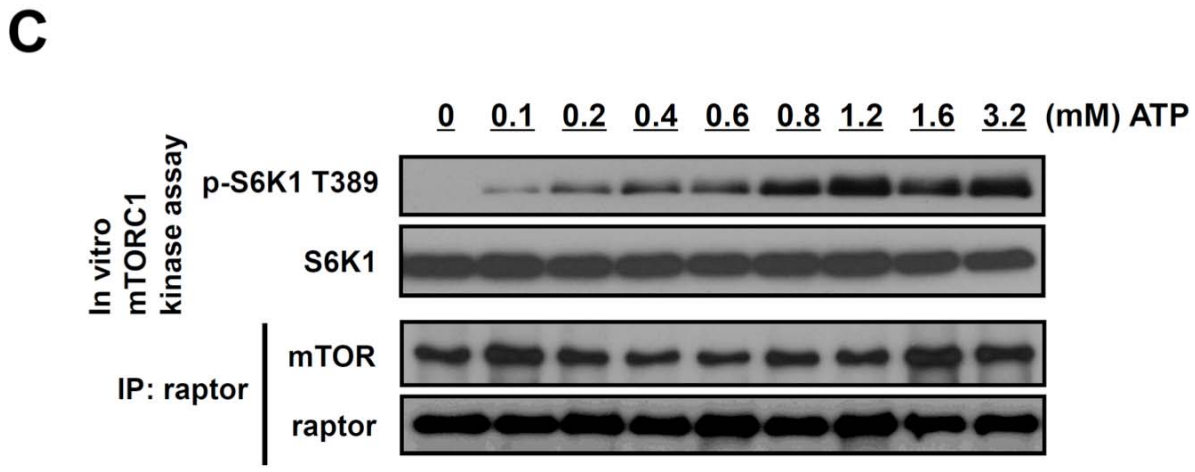

Figure 4-7. The activity of the mTOR kinase complexes is sensitive to ATP concentration.

(C) For mTORC1 kinase reaction, raptor immunoprecipitates prepared from lysates of MDA-MB-435 cells were used for *in vitro* kinase assays with full-length WT-S6K1 as the substrate and different amount of the ATP (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.6, and 3.2 mM, respectively). Both kinase complexes require at least 1.2 mM ATP for full kinase activity.

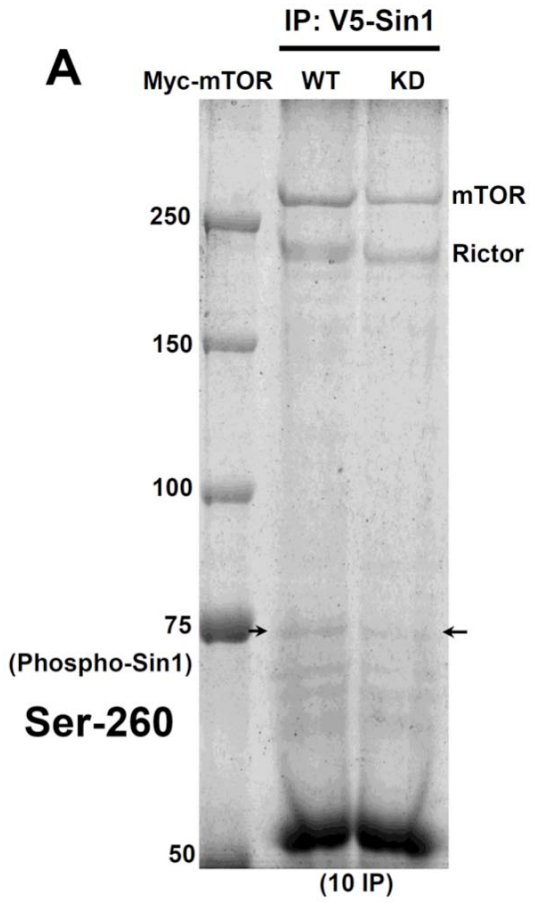

Figure 4-8. Identification of the mTOR-dependent SIN1 phosphorylation site.

(A) Xpress-rictor, SIN1-V5, and HA-mLST8 constructs were cotransfected with Myc-mTOR (wt) or Myc-mTOR (KD) in HEK-293T cells, respectively. Immunoprecipitates prepared from lysates of HEK-293T cells with anti-V5 antibody were resolved in SDS-PAGE and visualized by Coomassie blue staining. The potential SIN1 gel bands were excised and sent for Mass spectrometric analysis.

spectrometric analysis was then carried out to determine the potential SIN1 phosphorylation site. Based on the mass spectrometry readings of the distinct SIN1 peptides, Ser-260 has been verified with a high probability as the SIN1 phosphorylation site (Figure 4-8B).

To study the role of this SIN1 phosphorylation site, the anti-SIN1 (Ser-260) antibody has been generated. The specificity of this antibody was validated by examining its interaction to the wild type SIN1 or its phospho-mutant (S260A). The phospho-SIN1 (Ser-260) antibody did not recognize its mutated epitope and exhibited high specificity (Figure 4-8C). We applied this specific phospho-SIN1 (Ser-260) antibody to analyze the SIN1 phosphorylation in response to glucose deprivation. We observed that phosphorylation of SIN1 at Ser-260 decreased after glucose starvation for 16 hours, but not at amino acid deprivation condition (Figure 4-8D). In this setting, we also detected the disassembly of mTORC2 after 16 hours glucose depletion (Figure 4-6B). Thus, phosphorylation of SIN1 Ser-260 may play an important role for maintaining mTORC2 integrity.

We analyzed the SIN1 protein sequence and found that Ser-260 is located within the highly conserved region, which was designated as the Box1 (residues 225–267 of the human SIN1 sequence; Figure 4-8E). Box1 lays within the larger region of unknown function referred as the **C**onserved **R**egion **I**n the **M**iddle (CRIM), a highly conserved domain (residues 140–267). It suggests that the Ser-260 phosphorylation site within CRIM is critical for SIN1 stability. On the other hand, mTOR is known to prefer to phosphorylate serine

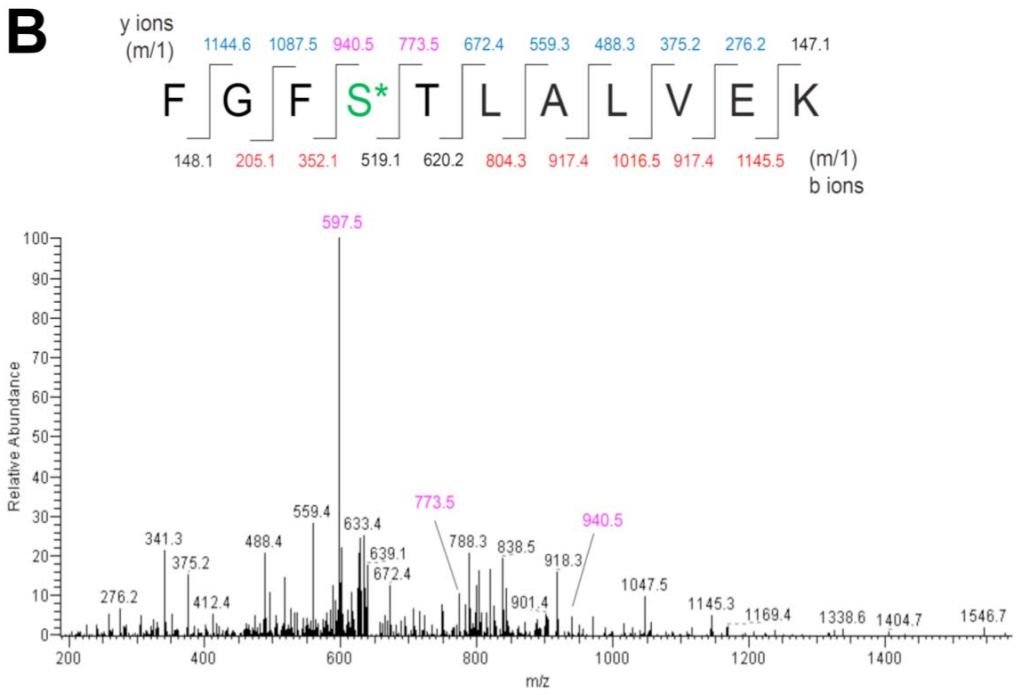

Figure 4-8. Identification of the mTOR-dependent SIN1 phosphorylation site

(B) Phosphorylation of Ser-260 in SIN1 identified by MS/MS. SDS-PAGE purification followed by enzymatic digestion and LC/MS/MS was used to isolate peptide containing phosphorylated serine residue in position 4 (S*). Expected masses for the y and b ions are listed above and below the peptide sequence. Ions that were positively identified are highlighted in blue and red. Y ions at 940.5 and 773.5 define the phospho-serine residue at position 4. The abundant ion at 597.5 is the neutral loss of 98 Da from the doubly charged parent ion.

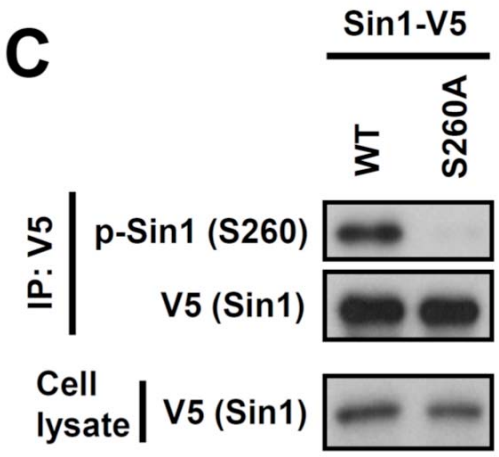

Figure 4-8.

(C) Cross validation of the p-SIN1 (Ser-260) antibody. The V5 tagged wild type or SIN1 Ser-260A phospho-mutant was expressed in HEK-293T cells and immunopurified using V5 antibody. The immunopurified wild type and phospho-mutant SIN1 proteins were analyzed by western blotting with the phospho-SIN1 (S260) and V5 antibodies.

