Identification and characterization of tak1 regulated signaling pathway

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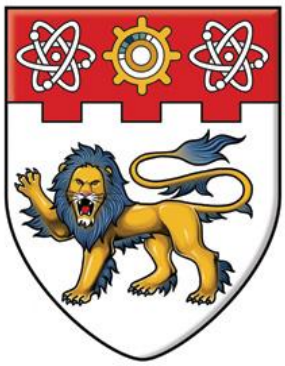

NANYANG TECHNOLOGICAL UNIVERSITY

IDENTIFICATION AND CHARACTERIZATION OF TAK1 REGULATED SIGNALING PATHWAY

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SCHOOL OF BIOLOGICAL SCIENCES

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Abstract

As the key kinase in innate immune defense, TGF β-activated protein kinase 1---TAK1 has been reported for its roles in mediating stimulation of LPS and proinflammatory cytokines, activating of NFκB and mitogen activated protein kinase (MAPK) as well as regulating cellular response to reactive oxygen species (ROS) and hyperosmotic stress. Due to the importance of TAK1 signaling pathway, the precise regulation of TAK1 signaling pathway in various physical conditions is outstandingly critical for cells to coordinate cellular responses appropriately. On the other hand, mTOR pathway is one of the most important regulatory machineries of cell growth. It regulates the assembly of translation initiation complex. One of issues which concern researchers is the tumorigenesis developed from chronic inflammation, which contains two aspects: the long term activation of inflammation signaling and out-of-control cell growth. Based the importance of TAK1 pathway and mTOR pathway in these two fields, respectively, we propose the molecular mechanism of tumorigenesis developed from chronic inflammation as that upregulation of mTOR pathway results from TAK1 activation during inflammation.

In this study, we spare no effort to investigate the molecular connections between inflammatory signaling and mTOR pathway. The molecular switch of mTOR pathway---TSC2 is identified as binding partner of TAK1 by IP-MS approach. TSC2 is also detected from immuno-purified complex of over-expressed and endogenous TAK1. This interaction is further substantiated by mapping the binding of aminol terminal TAK1 to TSC2. Phosphorylation of TSC2 is detected upon LPS stimulation, which is altered after λ phosphatase treatment or TAK1 inhibitor. TSC2 is also phosphorylated when it is over-

expressed with TAK1 complex in mammalian cells. Phospho antibodies recognize the phosphorylation of TSC2 on Thr1462 upon LPS, TNFa and IL-1β stimulations. By IP-MS approach, phospho-site on TSC2 is identified as Ser1365. Activation of mTOR, as indicated by phosphorylation of p70S6K1 on Ser389 and 4EBP1 on Thr70, is observed upon LPS, TNFα and IL-1β stimulations. mTOR activation in these conditions is associated with TAK1 kinase activity. Suppression of TAK1 by shRNA attenuates activation of mTOR pathway upon LPS and TNFα stimulations. Inhibition of TAK1 kinase activity by chemical inhibitor and genetic modification reduces mTOR response towards LPS, TNFα and IL-1β stimulations. These data drive us to the conclusion that TAK1 interacts with TSC2 and phosphorylates TSC2 to activate mTOR pathway, which subsequently regulates cellular responses to LPS, TNFα and IL-β.

In this report, we also characterize regulation of TAK1 signaling pathway mediated by HIPK2. HIPK2 interacts with TAB2 and TAB3, instead of binding to TAK1. HIPK2 also stabilizes TAB2 and TAB3 as TAK1 does. Suppression of HIPK2 by shRNA elevates the activation of TAK1 and its downstream signalling, as well as the poly-ubiquitin binding to TAK1 complex. Moreover, IL-1β stimulation induces HIPK2 activation, as indicated by phosphorylation of p53 on Ser46. With these results, we propose that HIPK2 negatively regulates TAK1 signaling pathway by interrupting the polyubiquitin binding of TAB2 and TAB3 upon various stimulations.

Abbreviation

4EBP1	Translation initiation factor 4E binding protein 1
AMP	Adenosine monophosphate
AMPK	5 prime adenosine monophosphate activated protein kinase
Akt/PKB	Protein kinase B
ATP	Adenosine-5'-triphosphate
AP-1	Activator protein 1
Asp or D	aspartic acid
Axin	a negative regulator of wnt signalling
Bcl-x1	myeloid cell leukaemia sequence 1
Brn3a	anti-apoptotic transcription factor
Cdks	Cyclin dependent kinases
c-jun	Oncogene jun, nuclear protein (a major component of AP-1)
c-myc	cellular progenitor of the v-myb oncogene
c-myb	Proto-oncogene myb (myeloblastosis) C.
CtBP	transcriptional co-repressor C terminal Binding Protein
CUE	Coupling of ubiquitin conjugation to ER degradation
CXCL10	C-X-C motif chemokine 10 also known as interferon gamma induced protein10
cyclin D1	Regulatory subunit to advance the G1 phase of the cell-cycle
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin-ligase
eIF	eukaryotic translation Initiation Factor
ERK	Extracellular signal-regulated kinase
FKBP12	FK506 binding protein 12 kDa
Flag	Flag epitope tag
GDP	Guanosine diphosphate
GEF	Guanine nucleotide Exchange Factor
GST	Glutathione S-transferase

GTP	Guanosine triphosphate
Gtr	the yeast homologous of GTPase Rag
GSK3	Glycogen synthase kinase 3
h	hour
HA	Hemaglutinin
HIPK	Homeodomain -Interacting Protein Kinase
HMD2	Human homology of murine double minute 2 (MDM2), a E3 ligase
HMGA1	High Mobility Group A1
Hox	transcription factor, a protein production of gene *hox*
hVps34	human homolog of Vacuolar protein sorting34
LEF-1	Lymphoid enhancer factor
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
IGF	Insulin-like Growth Factor
IκB	Inhibitor of NFκB
IKK	Iκ-B kinase
IL	Interleukin
IL-12 p40	Interleukin 12 protein 40 kDa (IL-12B)
IL-12 p35	Interleukin 12 protein 70 kDa (IL-12A)
IP	Immunoprecipitation
IRAK	IL-1 receptor associated protein kinase
IRFs	IFN regulatory factors
IRS	Insulin Receptor Substrate
IVK assay	*in vitro* Kinase assay
JNK	Jun N-terminal kinase
K or lys	Lysine
kDa	kilo Dalton (molecular weight of the protein)
Km	Michaels Menden Kinetics
LPS	lipopolysaccharide
MAP4K3	ste-20 related MAP kinase kinase kinase kinase
MAPK	Mitogen activated protein kinase
MAPKAP-K2	MAPK-Activated Protein Kinase 2, also known as MK2

MBP	Maltose Binding Protein
MDM2	murine double minute 2, an E3 ligase
MEFs	Mouse Embryonic Fibroblasts
MEKK1	MAPK kinase kinase 1
MKK3/6	Mitogen-activated protein Kinase Kinase 3/6
MKK4/7	Mitogen-activated protein Kinase Kinase 4/7
mLST8	mammalian lethal with SEC13 protein 8, also known as GβL
mRNA	massager Ribonucleic Acid
mTOR	mammalian Target of Rapamycin
mTORC1/2	mammalian Target of Rapamycin Complex 1/2
NFκB	Nuclear Factor kappa B
Nkx	homeodomain transcription factor
Myc	Myc epitope tag
MyD88	Myeloid differentiation primary response protein 88
NZF	Nuclear protein localization 4 Zinc Finger
p53	tumour suppressor, a 53 kiloDalton protein
p63	tumour protein 63, a transcription factor, homologue of p53
p73	tumour protein 73, homologue of p53
p300	histone acetyltransferase
p70S6K1	70 kiloDalton ribosomal protein S6 kinase
PCR	Polymerase Chain Reaction
PEST	a sequence rich in P(proline), E(glutamatic acid), S(serine) and T(threonine)
PDCD4	Programmed Cell Death 4
PDGF	Platelet-derived growth factor
PDK	phosphoinositide-dependent kinase
PI3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PMA	phorbol 12-myristate 13-acetate
PML	pro-myelocytic leukemia protein
PPARδ	Peroxisome proliferators activated receptor δ

PP1y	phosphatase 1 y
RAG	Ras-related small GTP binding protein
Raptor	regulatory associated protein of mTOR
Rheb	Ras homology enriched in brain
Rictor	rapamycin insensitive companion of mTOR
RIP	Receptor interacting protein kinase 1
RMP	RPB5-mediating protein
RNA	Ribonucleic Acid
Smad	a group of proteins homology with both *C.elegans* protein SMA and the *drosophila* protein MAD.
S6K	Ribosomal protein S6 Kinase
Sin1	stress-activated MAP kinase interacting protein1, also called Mip1
ski	nuclear proto-oncogene protein
siah1/2	ubiquitin ligase E3, encoded by gene siah1/2
S or Ser	Serine
SAPK2a/p38a	Stress-activated protein kinase 2a
T or Thr	Threonine
TAB1	TAK-1 binding protein 1
TAB2	TAK-1 binding protein 2
TAB3	TAK-1 binding protein 3
TAB4	TAK-1 binding protein 4
TAK1	TGF-β activated kinase 1
TCF	T cell factor
TGF-β	Transforming growth factor- beta
TIMP-2	Tissue inhibitor of metalloproteases 2
TIP	Type 2A phosphatase interacting protein
TLR	Toll like receptors
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TOR	target of rapamycin
TRAF	Tumour necrosis factor receptor associated factor
TrkA	neurotrophic Tyrosine kinase receptor type1
TSC1/2	tuberose sclerosis complex 1/2

Ub	Ubiquitin
Ubc	Ubiquitin carrier protein
Uev	Ubiquitin conjugating enzyme variant
UTR	UnTranslated Region
Vam6	guanine nucleotide exchange factor, a homologous of human Vps34
Vps34	Vacuolar protein sorting 34 or Class III PI3K
Wnt	a family of secreted signaling proteins
WSB-1	WD repeat and SOCS box-containing protein1, a E3 ligase
Y or Tyr	Tyrosine

Chapter I Introduction

1.1 TAK1 and inflammatory signaling cascade

1.1.1 TAK1 activation by IL-1β, TNFα and LPS

Transforming Growth Factor-beta Activated Kinase 1 (TAK1) was first identified in 1995 and belongs to the mitogen-activated protein kinase family. As the name suggests, TAK1 is activated by TGF-β but is more widely known as a protein kinase that is activated by the cytokines: interleukin-1 (IL-1) and tumour necrosis factor alpha (TNFα), and by lipopolysaccharide (LPS), a component of the bacterial cell wall [1]. Engagement of agonists to the IL-1 and Toll-like receptors leads to recruitment of the adaptor protein MyD88 and IL-1 receptor I associated protein kinases (IRAKs). Subsequently, IRAKs recruit an E3 ubiquitin ligase called tumour necrosis factor (TNF) receptor associated factor 6 (TRAF6) followed by its oligomerization [2]. The ubiquitin binding domains found on the TAK1 binding proteins TAB2 and TAB3 recognize the lysine-63 linked polyubiquitin chains synthesized by TRAF6, which leads to activation of TAK1. The activated TAK1 mediates phosphorylation of MKK3/6 and MKK4/7 which activate the p38 and JNK MAPKs respectively. The activated TAK1 also mediates activation of Inhibitor of NFκB Kinase (IKK) complex, resulting in the phosphorylation and degradation of Inhibitor of NFκB (IκB), followed by the release and activation of nuclear factor κB (NFκB). The downstream result of this signalling cascade is the activation of transcription factors which in turn regulate transcription of pro-inflammatory genes, as shown in Figure 1.1. TNFα activates TAK1 via a similar pathway but lysine-63

linked polyubiquitin chains are synthesized by different ubiquitin E3 ligase TRAF2.

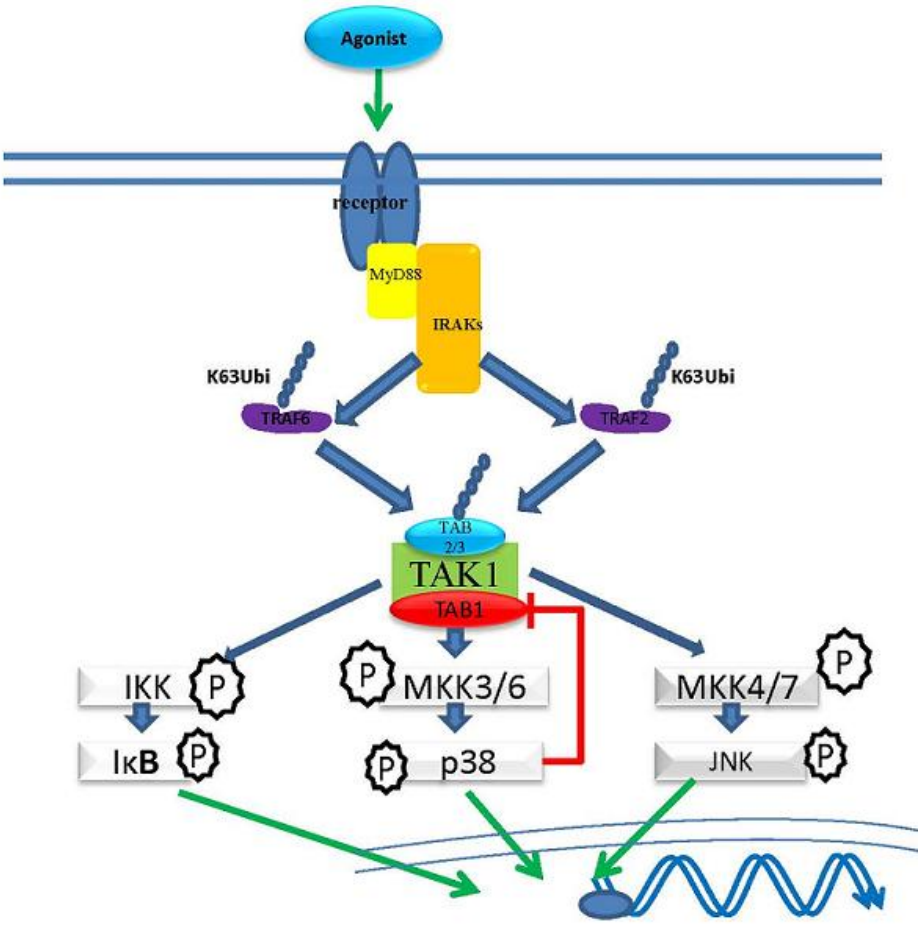


Figure 1.1 TAK1 activation in the IL-1, LPS and TNFα signalling pathways.