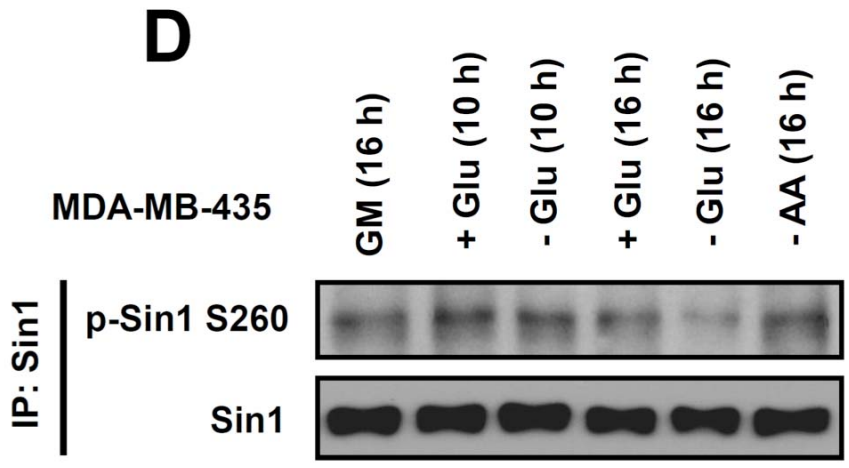

Figure 4-8.

(D) Glucose starvation results in dephosphorylation of SIN1 Ser-260 in MDA-MB-435 cells. MDA-MB-435 cells were incubated in plus or minus glucose medium for 10 and 16 hours. The cells were also incubated in 10 % serum medium or amino acid-free medium for 16 hours as controls. The SIN1 protein was immunopurified with anti-SIN1 antibody. The dephosphorylation of the endogenous SIN1 in MDA-MB-435 cells occurs after 16 hours of glucose starvation.

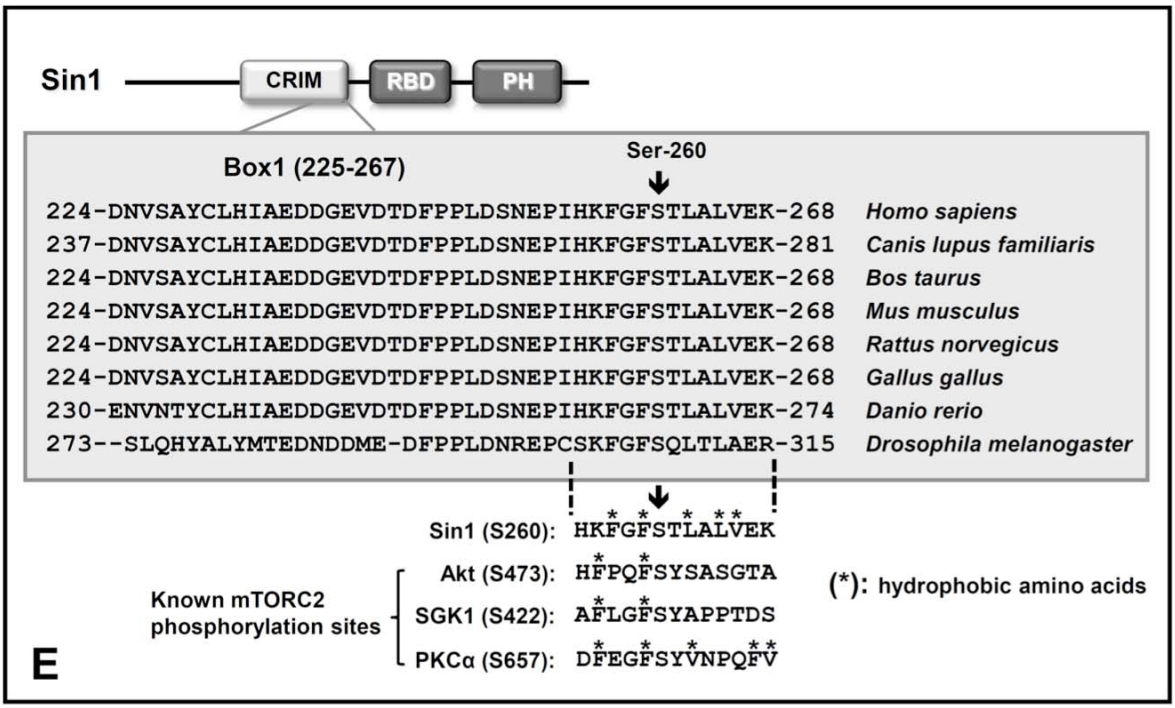

Figure 4-8.

(E) SIN1 protein domains: **CRIM**: conserved region in the middle; **RBD**: Raf-like Ras-binding domain; **PH**: pleckstrin homology. Box1 (amino acids 225-267) is the highly conserved region located within the CRIM domain. mTOR kinase-dependent SIN1 site (Ser-260) resides in the Box1, implying the significant role of this phosphorylation site. The Serine residue of SIN1 and the known mTORC2 substrates: Akt, SGK1 and PKCα are surrounded by hydrophobic amino acids.

***** indicates the hydrophobic amino acids.

residues within the hydrophobic motif. For instance, the well-studied mTORC2 substrates: Akt Ser-473, SGK1 Ser-422 and PKCα Ser-657 are located in the regions containing hydrophobic amino acids. The SIN1 Ser-260 site also represents the hydrophobic motif suggesting that mTOR might be a potential kinase of this site (Figure 4-8E).

To prove that mTOR is the SIN1 Ser-260 kinase, we studied the SIN1 phosphorylation by the *in vitro* mTORC2 reconstitution as described in Figure 4-3B. After co-incubation of the FLAG-mLST8/myc-mTOR and myc-rictor/V5-SIN1, these two heterodimers have assembled into functional mTORC2. Also, the increased SIN1 Ser-260 phosphorylation after mTORC2 assembly indicated that SIN1 is the substrate of mTOR kinase (Figure 4-8F).

4.9. The SIN1 Ser-260 site is ATP dependent and regulates SIN1 degradation and mTORC2 assembly

We have shown that the ATP depletion by the prolonged glucose starvation led to SIN1 dephosphorylation and its degradation. To test other physiological conditions that cause ATP depletion, we treated the MDA-MB-435 cells with 5 mM 2-DG and 10 μM rotenone in the time-dependent manner and found that SIN1 degradation occurred after 2 hours of drugs treatment (Figure 4-9A). We applied the lysates from cells with 1 hour drugs treatment for SIN1 immunoprecipitation and observed the decreased SIN1 Ser-260 phosphorylation and the disassembly of the mTORC2 (Figure 4-9B). In this condition, the treatment of 2-DG and rotenone led to acute ATP depletion (White et al., 2002)

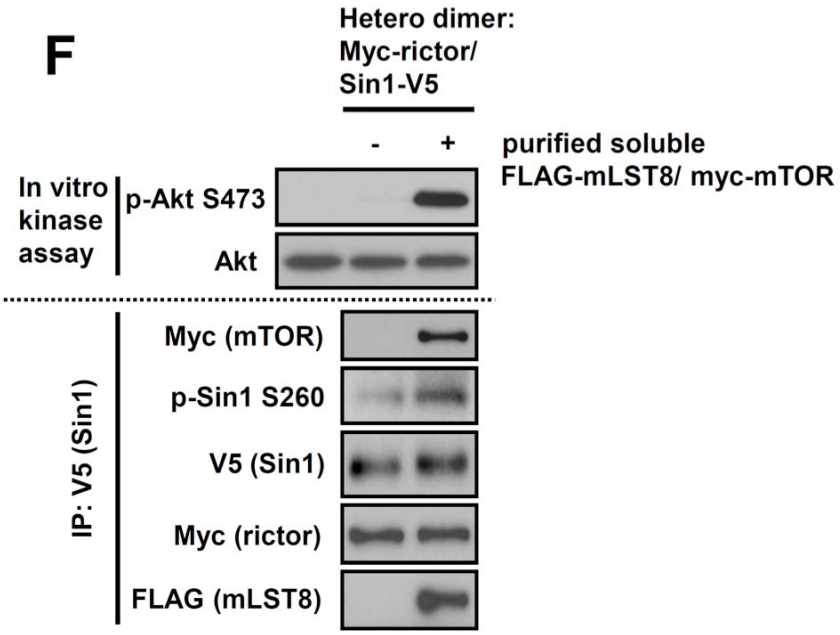

Figure 4-8. mTOR is the kinase for SIN1 Ser-260 phosphorylation.

(F) *In vitro* mTORC2 assembly was performed by incubation of two heterodimers, rictor/Sin1 and mTOR/mLST8 as mentioned in Figure 4-3B. The result showed the *in vitro* assembly of two heterodimers (rictor/Sin1 and mTOR/mLST8) and direct phosphorylation of SIN1 (slower migratory form and detection of the phospho-SIN1 S260 signal) by mTOR. The *in vitro* assembled mTORC2 is a functional complex that contains the kinase activity toward Akt and SIN1.

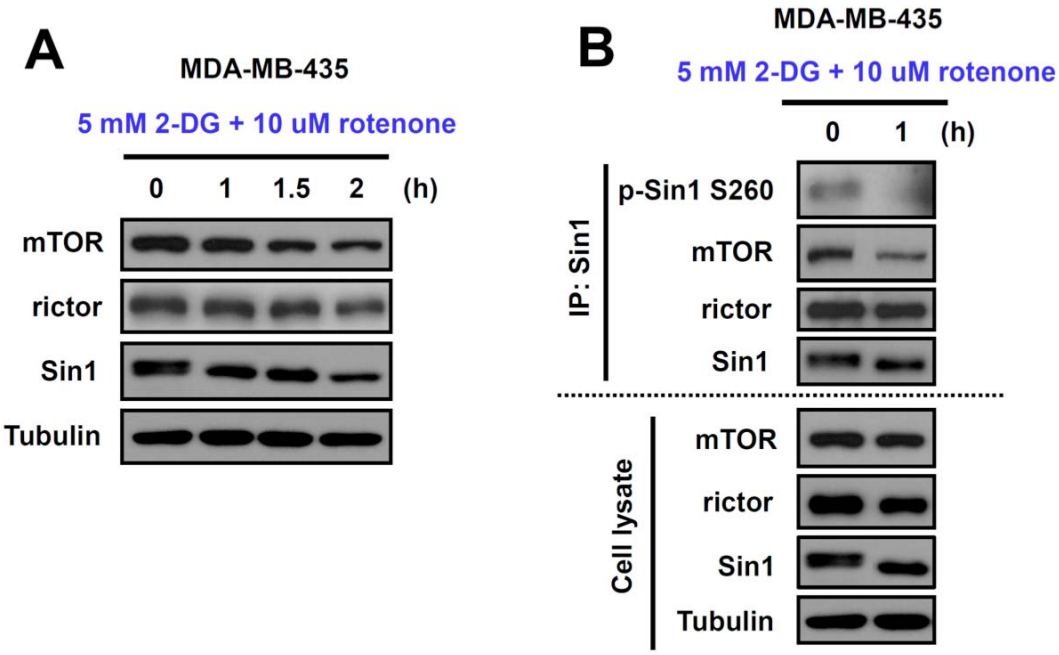

Figure 4-9. ATP depletion causes dephosphorylation of SIN1 Ser-260, mTORC2 complex dissociation, and SIN1 degradation.