Engagement of agonists (IL-1β, TNFα and LPS) to the receptors leads to recruitment of the adaptor protein MyD88 and IRAKs. Subsequently, IRAKs recruit an E3 ubiquitin ligase TRAF6/TRAF2 followed by its oligomerization. The ubiquitin binding domains found in TAB2 and TAB3 recognize the lysine-63 linked polyubiquitin chains synthesized by TRAF6/TRAF2 to activate TAK1. The activated TAK1 mediates phosphorylation of MKK3/6 and MKK4/7 which activate the p38α and JNK MAPKs respectively. The activated TAK1 also mediates activation of inhibitor of NFκB Kinase (IKK) complex, resulting in the phosphorylation and degradation of inhibitor of NFκB (IκB), followed by the release and activation of nuclear factor κB (NFκB). At the end, transcription factors under this pathway are activated to regulate transcription of pro-inflammatory genes.

1.1 .2 Activation of TAK1 by other stimuli

TAK1 is also activated by hyperosmotic stress when cells are exposed to high concentrations of sorbitol or NaCl in an oligomerization dependent manner [3]. In addition, TAK1 has also been shown to be activated by the non-canonical Wnt5a pathway, leading to suppression of the canonical Wnt pathway. In this pathway, TAK1 along with NLK (Nemo-Like Kinase) antagonizes the activation of the transcription factors TCF4, LEF1 and c-Myb by the Wnt-GSK3 pathway [4-7].

1.1.3 The phosphorylation of TAK1 and its kinase activity.

The kinase domain of TAK1 is crucial for its catalytic activity and autophosphorylation of several key amino acids (Thr184, Thr187 and Ser192) within the catalytic domain is required for TAK1 activation, as shown in Figure 1.2. Mutation on Ser192 causes the loss of TAK1 kinase activity and phosphorylation of Thr187 [8, 9]. Thr184 is also considered as an essential residue for TAK1 activation since IL-1β induces the endogenous TAK1 phosphorylated on Thr184 [10]. When TAK1 is co-expressed with TAB1, Thr187 of TAK1 is autophosphorylated leading to increased TAK1 kinase activity. Moreover, mutation on Thr187 results in the loss of TAK1 kinase activity and downstream NF-κB and AP-1 transcription activities [8, 10]. Thus, phosphorylation on Thr187 is an indicator of TAK1 activity.

1.1.4 TAK1 binding proteins

In cells, TAK1 can exist as the catalytic component of two different complexes: TAK1-TAB1-TAB2 or TAK1-TAB1-TAB3. TAB1 is the first characterized binding partner for TAK1 and identified by the yeast two-hybrid system. TAB1

consists of 504 residues and its carboxyl-terminal region (480-495) interacts strongly with the N-terminal region of TAK1 [11]. TAB1 is constitutively bound to TAK1.

Structural studies on the TAK1/TAB1 complex shed light on the physical basis for their interaction [12]. A fused chimera containing the N-terminal (31-303) of TAK1 and C- terminal (468-504) of TAB1 was crystallized in 2005 by Brown, K. *et al*. Structural analysis reveals a novel binding pocket on the TAK1 kinase domain whose shape complements that of a unique α-helix in TAK1-binding domain of TAB1, suggesting a hydrophobic association between TAK1 and TAB1. These reports confirm that TAB1 is an important binding partner of TAK1 and also regulates TAK1 kinase activity.

TAB2 was also identified as a TAK1 binding partner using the yeast two-hybrid system in 2000 [12], while TAB3 was discovered by homology searching of the human genome database for proteins related to TAB2 in 2004 [13]. The C-terminal region of TAB2 (401-693) and TAB3 (393-712) bind to TAK1 C-terminal (488-579) [13, 14]. According to studies from two independent groups: Cheung, C. F. *et al* [14] and Ishitani, T. *et al*. [15], these two proteins probably play redundant roles with each other in two distinct complexes: TAK1-TAB1-TAB2 and TAK1-TAB1-TAB3. They bind the lysine 63 linked poly-ubiquitin chain synthesized by the E3 ligases TRAF6 or TRAF2 and act as adaptor proteins to activate TAK1 [2].

TAB4 was recently discovered as a binding partner of TAK1 and also known as Type 2 phosphatase-interacting protein 41 (TIP41) [16]. It binds TAK1 directly and enhances polyubiquitin chain binding to TAK1, leading to increase of TAK1 activity. Further work is required to establish the role of TAB4.

1.1.5 TAK1 and TAB1 interaction

In over expression studies, TAB1 activates TAK1 and induces increased expression of TGF-β responsive genes even in the absence of TGF-β, implying that TAB1 plays a positive role in TAK1 activation [11]. Over-expressed TAB1 leads to autophosphorylation of TAK1 and a concomitant increase of TAK1 kinase activity [8]. Recently, Inagaki, M. *et al* [3] found that TAK1 from TAB1 deficient cells can still respond to the cytokines IL-1β or TNFα but not to hyperosmotic stress. Oligomerization of the TAK1-TAB1 complexes in a cell free assay results in activation of TAK1, which Inagaki, M. proposed also happens inside cells in hyperosmotic situation. Cheung, C.F. *et al* [17] reported the negative feedback control of TAK1 mediated by TAB1. The downstream p38α, which is activated after TAK1, phosphorylates TAB1 at Ser423 and Thr431 to attenuate TAK1 activation. In cells treated with SB203580 (a p38α specific inhibitor), TAB1 phosphorylation at Ser423 and Thr431 is undetectable and TAK1 activity is enhanced.

1.1.6 TAK1 isoforms

Bioinformatic analysis has shown that four TAK1 mRNA transcripts can be produced by alternative splicing of exons in the TAK1 gene and distributed among different tissues [18]. TAK1A consists of 579 residues while TAK1B, TAK1C and TAK1D are predicted to consist of 607, 518 and 491 residues respectively, as shown in Figure 1.3. The majority of studies on TAK1 have been carried out using the TAK1A isoform and little is known about other TAK1 isoforms. To date, a few reports have provided evidence showing the existence of TAK1B, TAK1C and TAK1D at the protein level. In the literature, TAK1A is commonly referred to as TAK1.

The TAK1A mRNA is widely expressed and found in most human tissues such as brain, heart, kidney, liver, lung, skeletal muscle, ovary and peripheral blood mononuclear cells [18]. TAK1A consists of three functional domains: the amino-terminal auto inhibition domain, the kinase domain, and the carboxyl domain, as shown in Figure 1.2. The N-terminal (1-21 amino acids) is also known as the autoinhibition domain for TAK1 kinase activity [1], deletion of this small N-terminal stretch renders TAK1 constitutively active. The kinase domain is from 31-300 amino acids, harbouring the catalytic domain and mediates the binding between TAK1 and TAB1. The carboxyl domain (479-553) mediates the interaction between TAK1 and its binding partners TAB2 and TAB3 [11].

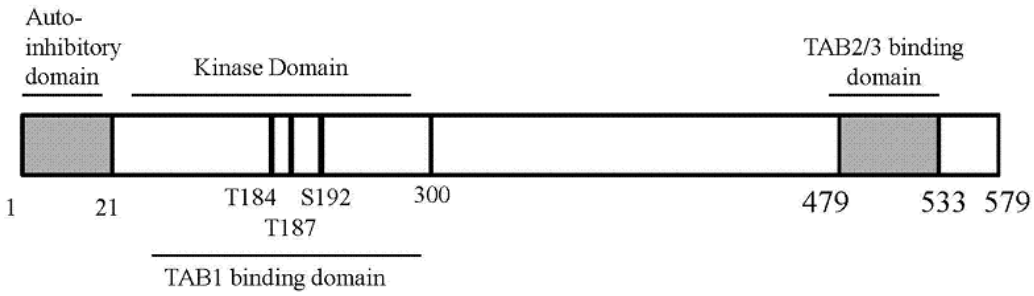


Figure 1. 2 Schematic map of TAK1A

TAK1 consists of autoinhibitory domain, kinase domain which also overlaps with TAB1 binding domain, and N terminal TAB2 and TAB3 binding domain. The length of functional domains and important residues are indicated.

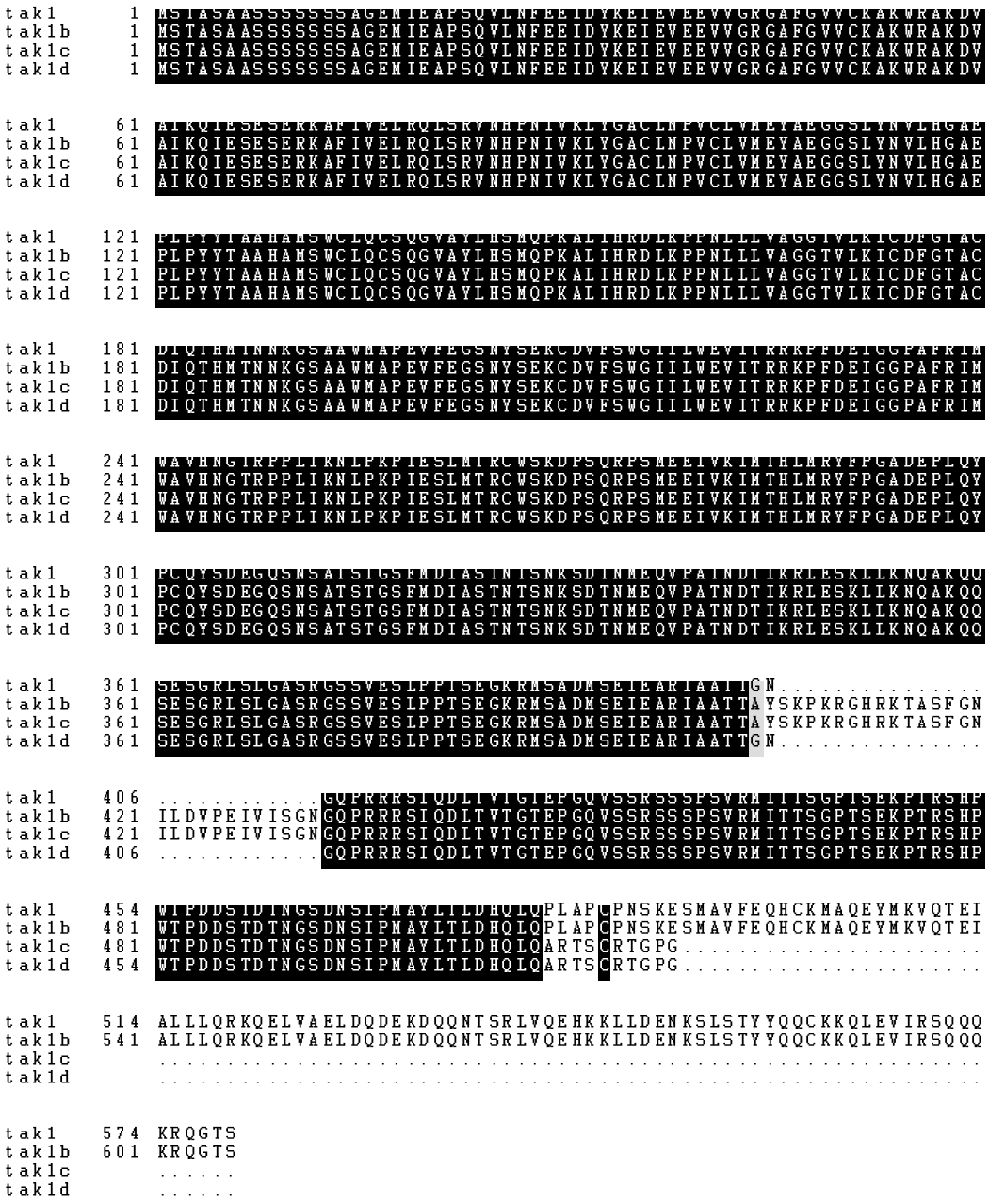


Figure 1.3 Amino acid sequence alignment of TAK1 isoform A, B, C, D.

The black shaded region indicates the identical residues among four isoforms and grey shaded region indicates the conserved residues.

1.2 mTOR pathway and mTOR signaling cascade

1.2.1 Tuberous Sclerosis Complex

Tuberous Sclerosis Syndrome (TSC) is defined as a multisystemic, autosomal dominant disorder characterized with the development of numerous benign tumors. It often causes disabling neurologic disorders, including epilepsy, mental retardation and autism, in addition to facial angiofibromas, renal angiomyolipomas and pulmonary lymphangiomyomatosis [19]. TSC has a wide clinical spectrum of disease and some patients may have minimal signs and symptoms with no neurologic disability.

TSC1 (Tuberous Sclerosis Complex 1, the gene product is called Hamartin or TSC1 [20]) and *TSC2* (Tuberous Sclerosis Complex 2, the gene product is called Tuberin or TSC2 [21]) genes were identified as the genetic loci responsible for tuberous sclerosis syndrome in 1997 and 1993, respectively. As shown in Figure 1.4, TSC1 protein contains 1164 residues, with a molecular weight of 140 kDa. It has no obvious functional domains but a TSC2 binding domain in the middle region between residue 302 to residue 430. Also shown in Figure 1.4, TSC2 contains 1807 residues, with a molecular weight at 200 kDa. It has a TSC1 binding domain at the N-terminus and a GTPase activating protein (GAP) domain at C-terminal region, leaving the middle region for interaction with and regulation by other proteins. There is no homology between TSC1 and TSC2 protein sequences. However, orthologues can be found in most eukaryotic cells, from fission yeast *Schizosaccharomyces pombe* to human.

The GAP domain on TSC2 is the only functional domain in TSC complex and is homologous to the GAP domain of Rap1GAP. The early searches for a GTPase target show that TSC2 has weak GAP activity *in vitro* towards both Rap1 and Rab5, two small G-proteins, demonstrating that this region of TSC2 is indeed a GAP domain [22, 23]. Missense mutations in TSC2 GAP domain have been found clinically in patients [24]. Cell culture and xenograft experiments suggest that this domain exerts the tumor suppressor activity of TSC2 [25].

The tumor suppression function of TSC complex is tightly related with its physical integrity [26, 27]. TSC1 and TSC2 associate with each other through the binding domain and form a heterodimeric complex. In this complex, TSC1 is required to stabilize TSC2 and prevent TSC2 from ubiquitin-mediated degradation [28-30]. Thus, dissociation of TSC1-TSC2 heterodimer may lead to degradation of TSC2 and tumorigenesis.

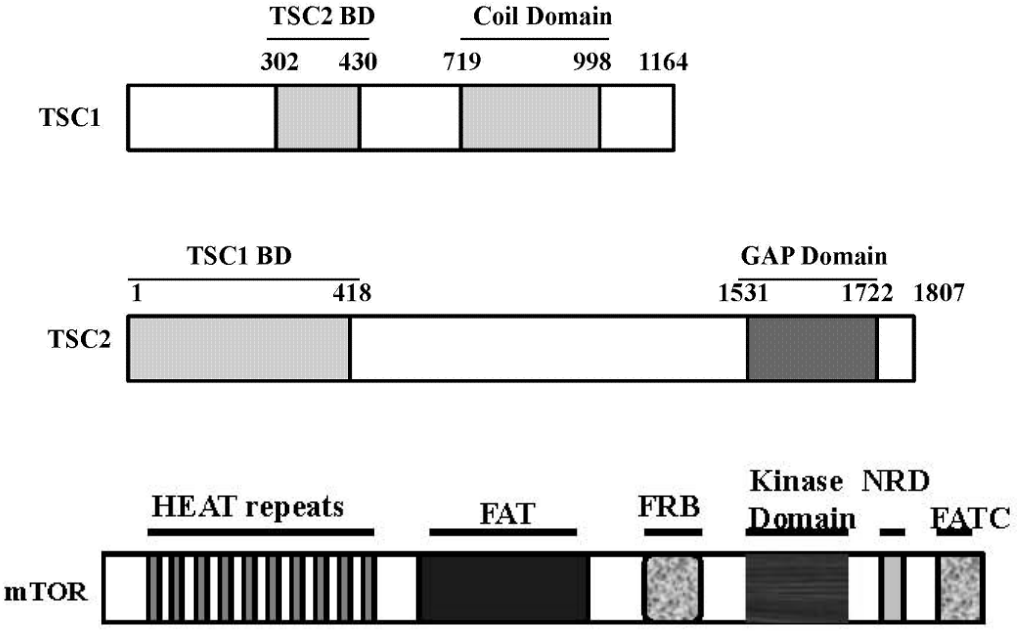


Figure 1.4 The schematic map of TSC1, TSC2 and mTOR

TSC1 contains 1164 residues. It has a TSC2 binding domain between residue 302 to residue 430 and a coiled-coil domain in the C-terminus. TSC2 contains 1807 residues. It has a TSC1 binding domain at N-terminus and a GTPase activating protein (GAP) domain at C-terminus, leaving the middle region for possible interaction with and regulation by other proteins. mTOR contains 20 HEAT repeats at N-terminal, FAT/ FRB/ Kinase domain/ NRD at middle region with FATC at C-terminal.

1.2.2 mTOR complex

TSC transduces environment signals to one of the most important cell growth mechanisms: mammalian target of rapamycin (mTOR) pathway. Target of rapamycin (TOR) is an atypical serine/threonine protein kinase, belonging to the phosphatidylinositol kinase related kinase (PIKK) family. There is only one protein product of *tor* in mammals: mammalian target of rapamycin (mTOR). The physiological importance of mTOR is demonstrated by the fact that knockout of mTOR in mice is embryonic lethal [31]. Structurally, mTOR possesses 20 tandem HEAT repeats (a protein-protein interaction region with two tandem anti-parallel α helices named after: Huntigin, Elongation factor3, pr65/A and TOR) at the amino-terminal region, followed by a FAT domain (name after FRAP, ATM and TRRAP), FRB domain (FK506-binding protein 12 kDa/Rapamycin Binding), kinase domain and FAT-C terminus. mTOR composes two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [32].

mTORC1 consists of mLST8 (mammalian lethal with SEC13 protein 8, also known as GβL), raptor (regulatory associated protein of mTOR) and mTOR. mLST8 binds tightly to mTOR and it is a non-enzymatic and not essential subunit of mTORC1 [33]. Raptor is an essential and non-enzymatic subunit of mTORC1. Knockout of raptor, similar to knockout of mTOR, is also early embryonic lethal, supporting the idea that raptor is indispensable for mTOR to function and activate downstream substrates. However, whether raptor is a

positive or negative regulator of mTOR activity remains ambiguous. A recent study reports that raptor mediates GTPase Rag to modulate mTOR activity in amino acid signaling [34], supporting the essential role of raptor in regulating mTORC1 function. Activated mTORC1 transduces signals down to various components of the translation initiation machinery in a coordinated manner through direct or indirect phosphorylation events.

mTORC2 consists of mLST8, rapamycin insensitive companion of mTOR (rictor), Sin1 (stress-activated MAP kinase interacting protein1, also called Mip1) and mTOR. Both rictor and Sin1 are unique subunits of mTORC2, whereas mTOR and mLST8 are also present in mTORC1. Rictor is the first identified subunit that is unique to mTORC2 [35]. The interaction between rictor and mTOR is not blocked by conditions which regulate mTORC1. The overall physiological importance of rictor is emphasized by the fact that rictor knockout mice die around E10.5 day, possibly due to defects in vascular development [33, 36, 37]. Knockdown of rictor by RNAi in cultured cells does not change the downstream signaling of mTORC1, suggesting that mTORC2 has different physiological functions from mTORC1. Functional studies of mTORC2 have shown that knockdown of rictor in HeLa cells increases F-actin accumulation, indicating that mTORC2 is important for cytoskeleton dynamics [35, 38]. mTORC2 is also identified as the long-sought PDK2, which phosphorylates Akt/PKB on the hydrophobic motif site Ser473 [39, 40].

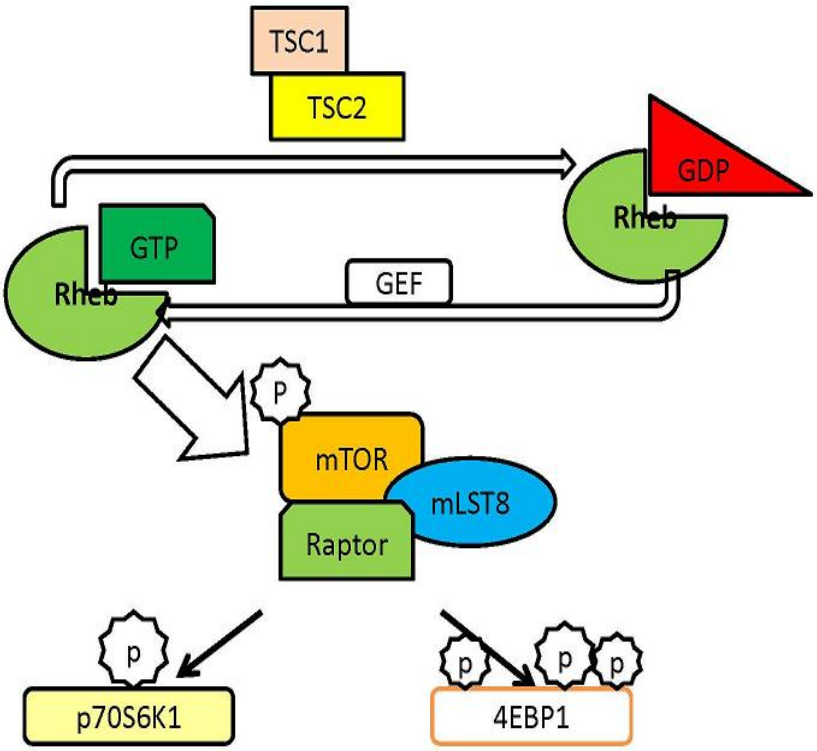
The physiological difference to separate mTORC1 from mTORC2 is the sensitivity to rapamycin. Rapamycin is a potent anti-fungal macrolide secreted by a bacterial strain *Streptomyces hygroscopicus*. To date, rapamycin has become an FDA approved drug for immunosuppression for organ

transplantation, prevention of restenosis post-angioplasty and chemotherapy for soft tissue and bone sarcomas [41-43]. The anti-proliferative properties of rapamycin are also a powerful tool to study cell growth regulation by mTOR. Upon entering cells, rapamycin binds a small protein receptor called FKBP12 (FK506 binding protein 12 kDa). The FKBP12/rapamycin complex specifically binds to the FRB domain of mTOR and potently inhibits its activity, reducing the phosphorylation of substrates of mTORC1 and causing cell growth arrest [32]. However, mTORC2 is rapamycin insensitive and some mTORC2 components may block the binding of rapamycin/FKBP12 complex to the FRB domain of mTOR.

With inhibition of mTORC1 function by rapamycin and impaired mTORC2 function in rictor knockout cells, functional studies on mTORC1 and mTORC2 are effectively separated. Because of the importance of cell proliferation, mTORC1 is more intensively studied than mTORC2. And mTOR complex mentioned below will be referred as mTORC1.

Figure 1.5 The upstream and downstream of mTORC1

In quiescent situation, TSC1 and TSC2 bind together and TSC2 exerts its GAP activity to convert Rheb from GTP loaded status to GDP loaded status. GDP loaded Rheb is then activated by GEF which changes GDP into GTP for Rheb. GTP loaded Rheb activated mTOR in mTORC1, which in turn recruits and phosphorylates p70S6K1 and 4EBP1.



1.2.3 The link between TSC and mTOR---Rheb

Activation of mTORC1 requires GTP loaded GTPase and Rheb (Ras homologue enriched in brain) is a GTPase that mediates TSC signal to regulate mTORC1 activity. Biochemical studies have shown that the Asp41 in the switch I region and Tyr54/ Lys56 within the constitutive effecter region of Rheb are required for Rheb to activate mTOR signaling [44]. The Asp41 residue within the putative switch I region is critical for binding between mTOR and Rheb. Tyr54 and Lys56 mediate proper farnesylation of Rheb so that mutation of these two sites reduces Rheb activity and the guanine nucleotides binding to Rheb.

In quiescent condition, TSC2 converts the Rheb from the GTP loading form to the GDP loading form. As the GDP bound Rheb cannot activate mTOR kinase; the translational machinery is arrested [45, 46]. Under conditions in which

nutrients and energy are plentiful or certain stimulations such as insulin are given, TSC2 is inactivated for its GAP role. Guanine nucleotide exchange factor GEF load Rheb with GTP. GTP bound Rheb interacts with and activates mTOR kinase [47].

1.2.4 Downstream of mTOR

There are two main substrates of mTOR: 70 kDa ribosomal protein S6 kinase (p70S6K1) and eukaryotic translation initiation factor 4E binding protein1 (4EBP1). The eukaryotic translation initiation factor complex 3 (eIF3) is a dynamic scaffold for mTORC1 and its substrates. In quiescent cells, p70S6K1 associates with the eIF3 complex, whereas mTORC1 does not. Upon stimulation, mTORC1 is recruited to eIF3 complex and phosphorylates p70S6K1 at Thr389 [48], which results in its disassociation from eIF3 and subsequent phosphorylation and activation by PDK1 at Thr229 [49]. At the same time, the mTORC1-eIF3 complex associates with mRNA 5' cap and brings mTORC1 into proximity with its other major target 4EBP1, phosphorylating 4EBP1 on Thr37, Thr46, Ser65 and Thr70 [50]. Both phosphorylation and activation of p70S6K1 and 4EBP1 are important for translation initiation.