(A) Acute ATP depletion leads to decreased SIN1 protein abundance. MDA-MB-435 cells were incubated with 5 mM 2-DG and 10 μM rotenone in glucose-free medium for 1, 1.5 and 2 hours.

(B) Acute ATP depletion leads to dephosphorylation of SIN1 Ser-260 and mTORC2 complex dissociation. MDA-MB-435 cells were incubated with 5 mM 2-DG and 10 μM rotenone in glucose-free medium for 1 hour. The SIN1 immunoprecipitates were prepared from cell lysates to examine the complex formation of the mTORC2.

and similar effects on mTORC2 that is caused by prolonged glucose starvation. These results indicate that the acute ATP-depletion is sufficient to cause dephosphorylation of SIN1 on Ser-260, SIN1 degradation, and deficiency of the mTORC2 integrity.

4.10. The mTOR-dependent phosphorylation of SIN1 at Ser-260 regulates SIN1 protein stability and maintains mTORC2 integrity

We hypothesized if Ser-260 in SIN1 is the main regulatory residue in the mTORC2 complex, mutation of this residue will alter SIN1 protein stability and mTORC2 integrity. We generated the SIN1 phospho-mutants by substituting the Ser-260 to alanine (S260A that prevents phosphorylation) or aspartic acid (S260D that mimics phosphorylation), and applied the mutant constructs to the mTORC2 reconstitution system. In the cotransfection with mTOR-WT, the SIN1 (S260A) mutant showed less protein abundance compared to wild-type SIN1 and S260D mutant (Figure 4-10A). In the co-expression with mTOR-KD, wild-type SIN1 that failed to be phosphorylated by functional mTOR kinase displayed low protein abundance, similar to the S260A mutant. However, S260D mutant of SIN1 that mimics the mTOR-dependent phosphorylation remained insensitive to the mTOR kinase activity (Figure 4-10A).

To further analyze mTORC2 assembly, we optimized SIN1 expression by co-expressing distinct amount of the cDNAs encoding wild-type SIN1 and its phospho-mutants (Figure 4-10B). To optimize expression of the unstable S260A mutant, we co-transfected with mTOR-WT only 30 % amount of the cDNAs

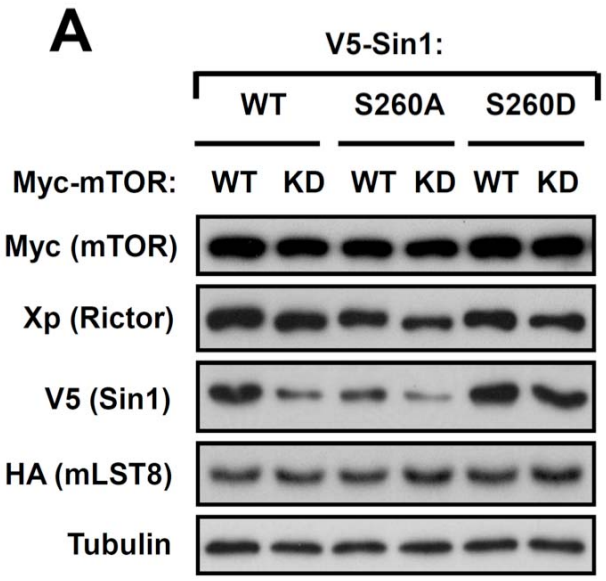

Figure 4-10. SIN1 Ser-260 phosphorylation is required for its protein stability.

(A) The V5 tagged wild type or phospho-specific SIN1 recombinant proteins (S260D and S260A) were co-expressed with myc-mTOR (wt) or myc-mTOR (KD), respectively, and another two mTORC2 components (Xp-rictor and HA-mLST8) in HEK-293T cells. The total cell lysates and immunoprecipitates purified using Myc antibody was analyzed by western blotting with the indicated tag antibodies.

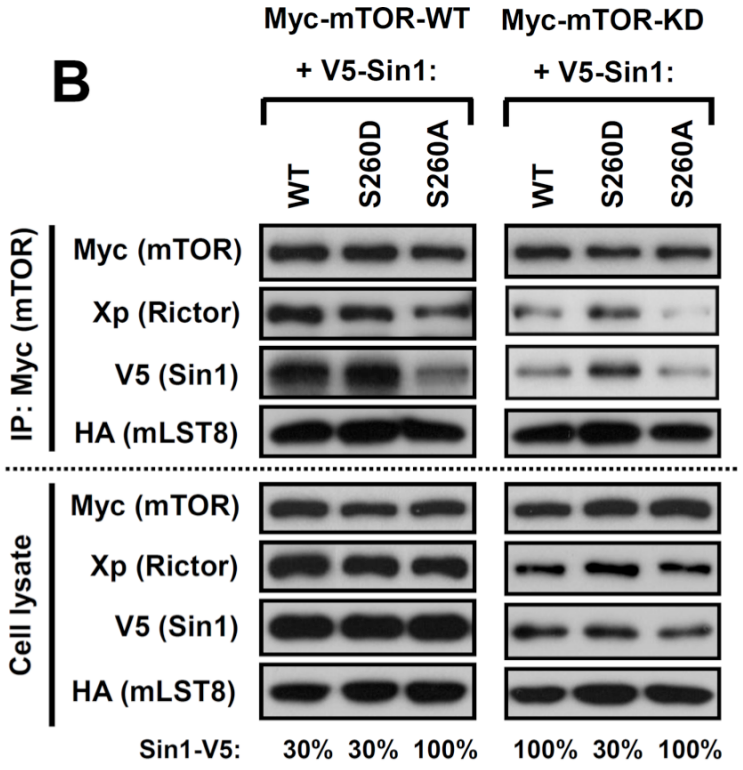

Figure 4-10. SIN1 Ser-260 phosphorylation is required for mTORC2 complex integrity

(B) The V5 tagged wild type or phospho-specific SIN1 recombinant proteins (S260D and S260A) were co-expressed with myc-mTOR (wt) or myc-mTOR (KD), respectively, and another two mTORC2 components (Xp-rictor and HA-mLST8) in HEK-293T cells. To optimize the SIN1 expression, the myc-mTOR-WT was co-transfected with 30 % of V5-SIN1-WT, 30 % of V5-SIN1-S260D or 100 % of V5-SIN1-S260A. The myc-mTOR-KD was co-transfected with 100 % of V5-SIN1-WT, 30 % of V5-SIN1-S260D or 100 % of V5-SIN1-S260A. The myc immunoprecipitates were prepared from cell lysates to examine the complex integrity of the mTORC2.

encoding wild-type SIN1 and S260D mutant. The immunoprecipitates with the comparable abundance of mTORC2 reconstituted with wild-type SIN1 or its phospho-mutants showed that SIN1 S260A mutant was not effective to form mTORC2. This suggested that phosphorylation of SIN1 at Ser-260 is required to retain complex integrity. In the cotransfection with mTOR-KD, only 30 % of the cDNA encoding SIN1 S260D mutant was co-expressed. Under this setting, wild-type SIN1 that was unable to be phosphorylated by functional mTOR kinase was incapable to assemble mTORC2, similar to the SIN1 S260A mutant (Figure 4-10B). In contrast, the SIN1 S260D mutant that mimics phosphorylation could prevent complex dissociation and still remained in the complex with the kinase dead mTOR. Collectively, the mTOR kinase-dependent phosphorylation of SIN1 at Ser-260 preserved the complex integrity of mTORC2 by stabilizing SIN1.

4.11. The mTOR-dependent phosphorylation of SIN1 at Ser-260 regulates cell proliferation

To further examine the biological impact of the mTOR-mediated SIN1 phosphorylation at Ser-260, we generated the stable MEFs by restoring similar amounts of SIN1-WT and its phospho-mutants into SIN1-deficient MEFs. We detected a higher basal Akt Ser-473 phosphorylation in SIN1 S260D mutant-restored stable MEF compared to those restored with SIN1-WT or S260A mutant (Figure 4-11A). We analyzed the complex integrity of mTORC2 in these stable MEFs by SIN1 immunoprecipitation and found that SIN1 S260D mutant is more effective in assembling mTORC2 compared to the wild-type SIN1

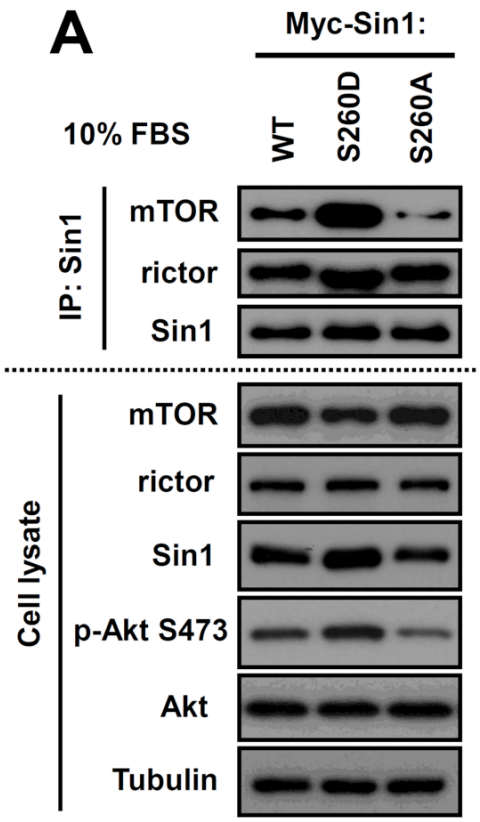

Figure 4-11.

(A) Stable expression of the SIN1 Ser-260D phospho-mutant showed high basal phosphorylation of Akt at Ser-473 and more mTORC2 complex. SIN1-null MEFs stably expressing WT-SIN1 or the phospho-mutants and grown in 10 % serum-containing medium. SIN1 immunoprecipitates from stable MEFs were used to examine the complex integrity of the mTORC2.

or its S260A mutant (Figure 4-11A). This supports our previous results that phosphorylation of SIN1 at Ser-260 preserved mTORC2 integrity and this complex carried a higher activity.

Since Akt controls cell proliferation, we hypothesized that SIN1 S260D mutation might more robustly promote cell proliferation compared to SIN1-WT or S260A mutant. To test this hypothesis, we examined the proliferation rate of the SIN1-restored stable MEFs after incubated in 10 % FBS-containing medium for 48 and 72 hours (Figure 4-11B). First, the proliferation rate of the SIN1-deficient MEFs increased by restoration of SIN1-WT (~1.6 times relative to unreconstituted SIN1-deficient MEFs). Restoration of the SIN1 S260D mutant had more potent effects on cell proliferation compared to that of SIN1-WT (~1.9 times compared to unreconstituted SIN1-deficient MEFs and 40 % higher than SIN1-WT-restored MEFs). The MEFs expressing the SIN1 S260A mutant did not proliferate to the same degree as those expressing either WT or S260D mutant (~1.3 times relative to unreconstituted SIN1-deficient MEFs and 60 % less than MEFs restored with SIN1-WT). These results are coherent with the effects of these mutants on mTORC2 signaling (Figure 4-11A). Those MEFs restored with SIN1 S260D mutant proliferated at the three times higher rate than those reconstituted with S260A mutant. It indicates that regulation of this site is critical for the mTORC2-dependent cell proliferation.

Because mTORC2 kinase activity is sensitive to cellular glucose levels, we studied effects of high (5.5 mM) and low (0.55 mM) glucose concentration on these SIN1 stable MEFs by analyzing cell proliferation. After incubation at 5.5

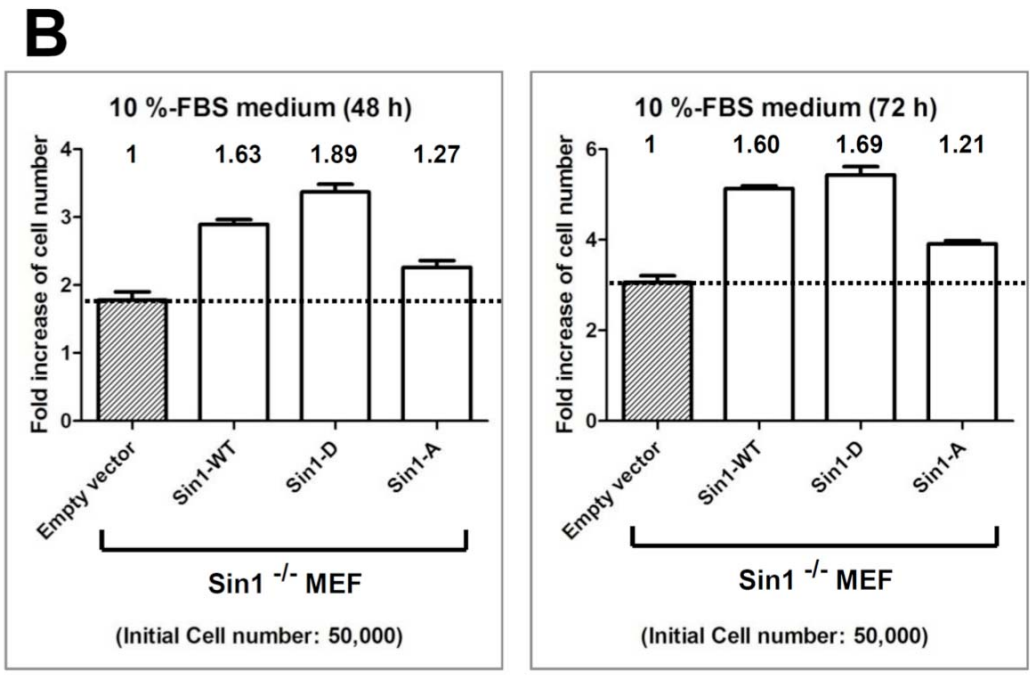

Figure 4-11.

(B) Phosphorylation of SIN1 at Ser-260 up-regulates cell proliferation. WT-SIN1 or the phospho-mutants were reintroduced into SIN1-null MEFs with the retroviral expression system. Cell proliferation measurements were performed by counting cells 48 and 72 hours after incubation in 10 %-FBS containing medium. The ratio of proliferation rate was graphed with GraphPad Prism 5 software.

mM glucose for 48 hours, we observed a higher basal Akt Ser-473 phosphorylation in the wild-type SIN1 and S260D mutant-expressing stable MEFs compared to those expressing the S260A mutant (Figure 4-11C). The Akt signaling activity is consistent with the higher proliferation rate of MEFs reconstituted with the wild-type SIN1 or S260D mutant (Figure 4-11D).

When the stable MEFs were incubated at 0.55 mM glucose for 48 hours, we detected a stronger Akt Ser-473 phosphorylation only in MEFs expressing the SIN1 S260D mutant that exhibited the higher SIN1 protein abundance (Figure 4-11C). The SIN1 S260D expressing MEFs displayed a higher proliferation rate compared to those reconstituted with the wild-type SIN1 and S260A mutant as shown in. Figure 4-11D. These results indicate that phosphorylation of SIN1 at Ser-260 maintains mTORC2 integrity and regulates cell proliferation in high or low glucose cell culture conditions.

4.12. mTOR-dependent phosphorylation of SIN1 at Ser-260 regulates tumor growth

To address a role of SIN1 phosphorylation *in vivo*, we studied the effects of SIN1 Ser-260 phosphorylation on tumor growth. To develop tumor growth, we transformed MEFs restored by WT or mutant SIN1 by overexpressing equal amount of H-Ras (Figure 4-12A). Mice injected with the wild-type SIN1 and S260D mutant-reconstituted MEFs had bigger tumors than those injected with MEFs restored with SIN1 S260A mutant (Figure 4-12A and B). These results show that mTOR kinase-dependent SIN1

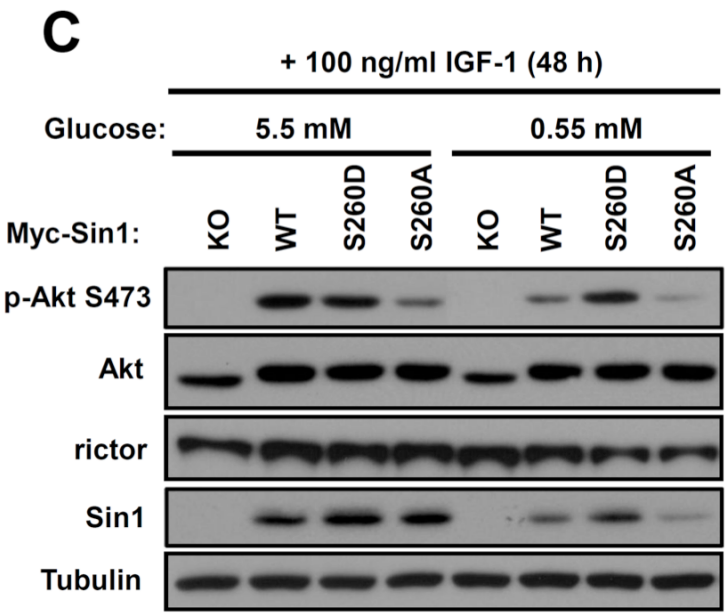

Figure 4-11.

(C) SIN1-null MEFs stably expressing SIN1 Ser-260D phospho-mutant showed high basal phosphorylation of Akt at Ser-473 in 5.5 mM or 0.55 mM glucose containing-mediums with the addition of 100 ng/ml IGF-1 for 48 hours. Immunoblotting was used to detect the indicated proteins in cell lysates.

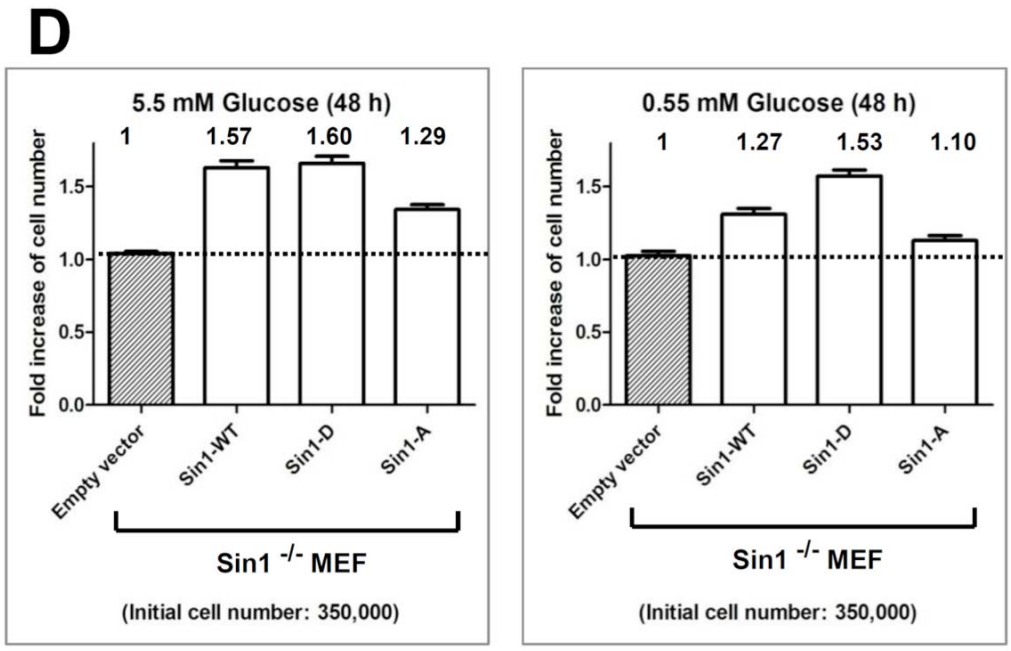

Figure 4-11.

(D) SIN1-null MEFs stably expressing SIN1 Ser-260D phospho-mutant exhibited higher proliferation rate in physiological and low glucose conditions. Cell proliferation measurements were performed by counting cells 48 hours after incubation in 5.5 mM or 0.55 mM glucose-containing mediums with the addition of 100 ng/ml IGF-1 for 48 hours. The ratio of proliferation rate was graphed with GraphPad Prism 5 software.