When mTOR is activated, it regulates various components of the translation initiation machinery in a coordinated manner through direct or indirect phosphorylation events. 4EBP1 tightly binds to eIF4E in hypophosphorylated status. When 4EBP1 is hyperphosphorylated by mTOR, 4EBP1 is dissociated with eukaryotic translation initiation factor 4E (eIF4E). Then, eIF4E recruits eIF4G and eIF4A to the 5' end of mRNA, which assemble eukaryotic

translation initiation complex 4F. Subsequently, eIF3, the small ribosomal subunit and the ternary complex are recruited to the cap, resulting in the assembly of the 48S translation pre-initiation complex, ribosome scanning and translation initiation [51, 52].

Another important substrate of mTORC1 is 70 kDa ribosomal protein S6K, including S6K1 and S6K2. p70S6K1 plays important roles in translation regulation. For example, p70S6K1 regulates the activity of RNA helicase during translation. When mTORC1 activates p70S6K1, it in turn phosphorylates eIF4B on Ser422. eIF4B phosphorylation affects its interaction with RNA helicase eIF4A[52], which promotes translation of mRNAs with long and structured 5'UTRs [53]. Meanwhile, activated p70S6K1 subsequently phosphorylates PDCD4 (programmed cell death 4), a tumor suppressor binding to eIF4A, to release eIF4A. eIF4A then unwinds the secondary structure of mRNA and promotes translation initiation[52].

1.2.5 mTOR senses the environmental signals.

mTOR has been considered as a switch board of cell growth and protein synthesis. Here, some of the important and well established mechanisms of regulating mTOR pathway are briefly introduced and summarized, as shown in Figure 1.6 and 1.7.

1.2.5.1 mTOR responds to amino acid shortage mediated by hVps34/Rag or MAP4K3

In mammals, 10 of the 20 amino acids are essential and must be supplied from exogenous sources, whereas the remaining 10 are produced by the organism.

For their essential function as building blocks in protein synthesis, intensively studies have been carried out to reveal the connection between amino acids and mTOR pathway activation. Initial studies show that p70S6K1 activity is largely protected from amino acid withdrawal in TSC2 or TSC1 deficient rodent cells as well as in *Drosophila* S2 cells where *d*TSC1 or *d*TSC2 levels are reduced [54]. It has been concluded that TSC complex antagonizes amino acids-TOR signaling. However, other researchers argued that amino acid withdrawal has no effect on binding of GTP and over expressed Rheb, proposing that amino acid signals reside on a pathway that functions in parallel with TSC- Rheb-mTOR axis [55].

According to the latest reports [55-57], Rheb is required but not sufficient for regulation mTOR by amino acid availability. In $TSC2^{-/-}TSC1^{-/-}$ cells and $TSC2^{+/+}TSC1^{-/-}$ cells, amino acid stimulation is still able to induce the phosphorylation of p70S6K1 [57]. Amino acid stimulation does not affect phosphorylation of p70S6K1 in TSC2 knockdown cells, either [58]. Thus, amino acid availability regulates mTOR signaling and cell growth via a TSC1/2 independent way.

Given the wortmannin sensitivity and class I PI3K independence of mTOR activity induced by amino acid stimulation, researchers searched for other components of the pathway. hVps34, human version of class III PI3K, is considered as a candidate to mediate mTOR activation in response to amino acid stimulation [57]. Takahiro, N. *et al*[59] reported that when hVps34 is knocked down in HEK293 cells, amino acid induced phosphorylation of p70S6K1 is reduced while insulin induced phosphorylation on p70S6K1 is

unaffected. Byfield, M. P. *et al* [60] also reported that hVps34 mediates amino acid availability and lies upstream of mTOR.

On the other hand, GTPase Rag (Ras related GTPase) is reported to play a role in amino acid signaling [34, 61, 62]. There are four members of Rag protein: RagA, RagB, RagC and RagD. RagA or B binds with RagC or D through carboxyl-terminus to form a heterodimer, which is only fully activated when RagA or B is GTP loaded while RagC or D is GDP loaded. Miss-load of nucleotide or the disruption of Rag heterodimeric complex abolishes its activity. The involvement of Rag in amino acid induced mTOR activity is identified in an extensive RNAi screen in which suppression of *d*Rag impairs activation of *d*S6K upon amino acid stimulation in *Drosophila* S2 cells. In mammalian cells, expression of inactive RagA/B mutant---$RagA^{TN}$/$RagB^{TN}$ inhibits mTORC1 activity in the presence of amino acids while expression of active RagA/B mutant---$RagA^{QL}$/$RagB^{QL}$ induces mTORC1 activity in the absence of amino acids [62]. The function of Rag in amino acid signaling is in parallel with other mTOR regulation mechanisms since active $RagA^{QL}$/$RagB^{QL}$ could not overcome other mTORC1 inhibitory signals while inactive $RagA^{TN}$/$RagB^{TN}$ suppresses insulin-stimulated phosphorylation of p70S6K1 in HeLa cells as amino acid starvation does. Although Rag heterodimer does not directly modulate the kinase activity of mTORC1 *in vitro*, it seems to play a critical role in mTOR translocation [34].

In 2009, studies of Binda, M. *et al* revealed the connection between Vps34, Rag and mTORC1 [63]. In budding yeast, guanine nucleotide exchange factor (GEF) Vam6 is homologous to human Vps34 while Gtr1 and Gtr2 are homolog of RagA/B and RagC/D, respectively. As the GTPase activator, GEF Vam6 senses

amino acid availability and activates GTPase Gtr1, leading to activation of TORC1. This study strongly suggests that in mammalian cells the Vps34 and RagA/B may mediate amino acid signal to mTORC1 in a coordinated way.

In 2009, with all this information, Li, L. and Guan, K. L. [64] proposed a model that Vps34 acts as GEP to activate GTPase RagA/B to regulate mTORC1 in response to amino acid stimulation, as summarized in Figure 1.6.

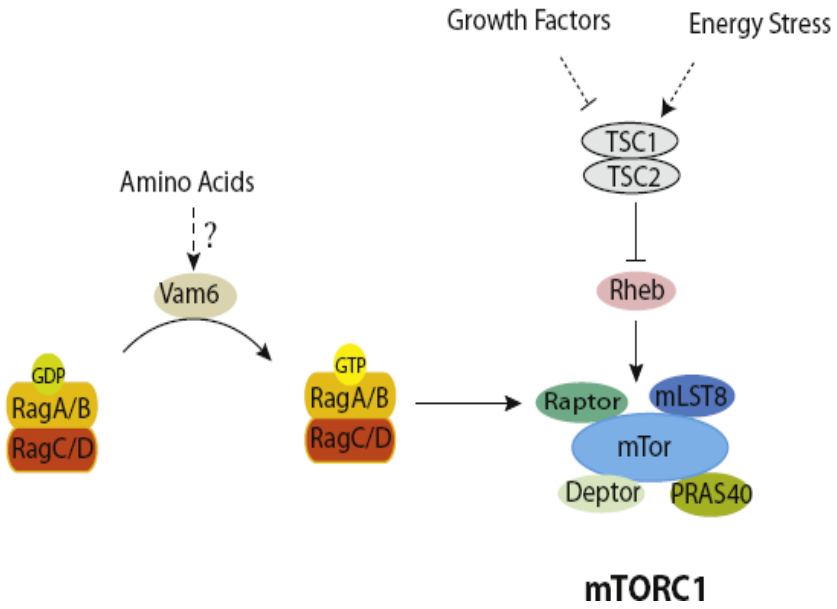


Figure 1.6 The flow chart of amino acid signaling, quoted from reference [64].

Amino Acids signals are firstly sensed by GEP Vam6/Vps34, which transfers the GDP loaded RagA(B)/RagC(D) to GTP loaded RagA(B)/RagC(D). The GTP loaded heterodimer in turn activates mTORC1. The amino acids signals may regulate mTOR activation in the pathway parallel with ones mediating growth factors and energy stress signals.

Findlay, G. M. *et al* [65] provided a different explanation for the amino acids signaling to mTOR. They considered that amino acids signal is wortmannin insensitive up to 200 nM while MAP4K3, a ste-20 related MAP kinase kinase kinase kinase, mediates amino acid availability to mTOR. In their study, suppression of MAP4K3 by RNAi inhibits both basal and amino acid-induced phosphorylation of p70S6K1 and 4EBP1 in *Drosophila* S2 cells and HeLa cells.

Suppression of MAP4K3 by RNAi in HeLa cells also results in reduced cell size in all cell cycle phases. Findlay, G. M. and his colleagues proposed a new mechanism by which mTOR responds to amino acid availability, that is a model in which MAP4K3 is downstream of a signal provided by amino acids, but upstream of mTORC1. However, there are still questions that need to be answered in their proposal such as: how MAP4K3 is activated in response to amino acid, what is the mechanism of MAP4K3 regulates mTOR, and whether Rheb is involved in this signaling.

1.2.5.2 TSC2 responds to insulin and growth factor mediated by PI3K/Akt pathway

As the switch of protein synthesis, mTOR is mainly regulated by insulin and growth factors like IGF and VEGF. In 2002 and 2003, several groups reported that the PI3K-Akt pathway mediates these signals to mTOR by phosphorylating and inhibiting TSC2 [66-69].

When insulin binds to its receptor on membrane, the receptor autophosphorylates and activates itself. It subsequently recruits and phosphorylates insulin receptor substrate 1 and 2 (IRS1/2) at multiple tyrosine residues. The specific phosphorylated residues on IRS1 and IRS2 serve as docking motifs for the class I phosphatidyinositol-3-kinase (PI3K) and leads to PI3K activation and production of phosphatidyinositide-3, 4, 5-P_3 (PIP3). Binding of PIP3 to PH domain of PKB/Akt recruits this kinase to cell membrane, where the phosphatidyinositol-dependent protein kinase1 (PDK1) phosphorylates PKB/Akt at Thr308 and activates PKB/Akt [70, 71]. Once it is activated, Akt phosphorylates TSC2 on Ser939, Ser1086, Ser1088 and Thr1462.

Phosphorylation of TSC2 causes destabilization of TSC2 and disrupts its interaction with TSC1. The disrupted TSC1/TSC2 complex thus loses its GTPase activity towards Rheb, which in turn activates mTORC1. This is followed by phosphorylation of p70S6K1 and 4EBP1, which are then able to activate the translation machinery and elevates protein synthesis. These events ultimately result in cell growth in response to insulin [66].

In 2003, Dan, H. C. *et al* [72] also reported that there are seven putative phosphorylation sites on TSC2 which fit in Akt phosphorylation-motif. However, they showed that the phosphorylation of TSC2 by Akt results in the degradation of both TSC1 and TSC2. Instead of using mTOR activation to report the function of TSC1-TSC2 on cell proliferation, they looked at cyclin-dependent kinase inhibitor $p27^{kip1}$ as downstream events of TSC2 phosphorylation, proposing that phosphorylation of TSC2 by Akt destabilizes $p27^{kip1}$ to promote cell cycle and cell proliferation. Their study provided a different view for insulin induced mTOR activation.

1.2.5.3 TSC2 responds to energy and nutrient deprivation mediated by AMPK.

Protein synthesis is a process which consumes approximately 20% to 25% of the total cellular energy[73], thus it must be tightly coordinated with cellular energy status. Previously, Dennis, P.B. *et al* proposed that mTOR may sense cellular ATP level because it has a high K_m (around 1 mM) for ATP [74]. However, this K_m value is still considerably lower than normal physiological ATP levels, thus only a drastic reduction in ATP would affect the mTOR activity, which is not a common physiological condition.

Inoki, K. *et al* [75] studied the coordination between cell growth and cellular energy availability. Because the AMP concentration in cell is much lower than ATP, a relevantly small decrease on ATP level will produce a relevantly greater change on AMP concentration. When the cellular energy level goes down, it is AMP that acts as a very sensitive indicator for cellular energy status. Moreover, increase of AMP activates the 5' AMP- activated protein kinase (AMPK). Therefore, Inoki, K. *et al* proposed the connection between TSC2 and AMPK for regulating cell growth according to energy availability [75]. They found that AMPK phosphorylates TSC2 on Ser1345 and Thr1227 to promote TSC2 GAP activity. The activated TSC2 leads to suppression of the mTOR pathway and inhibition of phosphorylation of p70S6K1 and 4EBP1. When TSC2 is knocked down in HEK293 cells under energy deprivation, the mTOR pathway is not suppressed. mTOR activation in TSC2 knockout cells is not inhibited by ATP depletion reagents while it is in the TSC2 present cells. In addition, TSC2 phosphorylation mediated by AMPK protects cells from apoptosis upon glucose deprivation and excessive growth during energy deprivation. The physiological significance of phosphorylation of TSC2 by AMPK is also validated by TSC2 mutant S1337A S1345A S1341A (TSC2 3A mutant), which cannot prevent cell growth and the development of apoptosis upon glucose deprivation.

Cell growth heavily depends on energy supply. TSC2 regulation by AMPK could effectively inhibit cell growth and enhance cell survival upon the energy deprivation situations through inhibiting translation. This is in line with the latest study on apoptosis control by TSC1-TSC2 upon endoplasmic reticulum stress [76], implying an important role of TSC1-TSC2 and Rheb in regulating inappropriate protein synthesis and cell survival.

1.2.5.4 TSC2 responds to Wnt signals mediated by GSK3 and AMPK

Wnt is a family of proteins which bind to cell membrane receptors and regulate gene transcription, cell growth, proliferation, polarity, differentiation and development [77, 78]. Dysregulation of Wnt pathway components has been reported in benign colorectal adenomas and cancer [79-81]. Inoki, K. *et al* [82] reported that the mTOR signaling may play a critical role in growth-stimulatory and tumorigenic effects of Wnt signaling via GSK3 mediated phosphorylation on TSC2.

In addition to accumulation of β-catenin and transcription activity upon the activation of the canonical Wnt signaling, Wnt also regulates translation under mTOR pathway as determined by the increased phosphorylation of p70S6K1, S6 and 4EBP1. Both *in vitro* and *in vivo* data support the regulation of mTOR by Wnt. While Wnt proteins (Wnt1, Wnt3a, Wnt10b), Wnt receptor (LRP6), inner membrane scaffold protein (Dishevelled-2), Wnt pathway regulator Axin and APC as well as glycogen synthase kinase 3 (GSK3) are required for regulation of mTOR, β-catenin seems to be dispensable in mTOR regulation by Wnt signaling [82].

Meanwhile, Mak, B.C. *et al* [83] reported physical interaction between TSC2 and Wnt signaling components Axin, Dvl as well as GSK3. The co-immunoprecipitation of TSC2 and GSK3 also lends further support to functional interaction between Wnt signaling and mTOR. It has also been reported that association between TSC2 and GSK3 occurs upon Wnt stimulation [84], confirming a role of GSK3 in Wnt signaling regulation on mTOR.

GSK3 is negatively regulated by Wnt signaling. GSK3 inhibits mTOR pathway when it is over expressed, as indicated by phosphorylation of p70S6K1 and 4EBP1. This inhibitory effect is released when GSK3α and GSK3β are knocked down or when GSK3 inhibitor is applied [82]. Examination of the TSC2 sequence shows four candidate sites for GSK3 phosphorylation: Thr1329, Ser1333, Ser1337 and Ser1341. GSK3 phosphorylation motif requires priming phosphorylation of the site which is three residues away from C-terminus of targeted phosphorylation site. Thus, the phosphorylation on Ser1345, which is mediated by AMPK, is considered important for regulation of TSC2 by GSK3. AMPK and GSK3 phosphorylate TSC2 in a hierarchical manner and they require each other for their full activity to control mTOR. Inoki, K. *et al* concluded that the coordinated phosphorylation of mTOR by GSK3 and AMPK is essential for mTOR pathway regulation by Wnt signaling [82].

These studies also support earlier reports on the phosphorylation and inhibition of GSK3 by Akt upon insulin stimulation[85]. Given its role as a negative regulator of mTOR pathway, inhibition of GSK3 could be an additional mechanism to upregulate mTOR in response to insulin.

1.2.5.5 TSC2 responds to mitogenic signals mediated by ERK1/2

A number of clinical studies have revealed a high level of ERK1/2 activity in both TSC1 and TSC2 associated brain lesions [86, 87], suggesting a potential role of ERK1/2 in tumor progression. In 2005, researchers reported that the phosphorylation of TSC2 on Ser1364 and Ser1798 are associated with ERK1/2 activation [88, 89]. The mitogen phorbol 12-myristate 13-acetate (PMA) is a potent cancer promoting agent and well known activator of the ERK1/2

signaling pathway. Upon PMA stimulation, TSC2 is phosphorylated, which is inhibited by MEK1 inhibitor UO126. On the other hand, overexpression of a constitutively active form of MEK1 [S218D/S222D] activates endogenous ERK1/2, which leads to phosphorylation of TSC2 [87]. The physical association between TSC2 and ERK1/2 is also demonstrated by co-immunoprecipitation. These data bring up the possibility that TSC2 may be a substrate of ERK1/2. The *in vitro* kinase assay confirms that TSC2 can be phosphorylated by recombinant ERK1/2. By bioinformatic searching, *in vitro* kinase assay and mass spectrometry, Ser664 and Ser540 on TSC2 are found to be phosphorylated by ERK [86]. Mutation of these two sites abrogates phosphorylation of TSC2 by ERK1/2 *in vitro* and in cells expressing constitutively active MEK1, too. This phosphorylation event on TSC2 causes the dissociation of TSC1/TSC2 complex and suppression of TSC2 function. It also inhibits the repression effect of TSC2 in cell proliferation and oncogenic transformation as well as tumorigenesis on the nude mice.

1.2.5.6 TSC2 responds to anisomycin and serum mediated by p38 and MK2

It has been reported that TSC2 interacts with trafficking protein 14-3-3 [90]. Given that Akt recognition sequences often overlap with putative 14-3-3 binding site and that TSC2 is a substrate of Akt [72, 91], researchers postulated that TSC2 and 14-3-3 may interact. In HEK293 cells, 14-3-3 can be co-precipitated with the TSC1-TSC2 complex when they are over expressed. TSC2 interacts with all endogenous isoforms of 14-3-3 (theta, zeta, gamma, and beta) in HEK 293 cells. The 14-3-3 binding domain in TSC2 has been mapped between residues 1101 to 1302. It is an established theory that the recognition motif for 14-3-3 is a phosphorylation site led consensus sequence. In TSC2, the

recognition sequence is led by phosphorylated Ser1210, according to both data base analysis and single site mutation studies. When Ser1210 is mutated into alanine, both full length TSC2 and truncated TSC2 (1102-1302) lose the ability to bind 14-3-3. According to the immunoprecipitation result from HEK293 cell, the interaction between TSC2 and 14-3-3 exists under basal resting conditions. However, the kinase which phosphorylates TSC2 on Ser1210 remained unknown till the identification of MK2.

It is upon anisomycin stimulation and serum treatment that the interaction between TSC2 and 14-3-3 is enhanced and TSC2 is phosphorylated [92]. Since both anisomycin stimulation and serum treatment activate p38α, a possible role of p38α is suggested for the interaction between TSC2 and 14-3-3. The phosphorylation of TSC2 and the interaction between TSC2 and 14-3-3 is increased by expression of an active form of p38α. And these phenomena are specifically blocked by p38α inhibitor. After revealing that phosphorylation site on TSC2 does not fall in substrate motif of p38α, Li, Y. *et al* scanned the downstream kinases of p38α, including MK2, PRAK and MNK1 as possible kinases for TSC2. However, only MK2 is able to significantly increase phosphorylation of TSC2 and interaction between TSC2 and 14-3-3 when it is over expressed. The constitutively active MK2 mutant also effectively activates TSC2 in the presence of p38α inhibitor. While the dominant negative MK2 efficiently blocks the interaction between TSC2 and 14-3-3 as well as phosphorylation of TSC2 in response to anisomycin stimulation and serum treatment. Further studies show that MK2 can directly phosphorylate TSC2 on Ser1210. These studies imply that MK2 is a regulator of TSC2 and interaction between TSC2 and 14-3-3, as shown in Figure 1.7.

1.2.5.7 TSC1 responds to cytokine signals mediated by IKKβ

As a key point of cell growth control, mTOR is regulated by normal growth conditions as well as immune signals which may induce either tumor or apoptosis. Compared with the studies on regulation of mTOR upon growth factor, nutrients and energy availability, investigation on details of the regulation of mTOR in the immune response is less extensive. One of the ground breaking studies is from Lee, D.F. *et al* [93], who reported the activation of mTOR mediated by IKKβ in response to TNFα stimulation.

The mTOR pathway is efficiently activated in response to inflammatory cytokines, such as TNFα and IL-1β, compared with stimulations of growth factor IGF, EGF and Wnt3a [93]. Instead of IKKα and IKKγ, it is IKKβ that bridges inflammatory signals to mTOR pathway. Given the *in vivo* interaction between IKKβ and TSC1, Lee, D.F. found that TSC1 is a substrate of IKKβ *in vitro* and *in vivo*. IKKβ phosphorylates TSC1 on Ser487 and Ser511, which in turn leads to the destabilization of TSC1 and translocation of TSC2. The degradation of the TSC1-TSC2 complex results in upregulated mTOR activity and phosphorylation of p70S6K1, as shown in Figure 1.7. IKKβ mediated phosphorylation of TSC1 also promotes tumor growth *in vivo*, as well as VEGF secretion. Most importantly, phosphorylation of TSC1 and activation of IKKβ is found highly relevant in human cancer samples. The importance of inflammatory signals in tumorigenesis is hence revealed.

The inflammatory microenvironment around tumors contains several types of inflammatory cells, especially tumor associated macrophages, which secrete proinflammatory cytokines, including TNFα, IL-1, IL-6 and IL-8. Given the

activation of IKKβ in response to proinflammatory cytokines and phosphorylation of TSC1 by IKKβ, the inflammatory signals would be able to induce mTOR activation, cell proliferation as well as angiogenesis and thereby promote tumor.

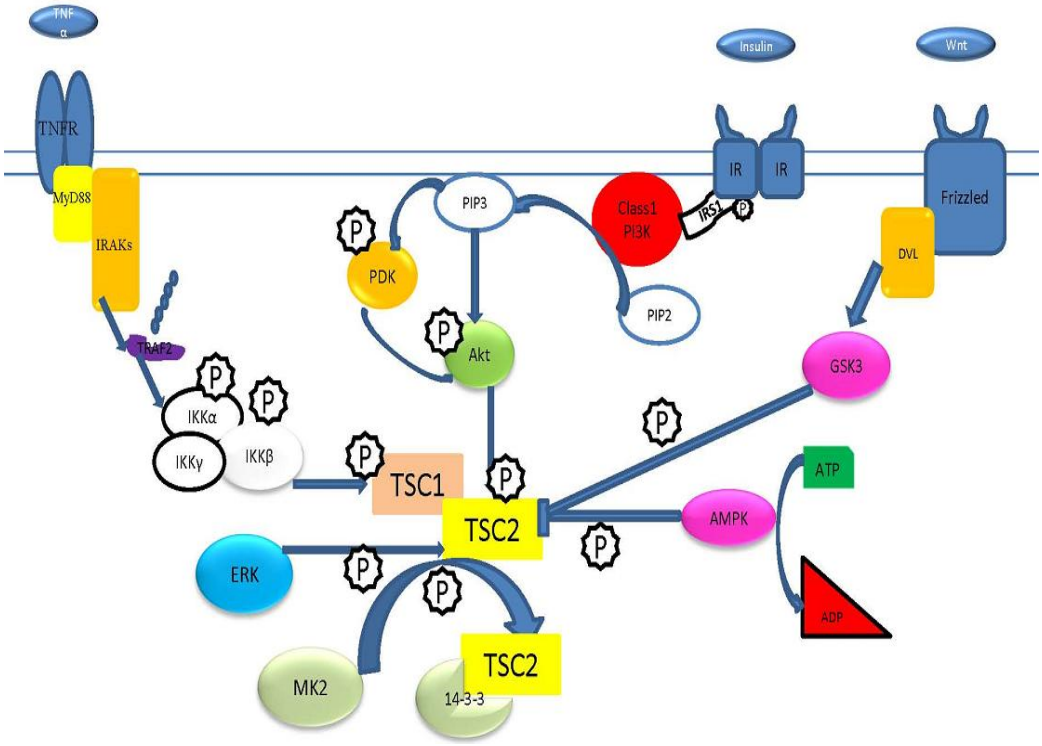


Figure 1.7 Regulation of TSC1-TSC2 complex

TSC1-TSC2 complex is an important regulator of mTOR. The regulation of TSC1-TSC2 thus is critical to mTOR pathway. Upon TNFα stimulation, activated IKKβ phosphorylates TSC1, resulting in the disassociation of TSC complex and relocalization of TSC2, by which the mTOR pathway is activated. Upon insulin stimulation, activated Akt/PKB phosphorylates TSC2, resulting the degradation of TSC2 and disassociation of TSC1-TSC2 complex, leading to the activation of mTOR pathway. Upon Wnt stimulation, GSK3 is inactivated and phosphorylation of TSC2 is reduced, followed by the activation of mTOR pathway. Upon the growth factor stimulation, ERK is activated and phosphorylates TSC2 to release mTOR pathway. Upon the growth factor stimulation, MK2 is also activated to phosphorylate TSC2, resulting in the association of 14-3-3 and TSC2, which disassociates TSC complex and release mTOR pathway. Upon energy deprivation, AMPK is activated to phosphorylate TSC2, which enhances the GAP activity of TSC2 and inhibits mTOR pathway. In Wnt signaling, GSK3 phosphorylated TSC2 in a manner coordinated with phosphorylation of TSC2 mediated by AMPK, which enhances the GAP activity of TSC2 to suppress mTOR pathway.

1.2.6 Feedback control of mTOR signaling pathway

Similar with other important signaling pathways, mTOR pathway is also under feedback control to prevent excessive activation.

As shown in Figure 1.8A, the first control pathway is through mTORC2 which phosphorylates Akt/PKB at Ser473 and facilitates phosphorylation of Akt/PKB on Thr308 by PDK1and enforces TSC2 phosphorylation and mTORC1 activation [39].

The second target of positive feedback controls on mTOR pathway is GSK3. As shown in Figure 1.8B, GSK3 phosphorylates TSC2 and, together with AMPK, suppresses mTOR in condition where energy is limiting [82]. However, GSK3 is phosphorylated and inhibited by Akt/PKB in insulin stimulation[85], cooperating with the mTOR activation in this condition. On the other hand, in TSC2 knockout cell and tumors with low Akt/PKB activity, both GSK3α and GSK3β are phosphorylated by p70S6K1 in a manner independent of PI3K and Akt/PKB [94]. Given the important role of GSK3 in cell growth, its inhibition by Akt/PKB and p70S6K1 could contribute to tumorigenesis.