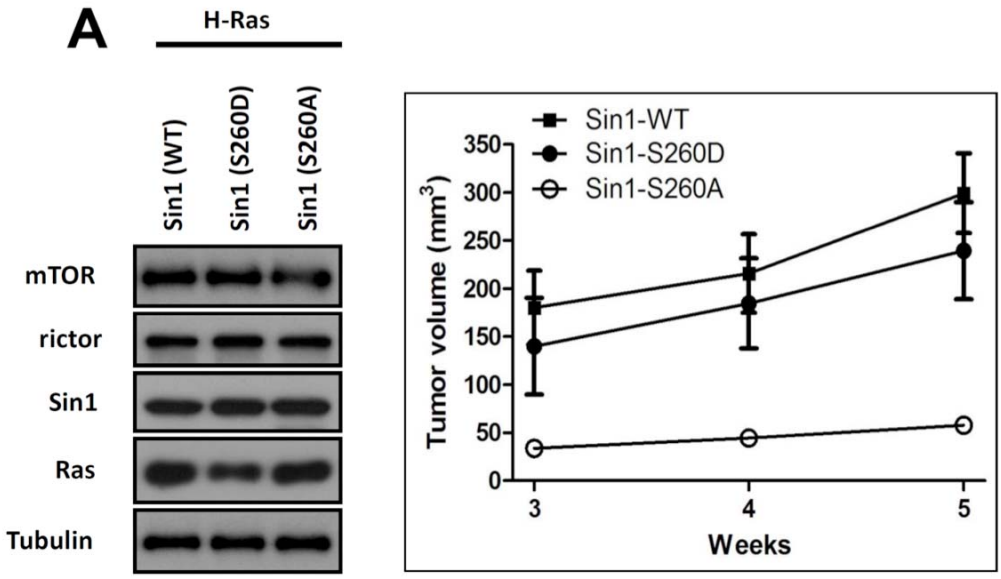

Figure 4-12. Blocking Ser-260 phosphorylation of SIN1 inhibits tumor growth.

(A) MEFs described in Figure 4-11A were transformed by overexpression of the oncogenic form of H-Ras and injected into 6-week-old immunodeficient nude mice (n = 5 for each group; 3×10^6 cells per mouse). Tumor size was measured after 3, 4 and 5 weeks; the tumor sizes were calculated and the volumes were presented by line graph.

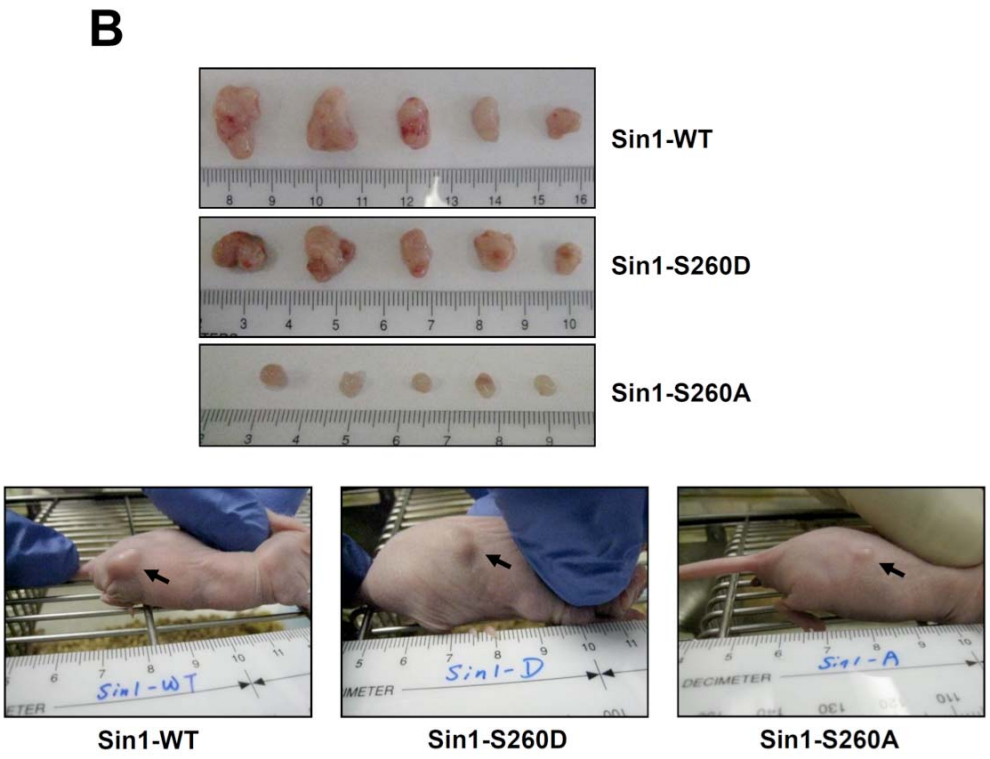

Figure 4-12. Blocking Ser-260 phosphorylation of SIN1 inhibits tumor growth.

(B) Mice injected with MEFs expressing each form of SIN1 were sacrificed and representative images of the excised tumors are shown.

phosphorylation at Ser-260 is pivotal in the regulation of cell proliferation and tumor growth.

4.13. Discussion

Our study demonstrates that the co-transfection of the essential components of mTORC2 (mTOR, mLST8, SIN1 and rictor) is able to assemble the functional complex with the growth factor-dependent kinase activity. We employed the reconstituted mTORC2 to study the role of the mTOR kinase domain within mTORC2 to execute Akt phosphorylation at Ser-473. The original intention was to eliminate the possibility that unknown kinase co-purified with mTORC2 may serve as the Akt Ser-473 kinase. As we expected, the substitution of the mTOR-WT with its kinase-dead form prevented mTORC2 kinase activity toward Akt. Our data distinctly demonstrates that mTOR kinase within the complex of mTORC2 is necessary for Akt phosphorylation at the regulatory Ser-473 site. This excludes a possibility of any other kinase that may associate with mTORC2 and phosphorylate Akt.

The analysis of the mTORC2 reconstitution system also results in the interesting finding implying the essential role of mTOR kinase in controlling mTORC2 integrity. Moreover, the mTOR kinase activity is required to preserve the protein stability of SIN1 by protecting it from lysosomal degradation. The reconstitution of mTORC2 with the mTOR-KD isoform leads to a rapid SIN1 protein turnover dependent on SIN1 phosphorylation as observed by the phosphatase-mediated SIN1 protein migration in the gel. The mTOR-mediated

SIN1 phosphorylation does not occur when only SIN1 and rictor are co-transfected in cells and form a heterodimer. Importantly, the mTOR-dependent SIN1 phosphorylation happens only after reconstitution of the functional mTORC2. It clarifies a specific role of the mTOR kinase on SIN1 phosphorylation. Most likely, SIN1 within mTORC2 complex is constitutively phosphorylated by mTOR kinase and this post-translational modification prevents the accelerated SIN1 protein degradation. Moreover, the mTOR-mediated SIN1 phosphorylation is independent on growth factor signaling since we do not observe mTORC2 dissociation or SIN1 degradation in cells under serum starvation. Thus, a basal mTOR kinase activity in this complex is sufficient to sustain SIN1 phosphorylation.

In addition to growth factors, mTOR activation is nutrient dependent (such as glucose and amino acids) (Guertin and Sabatini, 2007). To identify the physiological conditions that affect mTOR-dependent SIN1 phosphorylation, we provided nutrients-depletion conditions by incubation of cells in glucose-free or amino acids-free cell culture conditions. We found that only glucose starvation caused SIN1 degradation and mTORC2 disassembly. Glucose is the essential energy source for cells to produce ATP. Therefore, the glucose starvation-mediated ATP depletion may suppress mTOR function. Indeed, we confirmed that mTOR kinase that either exists in mTORC1 or mTORC2 requires at least 1.2 mM ATP for its full activity indicating its low ATP binding capacity. In contrast, other common kinases need only 0.1 mM ATP for their activity (Dennis et al., 2001). It emphasizes that mTOR kinase activity is highly sensitive to the low cellular ATP concentration associated with metabolic stress. These results

explained why glucose starvation-dependent ATP deprivation led to mTOR inactivation and compromised SIN1 phosphorylation causing a potent negative impact on the mTORC2 integrity.

Our biochemical study of SIN1 phosphorylation identified the potential SIN1 phosphorylation site by mTOR as Ser-260. This site carries two important features. First, SIN1 possesses a highly conserved region at Box1 of the Conserved Region in the Middle (CRIM) domain that is homologous from human to drosophila. The Ser-260 site locates within the evolutionally conserved Box1 and may play a significant role for SIN1 function. Second, the Ser-260 site locates within the hydrophobic motif of SIN1. Previous studies showed that mTOR kinase phosphorylates the serine residue within the hydrophobic motifs, identified in its known substrates: Ser-473 of Akt, Ser-422 of SGK1 and Ser-657 of PKCα (Alessi et al., 2009). By performing the *in vitro* assembly of mTORC2, we found that SIN1 as the component of mTORC2 is the substrate of mTOR. On the other hand, we developed a specific phospho-antibody against Ser-260 phosphorylation of SIN1 and showed that phosphorylation of SIN1 at Ser-260 reduced upon mTOR inactivation that was caused by ATP depletion. Thus we identified that mTOR phosphorylates SIN1 on the Ser-260 site.

Our work showed the significance of SIN1 phosphorylation at Ser-260 by expressing its phospho-mutants (S260D and S260A) in SIN1 null cells. The SIN1 S260D mutant that mimics phosphorylation could preserve the complex integrity by preventing SIN1 protein degradation. Importantly, a substitution by the wild type SIN1 to its S260D phospho-mutant in MEFs displayed a higher mTORC2

activity, cell proliferation and tumor growth rates. Our results point out an auto-regulatory mechanism of mTORC2 by constitutive phosphorylation of SIN1 at Ser-260 by mTOR (Figure 4-13). The physiological ATP concentration (1~5 mM) is sufficient to maintain basal mTOR kinase activity. Once cells encounter a metabolic stress causing a dramatic decrease of ATP level, the ATP-sensitive mTOR kinase fails to sustain SIN1 phosphorylation causing SIN1 degradation and mTORC2 dissociation. Therefore, the auto-regulatory mechanism temporarily shut down the ATP-consuming cell proliferation machinery and anabolic processes by disrupting mTORC2 under metabolic stress.

Phosphorylation of SIN1 at Ser-260 prevents its protein degradation and preserves the complex integrity of mTORC2 that further triggers the Akt-dependent anabolic processes and inducing cell proliferation. Thus, our findings provide a novel insight how the homeostatic ATP sensor regulates the mTORC2 integrity by SIN1 phosphorylation on Ser-260.