The third target is insulin receptor substrate IRS1, which mediates both positive and negative feedback to mTORC1. IRS1 facilitates PI3K activation and is an important mediator in insulin induced mTOR activation. However, in $TSC2^{-/-}$ cells, Rheb-mTOR loses control from TSC complex and the over-active p70S6K1 in turn suppresses the synthesis of IRS1 mRNA and phosphorylates IRS1 at Ser302, which reduces its affinity with insulin receptor [95]. The impaired IRS1 expression and function result in insulin resistance and reduced activation of PI3K-Akt upon growth factor stimulation. Zhang, J. *et al* [96] also

reported that, upon TNFα stimulation, p70S6K1 directly phosphorylates IRS1 on Ser270, leading to insulin resistance. Therefore, the low malignant potential of tumor arising from TSC1-TSC2 dysfunction may be explained with the negative feedback control of IRS1 mediated by p70S6K1 and the failure to activate PI3K upon growth factor stimulations [95].

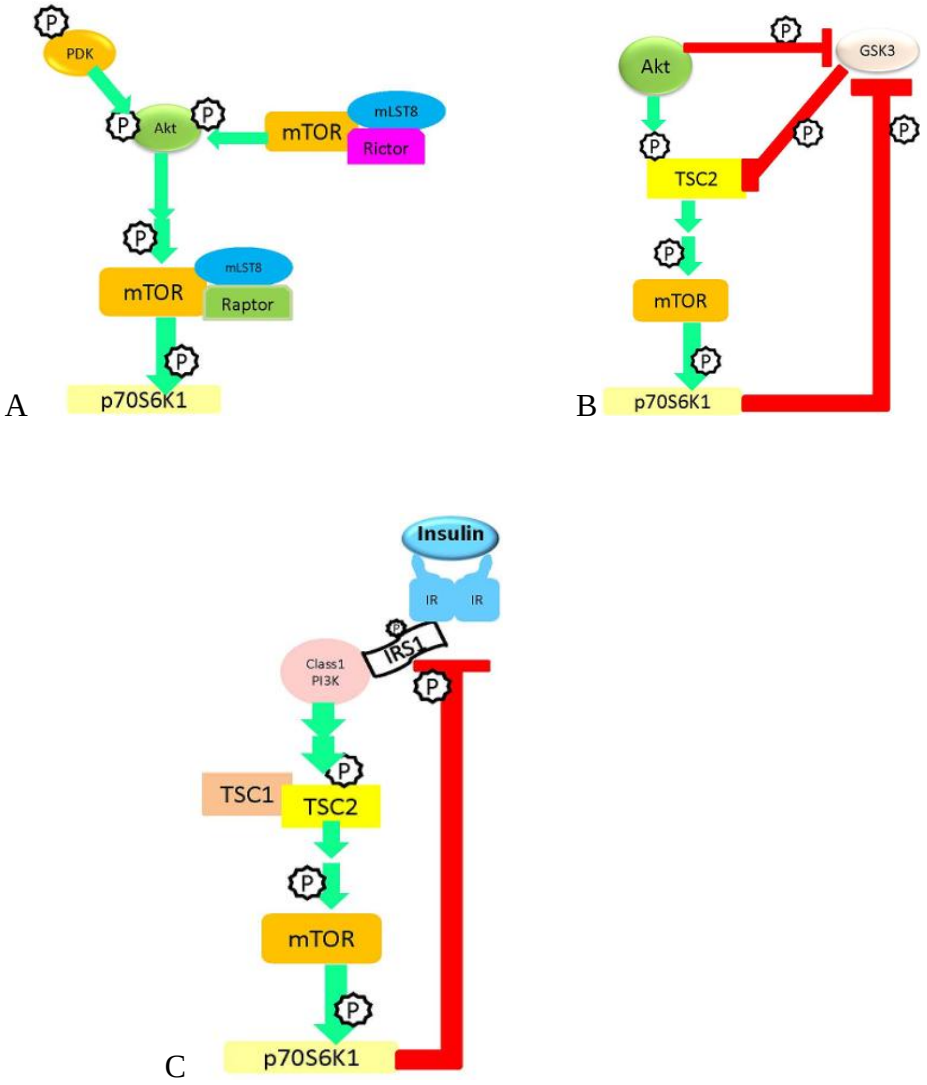


Figure 1.8 Feedback control of mTOR signaling

mTOR pathway is precisely regulated by several feedback loop. A, mTORC2 (mTOR with rictor and mLST8) phosphorylates Akt at T473 to enforce the activation of mTORC1. B, GSK3 phosphorylates TSC2 and inhibits mTOR activity; however, Akt/PKB and p70S6K1 both phosphorylate GSK3, resulting in the degradation of GSK3 and released mTOR activity. C, p70S6K1 phosphorylates IRS1 at S302, leading to the degradation of IRS1 and reduced mTOR activity.

1.3 The clinical significance of mTOR activation

1.3.1 mTOR activation and insulin resistance

The initial study on mTOR pathway suggests that mTOR is activated by insulin and insulin like growth factor. It functions in downstream of IRS1, PI3K and Akt/PKB while upstream of p70S6K1 and 4EBP1. In the case that mTOR pathway is persistently activated, such as in $TSC2^{-/-}$ cells, p70S6K1 phosphorylates IRS1 on S302 and impairs expression of IRS1. This is followed by cell insensitivity to insulin and reduced PI3K-Akt activation [95], which is considered as insulin resistance, as mentioned in section 1.2.6.

On the other hand, free fatty acids also induce insulin resistance and impaired mTOR activation [97]. The exciting study from Hang Shi, et al suggests that free fatty acids induce the expression of pro-inflammatory cytokines like TNFα and Il-6, which is associated with reduced phosphorylation of IRS1 at tyrosine 187 and impaired affinity of p85 to IRS1, suggesting insulin resistance and impaired mTOR activation. In this study, the role of TLR4 in free fatty acids induced insulin resistance is underlined. TLR4 is a receptor recognizing lipopolysaccharide (LPS) found on the cell wall of gram-negative bacteria and transfers the bacteria invasion signal to activate immune response [98, 99]. Their data show that free fatty acids induces NF-κB signaling activation and inflammatory cytokines secretion, which are both blocked in $TLR4^{-/-}$ cells [97]. The free fatty acids induced insulin resistance is also attenuated in $TLR4^{-/-}$ mice, indicating that TLR4 is required in insulin resistance and impaired mTOR activation in this context. This study strongly suggests that TLR4 involves in inhibition of mTOR activity in free fatty acids infusion [97].

1.3.2 mTOR related tumorigenesis and clinical research

Due to the important role of mTOR and p70S6K1 in cell growth and metabolism, it is reasonable to predict an association between mTOR pathway activity and aberrant forms of growth, including cancer. It has been reported that several downstream and upstream components of the mTOR pathway are mutated in tumorigenesis. Upregulation or mutation of class I PI3K and PKB/Akt, loss of PTEN (phosphatase and tensin homolog), the protein tyrosine phosphatase that inhibits phosphorylation of PI3P and Akt/PKB, mutation of the *TSC* genes and abnormal activation of p70S6K1 and eIF4E have all been identified in specific cancers [71, 101, 102].

The clinical trials against cancer focusing on mTOR pathway have been carried out. Firstly, rapamycin and its derivatives RAD001 have been developed and pursued in several clinical settings as monotherapy or in combination with other anti-cancer agents. Promising results have been revealed in Phase II trials for breast cancer [103] and renal cell carcinoma [104]. In specific settings, such as tuberous sclerosis complex, rapamycin and its derivatives have a pronounced effect [105]. Even though, the fact that in nutrient-depleted conditions rapamycin and its derivatives are largely cytostatic but not cytotoxic heavily affects their clinical potential in cancer treatment as monotherapy. In 2004, Novartis launched the rapamycin derivative RAD001, also called Everolimus, as a cancer treatment. RAD001 sensitizes tumor cells to DNA-damage-induced apoptosis through inhibition of translation of cyclin-dependent kinase inhibitor p21 [106], providing the basis for testing in phase II clinical trials. Another completed Phase II clinical trial in which breast cancer patients have been treated with the aromatase inhibitor Letrozole either alone or in combination

with rapamycin displayed a better progression-free survival rate in combination therapy [107].

A recent study from OSI Pharmaceuticals showed a synergistic effect of Erlotinib (Tarceva), an EGF receptor inhibitor, and rapamycin on the activation of Akt/PKB and p70S6K1 in various tumor-derived cell lines [108]. In agreement with this, it is reported that HERB inhibitor herceptin also shows synergistic effects with rapamycin in slowing the growth of breast cancer cells with high expression of ERB2 and in reducing tumorigenesis in xenograft models [109, 110]. Meanwhile, a dual classI PI3K and mTOR inhibitor has been developed with the effect of arresting growth of glioma-xenografted tumors [111]. These studies shed light on further application of rapamycin in a combination treatment in cancers not derived from mTOR mutations. The involvements of mTOR in tumorigenesis are the major focus of clinical research on mTOR pathway.

1.3.3 mTOR pathway plays a role in inflammatory

The potent immunosuppressive action of rapamycin is commonly attributed to inhibition of growth factor-induced T cell proliferation, but recent studies suggest that mTOR plays an important role in the modulation of both innate and adaptive immune response. mTOR regulates diverse functions of antigen-presenting cells, activation of effecter T cells, as well as functions and proliferation of regulatory T cells [112]. As listed in Table1, most of the phenomena were observed from rapamycin treated samples.

Table 1 The impact of mTOR pathway on adaptive immunity

Adaptive immunity event	Phenomenon
Antigen presenting	1)Rapamycin suppresses the differentiation and maturation of DCs; 2)Rapamycin impairs antigen uptaken---macropinocytosis and endocytosis; 3) Rapamycin enhances antigen presenting by inducing autophagy in mouse DCs; 4)Rapamycin reduces the viability of DCs; 5)Rapamycin increases the migration of human DCs in response to CCL19; 6) mTOR suppression elicits the production of IL-1 and IL-12 of DCs
T cell response	1)Rapamycin reduces T cell viability without affecting the number of T_{reg} cells 2)Rapamycin treatment shows immunosuppressive effect to T cell activation; 3)Rapamycin treatment keeps T cell anergy for longer period; 4)Rapamycin helps the development of T_{reg} cells
Therapeutic immunosuppression	1)Rapamycin is used for transplant tolerance; 2)Rapamycin is used for autoimmune disease.

1.4 HIPK2 and TAK1

1.4.1 HIPK family

HIPK, termed as Homeodomain-Interacting Protein Kinases, was first identified in 1998 [113] as co-repressors for homeodomain transcription factors. HIPK1, HIPK2 and HIPK3 are originally identified through their interaction with homeobox factors and show a conserved protein structure with more than 90% homology in the kinase domain and about 70% in the homeobox-interacting domain. HIPK4 is the less conserved and is identified according to its homology with the other members of the family during the human genome sequencing project [114, 115]. Functional redundancy among the members has been proposed. Especially, HIPK1 and HIPK2 show redundancy in embryo development [116-118]. So far, much of the information about HIPKs comes from studies on HIPK2, which has been used as a model for the description of the members' characters.

1.4.2 HIPK2 structure

As shown here, murine HIPK2 is a protein consisting 1189 residues, with an amino-terminal region containing a sumoylation site and a kinase domain followed by a homeobox-interacting domain, a PEST-sequence-containing region which overlaps with speckle-retention signal, a putative autoinhibitory domain and an ubiquitylation site at carboxyl domain.

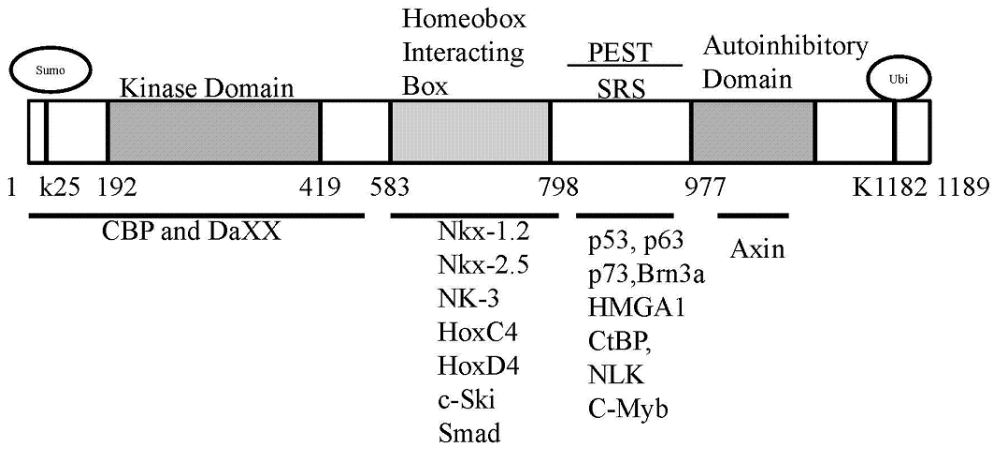


Figure 1.9 The schematic map of HIPK2

The schematic map of murine HIPK2. HIPK2 consists of a kinase domain, a homeobox-interacting domain, a PEST-sequence-containing region where speckle-retention signal locates, and a putative autoinhibitory domain. A sumoylation site locates at K25 while an ubiquitylation site locates at K1182. The proteins binding to each specific domain are indicated.