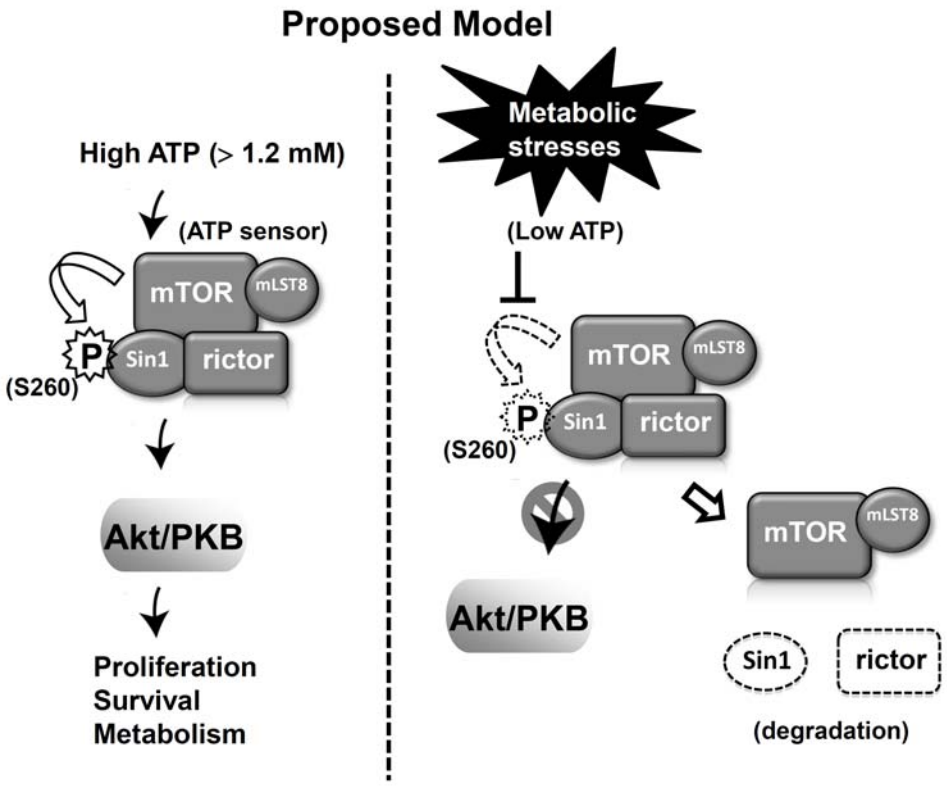

Figure 4-13. ATP-sensitive mTOR kinase-mediated phosphorylation of SIN1 at Ser-260 is required to preserve complex integrity of mTORC2 by preventing SIN1 degradation

The mTOR kinase has lower ATP binding affinity and requires sufficient cellular ATP concentration. The physiological ATP concentration is 1~5 mM that is enough to maintain the full mTOR kinase activity. In addition, the mTOR kinase activity is required for SIN1 phosphorylation at Ser-260, which is critical for maintaining complex integrity and SIN1 stability. When cells encounter metabolic stresses that decline cellular ATP level, deficient mTOR kinase fails to maintain the phosphorylation of SIN1 at Ser-260, which results in mTORC2 complex dissociation and SIN1 degradation.

Chapter 5

Future Directions

Our findings identify two distinct regulatory mechanisms of the anabolic signaling complex mTORC2 under ER stress and metabolic stress. In Chapter 3, our study provides a novel insight in the regulation of mTORC2 under ER stress that defines the overlooked balance between two kinases, Akt and GSK-3β. Initially, GSK-3β has been identified as a substrate of Akt. In our study, we showed that ER stress-inducible GSK-3β becomes the stress inducible upstream regulator of the mTORC2-Akt signaling. The phosphorylation of rictor on Ser-1235 by GSK-3β impairs the substrate binding of mTORC2 and inhibits its kinase activity.

Under ER stress, the GSK-3β kinase is activated as detected by the phosphorylation of its substrate glycogen synthase (Ser-641) and auto-phosphorylation site (Tyr-216). However, how ER stress regulates the activity of GSK-3β remains unknown. To identify the regulatory components of GSK-3β, we will apply several biochemical approaches:

(1) Immunoprecipitation: we will immunopurify GSK-3β from cells either under ER stress or with treatment of GSK-3β inhibitor, and then subject the samples to silver staining analysis. Following identification of the potential regulators by Mass Spectrometry analysis, we will study the impacts of the novel GSK-3β regulators on the activity of GSK-3β by expression of the recombinant proteins and specific shRNAs.

(2) Size exclusion chromatography: the biochemical purification of the GSK-3β regulators is the most challenging and difficult task. We have preliminary data based on the enrichment of the GSK-3β through size exclusion chromatography.

GSK-3β is located in the fractions of the molecular weight more than 60 kDa while the molecular weight of GSK-3β monomer is 46 kDa. This finding indicates that regulator might associate with GSK-3β in a complex. In this co-purification study the different types of detergents and chemical cross-linkers will be applied to stabilize the GSK-3β complex.

As an alternative approach to enrich the regulators we will perform the differential centrifugation prior to size exclusion chromatography. Differential centrifugation is a useful technique to separate the organelles from a homogenous solution of particles based on their densities. In this case, we can pre-isolate the cell fractions containing the GSK-3β effectors before the next-step purification. Moreover, we will conduct the ionic exchange chromatography to further purify and enrich the effectors. When we obtain the relatively pure fraction, we will perform mass spectrometric analysis to identify the specific GSK-3β regulators.

The biological relevance of mTORC2 regulation by ER stress is an important part of our future studies. ER stress is highly associated with metabolic diseases including type II diabetes. The initial stage of the type II diabetes is characterized by insulin resistance-- the peripheral tissues (muscle, fat and liver) fail to respond to insulin, leading to deficient glucose uptake. Previous mouse genetic models also linked GSK-3β with insulin resistance. GSK-3β is linked to insulin signaling by phosphorylating glycogen synthase known to lead to inhibition of glycogen synthesis and decrease of the cellular sensitivity to insulin. In our study, the negative regulation of Akt by GSK-3β may represent another

mechanism that contributes to insulin resistance.

To study the effects of rictor Ser-1235 phosphorylation on Akt-dependent glucose metabolism, we are in a process of generating rictor “phospho-mutants-knock-in” mice and analyze their phenotypes. It becomes clear that obesity causes ER stress and contributes to the development of type II diabetes. We will feed the rictor “phospho-mutants-knock-in” mice with high-fat diet (HFD) to induce obesity and ER stress. Then, we will conduct glucose tolerance tests (GTT) and insulin tolerance tests (ITT) in these mice to examine their systemic insulin sensitivity and glucose homeostasis.

To follow up the studies of the mouse phenotype, we plan to apply MEFs from the rictor “phospho-mutants-knock-in” mouse embryos, and study the impact of GSK-3β-mediated rictor phosphorylation on glucose uptake. To dissect the effects of GSK-3β on glucose uptake, we will first treat the MEFs with ER stress inducers (thapsigargin or tunicamycin) to activate GSK-3β. Subsequently, we will apply 2-NBDG (a new fluorescent derivative of glucose) and measure the rates of glucose uptake. Alternatively, we will test other insulin-sensitive tissues, such as skeletal muscle, adipose or liver tissues, to observe more significant glucose uptake.

Our studies also define the ATP-dependent auto-regulation of mTORC2. In Chapter 4, our results indicate that mTOR is a highly sensitive kinase to the cellular ATP level under metabolic stress induced by glucose deprivation or acute ATP depletion. We found that mTOR is the Ser-260 kinase of SIN1. Without

phosphorylation at Ser-260 by functional mTOR kinase, SIN1 remains unstable and undergoes lysosomal degradation. Our data indicate that SIN1 Ser-260 phosphorylation is required to maintain SIN1 stability and complex integrity. Does this phosphorylation event directly control mTORC2 assembly or indirectly preserve complex integrity by simply protecting SIN1 from lysosomal degradation?

To test the first possibility, we will apply SIN1-WT and its phospho-mutants to the *in vitro* mTORC2 reconstitution system (Chen and Sarbassov dos, 2011). If the SIN1 mutants do not affect the complex assembly, then we will examine the second possibility. To check if SIN1 phosphorylation indirectly maintains mTORC2 integrity, we will treat the SIN1-WT, S260D, and S260A-restored MEFs with the lysosomal inhibitor (chloroquine), and analyze the complex by SIN1 immunoprecipitation. If the inhibition of SIN1 degradation can enrich the complex level of mTORC2 containing SIN1-S260A mutant, then the second possibility is correct.

Moreover, how SIN1 phosphorylation prevents its lysosomal degradation is an intriguing question. Autophagy is a cellular catabolic process that integrates with lysosome for protein degradation. To study if autophagy is involved, we can knock down the critical components of the autophagic pathway (ATG5 and ATG7) using the shRNA lentiviral system. To further analyze SIN1 transportation to lysosomes for degradation, we will purify the lysosomal fractions from metabolic stressed cells treated with or without chloroquine, and determine SIN1 protein amount. This biochemical study will validate if SIN1 protein is directly

targeted to lysosome for degradation.

The SIN1 Ser-260 phospho-mutants will be highly valuable because the SIN1 S260D mutant is much more stable form and targeting of SIN1 for degradation is prevented by its Ser-260 phosphorylation. We propose that assembly of the SIN1 lysosomal targeting complex will be actively formed with the wild type or its S260A mutant but not with its phospho-mimetic S260D mutant. Based on this assumption, we will focus on identification of the proteins co-purified with the wild type SIN1 or S260A mutant. We will extend this study by characterizing the SIN1 complex in the lysosomal fractions that might lead to understanding of the selective lysosomal degradation pathway. The identified components of the SIN1 degradation complex will be validated by expression of the recombinant proteins and also specific shRNAs.

In our study, the ATP-sensing mechanism of mTORC2 explains how this anabolic signaling complex disintegrates under metabolic stress. Another stress responsive kinase, AMPK (AMP-activated protein kinase), is activated by low cellular ATP level. It is possible that AMPK may involve in the regulation of the mTORC2 under metabolic stress. To test this possibility, we will apply the AMPK inhibitor (Compound C) in cells under glucose starvation or acute ATP depletion conditions and study the mTORC2 activity, integrity, and SIN1 stability. Alternatively, we may employ the AMPK-null MEFs to see if AMPK contributes to mTORC2 regulation.