The kinase domain is a p38 MAPK-like domain highly conserved among the members of the HIPK family and throughout evolution [119]. HIPK2 has been shown to phosphorylate itself *in vitro* and several of its targets both *in vitro* and *in vivo* [120, 121]. Kinase activity is frequently, but not always, required for HIPK2-mediated regulation of gene transcription [119].

The homeobox-interacting domain is responsible for the interaction with the homeodomain transcription factors of Nkx and Hox families, the proto-oncogenec-Ski, and the transcriptional activators Smad1-4 [119].

The PEST-sequence-containing region in HIPK2 mediates the interactions with most of the non-homeotic factors that have been reported so far, such as p53, p63, p73, CtBP and Brn3a [119].

The speckle-retention signal is involved in HIPK2 subnuclear localization. It has been shown that HIPK2 is recruited into speckled subnuclear structures

[120, 121]. Some of these structures are typical nuclear bodies that contain the distinctive PML (pro-myelocytic leukaemia protein) and nuclear antigen Sp100 as well as the tumour suppressor p53 [120-124]. Another type of speckle structure is the polycomb group of proteins in which HIPK2 co-localizes with HIPK3 [125]. The co-localization of HIPK2 and HIPK3 is involved in HIPK2-mediated transcriptional repression.

The putative inhibitory role of the auto inhibitory domain stems from the increased HIPK2 kinase activity after deletion of this domain [126, 127]. It has been reported that Axing physically occupies the putative autoinhibitiory domain, thus allowing the activation of HIPK2 [126]. The existence of the autoinhibitory domain is further supported by the finding that the same region is cleaved by caspases in apoptosis and caspase-resistant mutants of HIPK2 induce apoptosis less efficiently [127].

In addition to the autophosphorylation, there are two post-translational modification sites defined and characterized in the HIPK2 protein: the sumoylation site at Lys25 [128-130] and the ubiquitination at Lys1182 [131]. Recently, reports have suggested that HIPK2 is widely modified by different E3 ligase to regulate cell response according to various stimulations [132-136].

1.4.3 The regulation of HIPK2

Recent studies show that HIPK2 activity is mainly controlled at the post transcriptional level through targeted proteolysis. Caspase-dependent processing triggers HIPK2 hyperactivation, whereas the ubiquitin-proteasome system keeps HIPK2 in check by targeting it for degradation. Both HIPK2

hyperactivation and HIPK2 degradation are under the control of transcription factor p53.

In response to adriamycin treatment, HIPK2 is processed at residues Asp916 and Asp977 in a caspase-dependent manner [127, 137]. Caspase-dependent cleavage removes autoinhibitory C-terminal domain from HIPK2, resulting in a hyperactive kinase, which in turn potentiates phosphorylation of p53 on Ser46 and activates the cell death machinery. However, HIPK2 processing requires caspase 6, which is a p53 target gene. Thus, p53 drives a positive feedback loop to potentiate HIPK2 apoptotic activity through its caspase-dependent processing in response to severe genotoxic stress [138].

p53 also negatively regulates HIPK2 function in response to sub-lethal DNA damage. Consistently, p53-deficient cells are found to express HIPK2 at elevated level and artificial expression of p53 drives proteasome mediated HIPK2 degradation [131, 139]. In line with these observations, HIPK2 is degraded in a manner depending on p53 and proteasome during the recovery phase from mild DNA damage. It is reported that p53-inducible E3 ubiquitin ligase MDM2 (murine double minute 2) acts as a p53-induced negative regulator of HIPK2, targeting HIPK2 for ubiquitination and degradation during mild DNA damage. MDM2 and its human homolog HDM2 are shown to interact with HIPK2 upon DNA damage and proposed to mediate HIPK2 poly-ubiquitination and degradation during sub-lethal DNA damage.

In addition, HIPK2 is reported as an unstable protein with a high turnover in unstressed cells due to ubiquitin mediated degradation, which is independent of p53 activity. There are three E3 ubiquitin ligases reported to regulate HIPK2

expression levels independent of p53 activity: the WSB-1 and the ring family ligases Siah-1 and Siah-2 [133, 135, 139]. Siah-1/2 associate with HIPK2 through their C-terminal region, which contains it substrate-binding domain, and directly mediates HIPK2 to poly-ubiquitylation. In addition, HIPK2 and Siah-1 co-localize under native and over-expression conditions in nuclear bodies, suggesting that HIPK2 ubiquitination may take place at nuclear bodies [132, 135, 139]. Similarly, WSB-1 also forms a complex with HIPK2 and is capable of triggering HIPK2 ubiquitination and degradation [133].

1.4.4 HIPK2 and TAK1

It is proposed that HIPK2 mediates Wnt-1 induced regulation of c-Myb in line with TAK1-NLK signalling [140-143]. In 2004, Chie, K.I. *et al* reported that HIPK2 interacts with NLK and c-Myb to form a complex. Upon Wnt-1 stimulation, TAK1 activates HIPK2, which in turn activates NLK and recruits c-Myb. In HIPK2-NLK-c-Myb complex, c-Myb is phosphorylated and subsequently degraded, resulting in the inhibition of c-Myb dependent transcription. Given the facts that TAK1-TAB1 complex phosphorylates HIPK2 *in vitro* and HIPK2 phosphorylates and activates NLK *in vitro*, Chie, K.I. proposed the TAK1-HIPK2-NLK signalling cascade axis in Wnt-1 stimulation. However, there has been no further progress on this topic. Instead, there have been reports that TAK1 complex may act directly above NLK, phosphorylating and activating NLK [142, 143]. Thus the physiological functions of NLK-HIPK2 and HIPK2-TAK1 interaction are unclear.

1.5 Objective

As the key kinase in LPS induced signaling cascade, TAK1 has been reported for its roles in NFκB activation, MAPK activation, as well as regulation response to reactive oxygen species and hyperosmotic stress. The regulation of TAK1 is extremely important for cell response to a variety of stimulations.

With increased understanding of the immune response, it has been reported that TAK1 carries on new roles to mediate proper cell response in physiological environment. More and more data suggest that the stimulations which activate immune response also activate mTOR pathway [93, 97, 144]. mTOR pathway is considered as one of the most important regulation machineries of cell growth. mTOR pathway has been studied for its critical clinical application in tumorigenesis and immune tolerant for long time. Given the importance of TAK1 in inflammation signaling activation and mTOR pathway in cell growth regulation, respectively, we propose the connection between inflammatory signaling induced TAK1 activation and the upregulation of mTOR pathway. By biochemical and molecular biology approaches, we carry on to investigate the connection between inflammatory signaling and mTOR pathway activation. We look forward to finding out the molecular joint points of these two pathways by Mass spectrometry and predict the post-translational modification among these molecules would be critical for the conversation. The physiological significance of merging of these two signaling pathway is also expected since TAK1 regulates cytokine synthesis while mTOR pathway control cell growth.

On the other hand, our knowledge on regulation of classic TAK1 pathway has also been developed in past few years. One of the reported regulation partner of

TAK1 is HIPK2, which is deeply involved in tumor suppression and apoptosis. Since the discovery of this interaction, its functional characterization has been puzzled. In this study, with shRNA approach to knock down HIPK2 and traditional UV-irradiation to activate HIPK2, we expect to extend the understanding the role of HIPK2 in regulating TAK1 mediated inflammatory signaling cascade.

Chapter II Materials & Methods

2.1 Materials

[^{32}Pγ]-ATP (150 mCi/ml), glutathione sepharose 4B were from Amersham (Little Chalfont, UK); Protein G agarose, Braford assay reagent, ECL reagent for western blot were from Pierce; 3-8% Pre-cast gel, plasmid Mini/Mega Prep kits and TRIzol® reagent were from Invitrogen, DMEM media and fetal bovine serum were from Hyclone; Nitrocellulose membrane was from Schleicher & Schuell (Dassel, Germany); Fugene®6 transfection reagent and protease inhibitor were from Roche; Reagent λ phosphatase was from New England Biolabs Inc; trypsin, G418, IGF, TAK1 inhibitor (5Z-7-Oxozeaenol), p38 inhibitor (SB203580), PD95058, rapamycin, Wortmamnine, BX759 and IKKβ inhibitor (BAY11-7082) were all purchased from Sigma; all other chemicals were purchased from either Merck (Darmstadt, Germany), Sigma or USB (Cleveland, Ohio, U.S.A.). EDTA free complete protease inhibitor was from Roche (Mannheim, Germany). LPS, TNFα and IL-1β were purified in our lab.

Buffers - Buffer A [50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 1% (w/w) Triton X-100, 1 mM Na_3VO_4, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol plus 1 tablet/50 ml of EDTA free complete protease inhibitor cocktail]. Buffer B [50 mM Tris/HCl pH 7.5, 0.27 M sucrose and 0.1% (v/v) 2-mercaptoethanol].

2.2 Antibodies

Ubiquitin antibody (P4D1), GST antibody (Z5), HA antibody (Y-11), Myc antibody (9E10) and TSC2 antibody (C20) were from Santa Cruz (Santa Cruz,

CA, USA); Flag antibody (M2) was from Sigma. TAK1 antibody has been described previously (Cheung, *et al.*, 2003). Sheep-, rabbit-, or mouse-specific secondary antibodies conjugated to horseradish peroxidase were from Pierce (Rockford, IL, USA). Antibodies recognising p38α, JNK1 & 2, IκB, Harmatin, and the phosphorylated forms of p38α (pT180/pY182) and IκB (pS32), p70S6K (pT389), 4EBP1 (pT70), Akt(pS308), Akt(pT473), PDK(pS241), TSC2(pS939), TSC2(pT1254), TSC2(pT1462), TSC2(pY1571) and TAK1 (pT187) antibodies were purchased from cell signalling (Beverly, MA, U.S.A.). Antibody recognising the phosphorylated forms of JNK 1&2 (pT183/pY185) was from BioSource (Carlsbad, California, U.S.A.). Sheep anti-TAK1 antibody recognizing the [CKKQLEVIRSQQQKRQGTS] at C terminus was custom made (Biogenes, Berlin, Germany).

2.3 DNA Plasmids

The following plasmids were used in the study of Result Part: pEBG2T- GST, pEBG2T-GST-TAK1, pEBG2T-GST-TAK1(345-579), pEBG2T-GST-TAK1(18-370), pEBG2T-GST-HIPK2, pEF6-HA-TAB2, pEF6-HA-TAB3, pCMV5-Myc-TAK1, pCMV5-FLAG-TAB1, pCMV5-Myc-p38α, pCMV5-Myc-IKKβ, pCMV5-Myc-HIPK2, pCMV5-Myc-NLK, pCMV5-FLAG-TSC2, pCMV5-FLAG-TSC1, pEF6-HA-TAB3(NZF deletion), pEF6-HA-TAB3 (NZF/CUE deletion), pEF6-HA-Ubi. All mammalian expression plasmids used in this study contain N-terminal FLAG, GST, HA or myc epitope tags. GST-TSC2-(910-1112), GST-TSC2-(1300-1400), GST-TSC2-(1397-1807) and MKK6-MBP were used for purification of protein in *E.coli*. pSUPER-GFP-TAK1(88) was constructed by cloning the sequence 5'-[ccgagatcgactaca aggagattcaagagatctccttgtagtcgatctcttttt]-3' into the BglII/HindIII site of

pSUPER-GFP. All plasmids were constructed using standard molecular biology techniques. The DNA templates encoding MKK6 (clone ID 4499772) was obtained from the IMAGE consortium (Open Biosystems, Huntsville, AL, USA). The templates of TSC1 and TSC2 are kindly provided by Dr. John Blenis (Department of cell biology, Harvard Medical School, USA). Plasmids encoding shRNA targeting HIPK2 were purchased from Sigma.

2.4 Cell culture and stimulation

HEK293, 3T3L1R, MCF7, wild type and TAK1 Deficient mouse embryonic fibroblast cells were cultured in DMEM supplemented with 10% FCS (Hyclone) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). The cells were incubated at 37°C with a humidified atmosphere of 5% CO_2. Prior to stimulation with TNFα and IL-1β, cells were incubated in starve media contain only DMEM and antibiotics. After 16 h, the cells were stimulated with 50 ng/ml of either TNFα or IL-1β. RAW264.7 cells were cultured in DMEM supplemented with 10% inactivated FCS (Hyclone) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). RAW264.7 cells were stimulated with LPS at 100 ng/ml. Cells were then harvested at different timepoints with buffer A and used immediately or stored at -20°C until required. HEK293, 3T3L1R, MCF7 cell lines were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Wild type and TAK1 deficient mouse embryonic fibroblasts are kindly provided by Dr. Shizuo Akira (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Japan)

2.5 Expression of proteins in HEK 293 cells by transfection

HEK293 cells were transfected by a modified calcium phosphate method[145]. Plasmids were used at 20 μg DNA/ 10 ml media per 10 cm Petri dish. Cells were harvested by lysis in buffer A. The expression of proteins was determined by resolving on SDS-PAGE and western blot analysis.

2.6 Generation of stable TAK1 knockdown cell lines

RAW 264.7 cells were transfected with shRNA by Fugene®6 transfection Reagent as described by manufacturer (Roche). Cells expressing shRNA targeting TAK1 or control GFP vector were selected with media containing G418 500 μg/ml for 7-10 days. Surviving single cells were picked as single colonies into 96-well plates and subcultured in selective media containing G418 for further experiments.