Overall, we identify two different regulatory mechanisms of mTORC2 by

the essential components rictor and SIN1. These two projects provide novel insights into the regulation of mTORC2 under cellular stress conditions. We will need the future studies to extend our knowledge in the field of the mTORC2-Akt signaling pathway.

Bibliography

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol *7*, 261-269.

Alessi, D.R., Pearce, L.R., and Garcia-Martinez, J.M. (2009). New insights into mTOR signaling: mTORC2 and beyond. Sci Signal *2*, pe27.

Bellacosa, A., Kumar, C.C., Di Cristofano, A., and Testa, J.R. (2005). Activation of AKT kinases in cancer: implications for therapeutic targeting. Adv Cancer Res *94*, 29-86.

Boulbes, D., Chen, C.H., Shaikenov, T., Agarwal, N.K., Peterson, T.R., Addona, T.A., Keshishian, H., Carr, S.A., Magnuson, M.A., Sabatini, D.M.*, et al.* (2010). Rictor phosphorylation on the Thr-1135 site does not require mammalian target of rapamycin complex 2. Mol Cancer Res *8*, 896-906.

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.

Cantley, L.C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.

Carrano, A.C., Eytan, E., Hershko, A., and Pagano, M. (1999). SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol *1*, 193-199.

Chen, C.H., and Sarbassov dos, D. (2011). The mTOR (mammalian target of rapamycin) kinase maintains integrity of mTOR complex 2. J Biol Chem *286*, 40386-40394.

Chen, C.H., Shaikenov, T., Peterson, T.R., Aimbetov, R., Bissenbaev, A.K., Lee, S.W., Wu, J., Lin, H.K., and Sarbassov dos, D. (2011). ER stress inhibits mTORC2 and Akt signaling through GSK-3beta-mediated phosphorylation of rictor. Sci Signal *4*, ra10.

Cohen, P., and Frame, S. (2001). The renaissance of GSK3. Nat Rev Mol Cell Biol *2*, 769-776.

Cohen, P., and Goedert, M. (2004). GSK3 inhibitors: development and therapeutic potential. Nat Rev Drug Discov *3*, 479-487.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785-789.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

del Peso, L., Gonzalez-Garcia, M., Page, C., Herrera, R., and Nunez, G. (1997). Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science *278*, 687-689.

Dennis, P.B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S.C., and Thomas, G. (2001). Mammalian TOR: a homeostatic ATP sensor. Science *294*, 1102-1105.

Dibble, C.C., Asara, J.M., and Manning, B.D. (2009). Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by S6K1. Mol Cell Biol *29*, 5657-5670.

Diehl, J.A., Cheng, M., Roussel, M.F., and Sherr, C.J. (1998). Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev *12*, 3499-3511.

Engelman, J.A., Luo, J., and Cantley, L.C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat Rev Genet *7*, 606-619.

Facchinetti, V., Ouyang, W., Wei, H., Soto, N., Lazorchak, A., Gould, C., Lowry, C., Newton, A.C., Mao, Y., Miao, R.Q.*, et al.* (2008). The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. EMBO J *27*, 1932-1943.

Fingar, D.C., and Blenis, J. (2004). Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene *23*, 3151-3171.

Fiol, C.J., Mahrenholz, A.M., Wang, Y., Roeske, R.W., and Roach, P.J. (1987). Formation of protein kinase recognition sites by covalent modification of the substrate. Molecular mechanism for the synergistic action of casein kinase II and glycogen synthase kinase 3. J Biol Chem *262*, 14042-14048.

Frame, S., and Cohen, P. (2001). GSK3 takes centre stage more than 20 years after its discovery. Biochem J *359*, 1-16.

Frias, M.A., Thoreen, C.C., Jaffe, J.D., Schroder, W., Sculley, T., Carr, S.A., and Sabatini, D.M. (2006). mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. Curr Biol *16*, 1865-1870.

Fruman, D.A., Meyers, R.E., and Cantley, L.C. (1998). Phosphoinositide kinases. Annu Rev Biochem *67*, 481-507.

Garcia-Martinez, J.M., and Alessi, D.R. (2008). mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem J *416*, 375-385.

Gingras, A.C., Raught, B., and Sonenberg, N. (2001). Regulation of translation initiation by FRAP/mTOR. Genes Dev *15*, 807-826.

Grimes, C.A., and Jope, R.S. (2001). The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. Prog Neurobiol *65*, 391-426.

Guertin, D.A., and Sabatini, D.M. (2007). Defining the role of mTOR in cancer. Cancer Cell *12*, 9-22.

Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J., Brown, M., Fitzgerald, K.J., and Sabatini, D.M. (2006). Ablation in mice of the

mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Dev Cell *11*, 859-871.

Harris, T.E., and Lawrence, J.C., Jr. (2003). TOR signaling. Sci STKE *2003*, re15.

Hoeflich, K.P., Luo, J., Rubie, E.A., Tsao, M.S., Jin, O., and Woodgett, J.R. (2000). Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature *406*, 86-90.

Holz, M.K., Ballif, B.A., Gygi, S.P., and Blenis, J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell *123*, 569-580.

Huang, W.C., Lin, Y.S., Chen, C.L., Wang, C.Y., Chiu, W.H., and Lin, C.F. (2009). Glycogen synthase kinase-3beta mediates endoplasmic reticulum stress-induced lysosomal apoptosis in leukemia. J Pharmacol Exp Ther *329*, 524-531.

Hughes, K., Nikolakaki, E., Plyte, S.E., Totty, N.F., and Woodgett, J.R. (1993). Modulation of the glycogen synthase kinase-3 family by tyrosine phosphorylation. EMBO J *12*, 803-808.

Ikenoue, T., Inoki, K., Yang, Q., Zhou, X., and Guan, K.L. (2008). Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. EMBO J *27*, 1919-1931.

Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol *4*, 648-657.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J., and Su, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell *127*, 125-137.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A., and Hall, M.N. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol *6*, 1122-1128.

Jeno, P., Ballou, L.M., Novak-Hofer, I., and Thomas, G. (1988). Identification and characterization of a mitogen-activated S6 kinase. Proc Natl Acad Sci U S A *85*, 406-410.

Julien, L.A., Carriere, A., Moreau, J., and Roux, P.P. (2010). mTORC1-activated S6K1 phosphorylates Rictor on threonine 1135 and regulates mTORC2 signaling. Mol Cell Biol *30*, 908-921.

Kerkela, R., Kockeritz, L., Macaulay, K., Zhou, J., Doble, B.W., Beahm, C., Greytak, S., Woulfe, K., Trivedi, C.M., Woodgett, J.R.*, et al.* (2008). Deletion of GSK-3beta in mice leads to hypertrophic cardiomyopathy secondary to cardiomyoblast hyperproliferation. J Clin Invest *118*, 3609-3618.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163-175.

Kim, D.H., Sarbassov, D.D., Ali, S.M., Latek, R.R., Guntur, K.V., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2003). GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell *11*, 895-904.

Kohn, A.D., Summers, S.A., Birnbaum, M.J., and Roth, R.A. (1996). Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem *271*, 31372-31378.

Lee, H.C., Tsai, J.N., Liao, P.Y., Tsai, W.Y., Lin, K.Y., Chuang, C.C., Sun, C.K., Chang, W.C., and Tsai, H.J. (2007). Glycogen synthase kinase 3 alpha and 3 beta have distinct functions during cardiogenesis of zebrafish embryo. BMC Dev Biol *7*, 93.

Lee, T.H., and Linstedt, A.D. (1999). Osmotically induced cell volume changes alter anterograde and retrograde transport, Golgi structure, and COPI dissociation. Mol Biol Cell *10*, 1445-1462.

Liang, M.H., and Chuang, D.M. (2006). Differential roles of glycogen synthase kinase-3 isoforms in the regulation of transcriptional activation. J Biol Chem *281*, 30479-30484.

Liang, M.H., and Chuang, D.M. (2007). Regulation and function of glycogen synthase kinase-3 isoforms in neuronal survival. J Biol Chem *282*, 3904-3917.

Liu, C., Li, Y., Semenov, M., Han, C., Baeg, G.H., Tan, Y., Zhang, Z., Lin, X., and He, X. (2002). Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism. Cell *108*, 837-847.

Liu, Q., Chang, J.W., Wang, J., Kang, S.A., Thoreen, C.C., Markhard, A., Hur, W., Zhang, J., Sim, T., Sabatini, D.M.*, et al.* (2010). Discovery of 1-(4-(4-propionylpiperazin-1-yl)-3-(trifluoromethyl)phenyl)-9-(quinolin-3-yl)benz o[h][1,6]naphthyridin-2(1H)-one as a highly potent, selective mammalian target of rapamycin (mTOR) inhibitor for the treatment of cancer. J Med Chem *53*, 7146-7155.

Loffing, J., Flores, S.Y., and Staub, O. (2006). Sgk kinases and their role in epithelial transport. Annu Rev Physiol *68*, 461-490.

MacAulay, K., Doble, B.W., Patel, S., Hansotia, T., Sinclair, E.M., Drucker, D.J., Nagy, A., and Woodgett, J.R. (2007). Glycogen synthase kinase 3alpha-specific regulation of murine hepatic glycogen metabolism. Cell Metab *6*, 329-337.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J., and Cantley, L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell *10*, 151-162.

Mussmann, R., Geese, M., Harder, F., Kegel, S., Andag, U., Lomow, A., Burk, U., Onichtchouk, D., Dohrmann, C., and Austen, M. (2007). Inhibition of GSK3 promotes replication and survival of pancreatic beta cells. J Biol Chem *282*, 12030-12037.

Oh, W.J., Wu, C.C., Kim, S.J., Facchinetti, V., Julien, L.A., Finlan, M., Roux, P.P., Su, B., and Jacinto, E. (2010). mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide. EMBO J *29*, 3939-3951.

Ozcan, U., Cao, Q., Yilmaz, E., Lee, A.H., Iwakoshi, N.N., Ozdelen, E., Tuncman, G., Gorgun, C., Glimcher, L.H., and Hotamisligil, G.S. (2004). Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science *306*, 457-461.

Patel, S., Doble, B.W., MacAulay, K., Sinclair, E.M., Drucker, D.J., and Woodgett, J.R. (2008). Tissue-specific role of glycogen synthase kinase 3beta in glucose homeostasis and insulin action. Mol Cell Biol *28*, 6314-6328.

Pearce, L.R., Komander, D., and Alessi, D.R. (2010). The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol *11*, 9-22.

Peterson, R.T., Beal, P.A., Comb, M.J., and Schreiber, S.L. (2000). FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. J Biol Chem *275*, 7416-7423.

Peterson, T.R., Laplante, M., Thoreen, C.C., Sancak, Y., Kang, S.A., Kuehl, W.M., Gray, N.S., and Sabatini, D.M. (2009). DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell *137*, 873-886.

Rayasam, G.V., Tulasi, V.K., Sodhi, R., Davis, J.A., and Ray, A. (2009). Glycogen synthase kinase 3: more than a namesake. Br J Pharmacol *156*, 885-898.

Ring, D.B., Johnson, K.W., Henriksen, E.J., Nuss, J.M., Goff, D., Kinnick, T.R., Ma, S.T., Reeder, J.W., Samuels, I., Slabiak, T.*, et al.* (2003). Selective glycogen

synthase kinase 3 inhibitors potentiate insulin activation of glucose transport and utilization in vitro and in vivo. Diabetes *52*, 588-595.

Roudabush, F.L., Pierce, K.L., Maudsley, S., Khan, K.D., and Luttrell, L.M. (2000). Transactivation of the EGF receptor mediates IGF-1-stimulated shc phosphorylation and ERK1/2 activation in COS-7 cells. J Biol Chem *275*, 22583-22589.

Ruel, L., Bourouis, M., Heitzler, P., Pantesco, V., and Simpson, P. (1993). Drosophila shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of Notch. Nature *362*, 557-560.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., and Sabatini, D.M. (2007). PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell *25*, 903-915.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol *14*, 1296-1302.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L., and Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell *22*, 159-168.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098-1101.

Scheid, M.P., Marignani, P.A., and Woodgett, J.R. (2002). Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. Mol Cell Biol *22*, 6247-6260.

Schlessinger, J., and Lemmon, M.A. (2003). SH2 and PTB domains in tyrosine kinase signaling. Sci STKE *2003*, RE12.

Schroder, W.A., Buck, M., Cloonan, N., Hancock, J.F., Suhrbier, A., Sculley, T., and Bushell, G. (2007). Human Sin1 contains Ras-binding and pleckstrin homology domains and suppresses Ras signalling. Cell Signal *19*, 1279-1289.

Seidensticker, M.J., and Behrens, J. (2000). Biochemical interactions in the wnt pathway. Biochim Biophys Acta *1495*, 168-182.

Shaw, R.J., and Cantley, L.C. (2006). Ras, PI(3)K and mTOR signalling controls tumour cell growth. Nature *441*, 424-430.

Shiota, C., Woo, J.T., Lindner, J., Shelton, K.D., and Magnuson, M.A. (2006). Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. Dev Cell *11*, 583-589.

Song, L., De Sarno, P., and Jope, R.S. (2002). Central role of glycogen synthase kinase-3beta in endoplasmic reticulum stress-induced caspase-3 activation. J Biol Chem *277*, 44701-44708.

Srinivasan, S., Ohsugi, M., Liu, Z., Fatrai, S., Bernal-Mizrachi, E., and Permutt, M.A. (2005). Endoplasmic reticulum stress-induced apoptosis is partly mediated by reduced insulin signaling through phosphatidylinositol 3-kinase/Akt and increased glycogen synthase kinase-3beta in mouse insulinoma cells. Diabetes *54*, 968-975.

Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G.F., Holmes, A.B., Gaffney, P.R., Reese, C.B., McCormick, F., Tempst, P.*, et al.* (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science *279*, 710-714.

Tanabe, K., Liu, Z., Patel, S., Doble, B.W., Li, L., Cras-Meneur, C., Martinez, S.C., Welling, C.M., White, M.F., Bernal-Mizrachi, E.*, et al.* (2008). Genetic deficiency of glycogen synthase kinase-3beta corrects diabetes in mouse models of insulin resistance. PLoS Biol *6*, e37.

Testa, J.R., and Bellacosa, A. (2001). AKT plays a central role in tumorigenesis. Proc Natl Acad Sci U S A *98*, 10983-10985.

Thomas, G. (2002). The S6 kinase signaling pathway in the control of development and growth. Biol Res *35*, 305-313.

Thoreen, C.C., Kang, S.A., Chang, J.W., Liu, Q., Zhang, J., Gao, Y., Reichling, L.J., Sim, T., Sabatini, D.M., and Gray, N.S. (2009). An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J Biol Chem *284*, 8023-8032.

Toker, A., and Newton, A.C. (2000). Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. J Biol Chem *275*, 8271-8274.

Treins, C., Warne, P.H., Magnuson, M.A., Pende, M., and Downward, J. (2010). Rictor is a novel target of p70 S6 kinase-1. Oncogene *29*, 1003-1016.

Vander Haar, E., Lee, S.I., Bandhakavi, S., Griffin, T.J., and Kim, D.H. (2007). Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat Cell Biol *9*, 316-323.

Viatour, P., Dejardin, E., Warnier, M., Lair, F., Claudio, E., Bureau, F., Marine, J.C., Merville, M.P., Maurer, U., Green, D.*, et al.* (2004). GSK3-mediated BCL-3 phosphorylation modulates its degradation and its oncogenicity. Mol Cell *16*, 35-45.

Viglietto, G., Motti, M.L., Bruni, P., Melillo, R.M., D'Alessio, A., Califano, D., Vinci, F., Chiappetta, G., Tsichlis, P., Bellacosa, A.*, et al.* (2002). Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. Nat Med *8*, 1136-1144.

Wang, Q.M., Fiol, C.J., DePaoli-Roach, A.A., and Roach, P.J. (1994). Glycogen synthase kinase-3 beta is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. J Biol Chem *269*, 14566-14574.

White, P., Gu, L., and Chen, J. (2002). Decreased actin solubility observed during ATP-depletion is mimicked by severing agents but not depolymerizing agents in isolated and cultured proximal tubular cells. Clin Physiol Funct Imaging *22*, 312-319.

Xu, C., Kim, N.G., and Gumbiner, B.M. (2009). Regulation of protein stability by GSK3 mediated phosphorylation. Cell Cycle *8*, 4032-4039.

Yang, Q., Inoki, K., Ikenoue, T., and Guan, K.L. (2006). Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes Dev *20*, 2820-2832.

Yang, W.L., Wang, J., Chan, C.H., Lee, S.W., Campos, A.D., Lamothe, B., Hur, L., Grabiner, B.C., Lin, X., Darnay, B.G.*, et al.* (2009). The E3 ligase TRAF6 regulates Akt ubiquitination and activation. Science *325*, 1134-1138.

Yip, C.K., Murata, K., Walz, T., Sabatini, D.M., and Kang, S.A. (2010). Structure of the human mTOR complex I and its implications for rapamycin inhibition. Mol Cell *38*, 768-774.

Zhou, B.P., Liao, Y., Xia, W., Spohn, B., Lee, M.H., and Hung, M.C. (2001a). Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol *3*, 245-252.

Zhou, B.P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M.C. (2001b). HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol *3*, 973-982.

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Education/Training

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National Taiwan University, Taiwan	B.S.	1995-1999	Chemistry
National Tsing Hua University, Taiwan	M.S.	1999-2001	Life Sciences
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A. Positions and Honors:

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2001-2003	Ensign, Navy (Compulsory military service, Taiwan)
2003-2005	Research Assistant, National Health Research Institute (Taiwan)
2005-2007	Research Intern, M. D. Anderson Cancer Center (USA)
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Research Awards

2010	Award for Poster Presentations (2nd place) at Cancer Biology Program Retreat, M. D. Anderson Cancer Center (USA)
2011	MD Anderson Alumni and Faculty Association Graduate Student Award in Basic Science (1st place) at Trainee Research Day, M. D. Anderson Cancer Center
2011	GSBS Travel Award, University of Texas at Houston
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2011	Andrew Sowell-Wade Huggins Scholarship, University of Texas at Houston

Selected Posters

2004	12th annual Symposium on Recent Advances in Cellular and Molecular Biology (Taiwan)

2010	Cancer Biology Program Retreat, M. D. Anderson Cancer Center (USA)
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B. Peer-reviewed publication:

1. Chu C.-L., Hsiao Y.-Y., **Chen C.-H.**, Van R.-C., Lin W.-J., Pan R.-L. Inhibition of plant vacuolar H^+-ATPase by diethylpyrocarbonate. ***Biochim. Biophys. Acta. Bioenerg***. 1506 (1): 12-22 (2001)

2. Boulbes D., **Chen C.-H.**, Shaiken T., Agarwal N., Peterson T., Addona T., Keshishian H., Carr S., Magnuson M., Sabatini D., Sarbassov D.D. Rictor phosphorylation on the Thr-1135 site does not require mTORC2. ***Molecular Cancer Research*** 8 (6): 896-906 (2010)

3. **Chen C.-H.**, Shaiken T., Peterson T.R., Aimbetov R., Bissenbaev A.K., Lee S.-W., Wu J., Lin H.-K., Sarbassov D.D. ER stress inhibits mTORC2 and Akt signaling through GSK-3β–mediated phosphorylation of rictor. ***Science Signaling*** 4 (161): ra10 (2011)

4. **Chen C.-H.**, Sarbassov D.D. The mTOR (mammalian target of rapamycin) kinase maintains integrity of mTOR complex 2. ***Journal of Biological Chemistry*** 286 (46): 40386-40394 (2011)

5. **Chen C.-H.**, Kazyken D., Bulgakova O., Sarbassov D.D. The homeostatic ATP sensor mTOR maintains the mTORC2 complex integrity. ***Molecular Cell*** (under review)

6. Aimbetov R., **Chen C.-H.**, Bulgakova O., Abetov D., Bissenbaev A.K., Bersimbayev R.I., Sarbassov D.D. Integrity of mTORC2 is dependent on the rictor Gly-934 site. ***Oncogene*** advance online publication 12 Sep: 1-6 (2011)

7. Agarwal N.K., **Chen C.-H.**, Cho H., Boulbes D.R., Spooner E., Sarbassov D.D. Rictor regulates cell migration by suppressing RhoGDI2. ***Oncogene*** (under review)

8. Yao Y., Suraokar M., Asano T., Shaiken T., **Chen C.-H.**, Sarbassov D.D., Abbruzzese J., Reddy S. Akt phosphorylation functions to suppress the expression of adipocyte differentiation inhibitor and promote adipogenesis. ***Science Signaling*** (under review)

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