2.7 GST pull down and immunoprecipitation assay

Cells were harvested by lysis buffer A. An aliquot of 1 mg of supernatant cell lysate was added to 10 μl glutathione-sepharose beads for GST pull down assay or 2 μg of antibody coupled with 10 μl of Protein G–sepharose for immunoprecipitation assay. Following the incubation for 1 h at 4°C, the suspension was centrifuged, the supernatant was discarded and the beads were washed twice with 1 ml of buffer A containing 0.5 M NaCl. Followed by two washes with 1 ml of buffer B, the proteins were boiled at 72 °C for 10 min in sample buffer and loaded on SDS-PAGE and identified by Western blot analysis.

2.8 *In vitro* kinase assay

The purified TSC2 truncates and MKK6-MBP were incubated at 30°C with 40 ml of 50 mM Tris-HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 0.1 mM EGTA, 0.1 mg/ml BSA, 10 mM magnesium acetate and 0.1 mM [$^{32}P\gamma$] ATP (10^6 c.p.m./nmol) and 2 µl of purified TAK1 complex. The total volume of 30 µl reaction was terminated for the time indicated by the addition of 10 µl sample buffer and resolved on SDS-PAGE. The phosphorylated substrates were visualized via autoradiography.

2.9 *In vitro* dephosphorylation assay

Following the immunoprecipitation with anti-TSC2 antibody, the bound proteins were treated with 400 units of λ-phosphatase (λ-PPase) which can remove phosphate groups from serine, threonine or tyrosine residues in proteins. The reaction was carried out with 1 µl of λ-PPase (400,000 units/ml) in 1 x λ-PPase Reaction Buffer Pack (50 mM Tris-HCl, 100 mM NaCl, 2 mM dithiothreitol, 0.1 mM EGTA, 0.01 % Brij 35, pH 7.5), 1 mM $MnCl_2$ for 60 min at 30°C. Then, the proteins were resolved on SDS-PAGE and visualized by Western Blotting.

2.10 Western blotting

The lysate was subjected to SDS-PAGE and gels were transferred to nitrocellulose membrane in transfer buffer (500 mM Glycine, 50 mM TrisHCl, 0.01% SDS, 20% methanol) at 70 V for 3 h using an electroblotting apparatus. Membranes were washed in TBS-T (50 mM TrisHCl, 100 mM NaCl, 0.1% Tween at pH 7.4) and then blocked with 5% non-fat milk extract in TBS-T for

1h. Membranes were exposed to primary antibody in 5% non-fat milk prepared in TBS-T for 3 h at 4°C for overnight. Membranes were washed with TBS-T three times for ten minutes. Then the membranes were incubated with appropriate secondary antibodies coupled to horse radish peroxidase (HRP) for 1h at room temperature. After washing the membranes three times for ten minutes, signals were visualized using Western Pico Super ECL reagent (Pierce).

2.11 Real time PCR

Total RNA was extracted from RAW264.7 cells using TRIzol® Reagent according to the manufacturer's instructions. 2 μg of total RNA was reversed transcribed into cDNA for 1 h at 50°C using oligo dT primer and reverse transciptase in the presence of RNAse inhibitor. Transcribed cDNA template (50 ng) was incubated with 200 nM primers in a total volume of 20 μl using KAPA SYBR FAST qPCR kit. The sequences of primers were listed in TABLE2. GAPDH was used as internal control to minimize the error of different input amount.

Every experiment was duplicated. The reading at time point zero without rapamycin treatment was considered as internal control: the one fold; readings at other time points with or without rapamycin treatment were compared with this standard to calculate the increased fold number.

Table2 The sequence of primers for every cytokine in RT-PCR

Number	Primer sequence	Cytokine	species
A1	GCGGCATGTTCTGGATTTGACTC	IL-2	Mouse
A2	CCACCACAGTTGCTGACTCATC	IL-2	Mouse
A3	ATCATCCCTGCGAGCCTATCCT	CXCL10	Mouse
A4	GACCTTTTTTGGCTAAACGCTTTC	CXCL10	Mouse
A5	GGATGTGACCTTCCTCAGACTC	IFNAlpha	Mouse
A6	ACCTTCTCCTGCGGGAATCCAA	IFNAlpha	Mouse
A7	GCCTTTGCCATCCAAGAGATGC	IFNBeta	Mouse
A8	ACACTGTCTGCTGGTGGAGTTC	IFNBeta	Mouse
A9	CAGCAACAGCAAGGCGAAAAAGG	IFNGamma	Mouse
A10	TTTCCGCTTCCTGAGGCTGGAT	IFNGamma	Mouse
A11	CGGGAAGACAATAACTGCACCC	IL10	Mouse
A12	CGGTTAGCAGTATGTTGTCCAGC	IL10	Mouse
A13	ACGAGAGTTGCCTGGCTACTAG	IL12A	Mouse
A14	CCTCATAGATGCTACCAAGGCAC	IL12A	Mouse
A15	TTGAACTGGCGTTGGAAGCACG	IL12B	Mouse
A16	CCACCTGTGAGTTCTTCAAAGGC	IL12B	Mouse
A17	ACGGCTGAGTTTCAGTGAGACC	IL1Alpha	Mouse
A18	CACTCTGGTAGGTGTAAGGTGC	IL1Alpha	Mouse
A19	TGGACCTTCCAGGATGAGGACA	IL1Beta	Mouse
A20	GTTCATCTCGGAGCCTGTAGTG	IL1Beta	Mouse
A21	TACCACTTCACAAGTCGGAGGC	IL6	Mouse
A22	CTGCAAGTGCATCATCGTTGTTC	IL6	Mouse
A23	GGTGCCTATGTCTCAGCCTCTT	TNFalpha	Mouse
A24	GCCATAGAACTGATGAGAGGGAG	TNFalpha	Mouse

(the odd number indicates the forward primers, the even number indicates the backward primers)

2.12 MTT assay

Cells were seeded at 96 well plate as 2500 cell per 100 μl media per well. 12 hour later, cells were treated with LPS, IGF, TNFα or IL-1β respectively at the indicated dose alone or in addition of 100 nM rapamycin for 48 hours. At the time point when stimulation is finished, 20 μl 5 mg/ml MTT solution was added into media and incubated with cells for 2 hour. After that, all the solution in plate was discarded; another 100 μl of DMSO was added into plate to dissolve MTT crystal. 20 minutes later, plates were read by plate reader for their absorbance at wavelength 590 nm.

2.13 Immunostaining

Cells were seeded on sterilized cover slip at 30% confluence in 6 well-plate. 24 hour later, cells were transfected with plasmids encoding interesting protein. 18 hour later, cells were changed into fresh media and cultured for another 30 hour. Then, cells were fixed with 1 ml 4% paraformaldehyde in PBS at room temperature for 10 minutes. Briefly washed with PBS, cells were permeabilized with 0.5 % Triton X 100 in PBS for 5 minute. After brief wash with PBS again, cells were blocked with 1% BSA at RT for 30 minutes, followed by being probed with the primary antibody against first target protein at 4°C for overnight and its secondary antibody at RT for 1 hour. After another round of brief wash with PBS, cells were continuously probed with primary antibody against second target protein at 4°C for overnight and its secondary antibody at RT for 1 hour. Cells were then stained with DAPI and covered with glass slides. The cover slip and glass slides were sealed and samples were kept in 4 °C in dark. The sample then is ready for being visualized under confocal laser scaning microscope (Zeiss Laser Scanning Microscope 710 system).

Chapter III Results

Part I TAK1 and mTOR pathway

3.1 Identification of TSC2 as TAK1 binding partner

3.1.1 TSC2 is identified as TAK1 binding partner by IP-MS approach

TAK1 is a critical protein in TLR4, TNFR and IL-1R mediated signalling pathways, playing an important role in innate immune response. Along with the increase of understanding on TAK1signaling pathway, it has been reported that TAK1 involved in other physical conditions by interacting with different proteins. To look for the novel signaling pathway in which TAK1 may be involved, Mass spectrometry approach was applied. HEK293 cells were transfected with GST-TAK1 or GST plasmids. After 36 hours, cells were harvested and cell lysate was subjected to GST pull-down with glutathione-sepharose beads. The pull down complex was then separated on SDS-PAGE and stained with Coomassie Brilliant Blue. Compared with pulldown complex from GST plasmid transfected cells, the protein bands specifically appeared in pulldown complex from GST-TAK1 but not GST expressing cells were excised and processed for liquid chromatography mass spectrometry (LC-MS), as shown in Figure 2.1.

The identities of proteins with their sequence coverage from selected bands are listed in Table3. In GST-TAK1 pulldown complex, the mTOR switch protein---Tuberous Sclerosis Complex 2 (TSC2) was identified. TSC2 is the upstream kinase of mTOR pathway. TSC2 tightly interacts with its partner TSC1 and functions as GTPase Activating Protein (GAP) to alter activity of GTPase Rheb, which only activates mTOR activity in its GTP-binding formation. Thus, TSC2

negatively regulates mTOR activation. The identification of TSC2 from GST-TAK1 pulldown complex indicated the potential connection between TAK1 and TSC2.

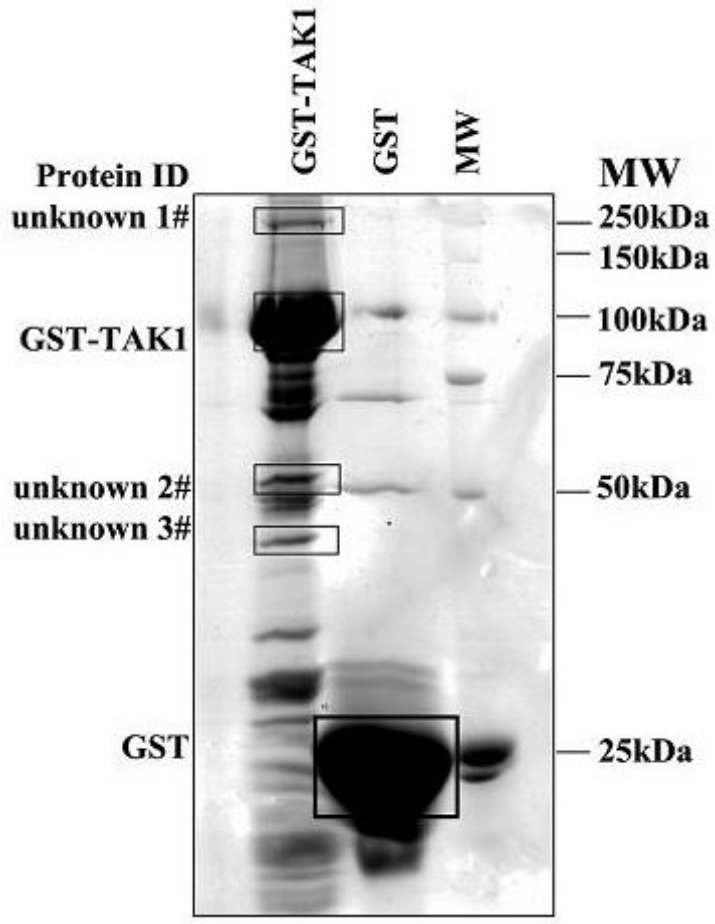


Figure 2.1 The interacting proteins from GST-TAK1 pulldown complex

HEK293 cells were transfected with either GST-TAK1 or GST alone. 36 hours after transfection, cells were harvest and lysate was subjected to GST pulldown. The pulldown complex was separated on SDS-PAGE. The gel was then stained with Coomassie blue. The protein bands from GST-TAK1pull down complex were processed for LC- MS.

Table.3 The identity of proteins which bind to GST-TAK1.

Band No.	Protein identified	Molecular Weight	Sequence Coverage (%)	Peptide Score	Notes
1	ACACA	267	14	690	ACACA is a biotin containing enzyme which catalyzes the carboxylation of acetyl-CoA, the rate-limiting step in fatty acid synthesis
1	SNRNP200	246	11	483	RNA helicase involved in second step of RNA splicing
1	TSC2	200	12	393	Is a tumour suppressor, negatively regulating mTORC1 signalling. Acts as a GTPase-activating protein for Rheb.
2	IMPDH2	52	12	51	Inosine monophosphoate dehydrogenase 2
3	DNAJA1	46	47(8)	863(89)	DnaJ Homolog subfamily A member1,co-chaperone
3	ILF2	43	50(28)	633(229)	Interleukin enhancer-binding factor 2 (NFAT), a transcription factor required for T-cell expression of the interleukin 2 gene

3.1.2 Over-expressed TSC2 is co-purified with TAK1-GST

To validate the interaction between TAK1 and TSC2, GST-TAK1 and Flag-TSC2 were over expressed in HEK293 cells. The cell lysate was pulled down with GST beads, immunoblotted with Flag antibody and GST antibody respectively. Flag-TSC2 was detected from GST-TAK1 pull-down complex, shown in Figure 2.2A. Immunoprecipitation was also carried out with a TSC2 antibody from HEK293 lysate in which Flag-TSC2 and GST-TAK1 were over-

expressed. By probing with GST antibody, GST-TAK1 was detected from TSC2 IP complex, shown in Figure 2.2 B. Above data supported the interaction between TAK1 and TSC2 in over expression study.

TAK1 exists in a large complex with its binding partners TAB1 and TAB2/TAB3. So far, studies have showed that TAB1 mediates a negative feedback loop for TAK1 kinase activity while TAB2/TAB3 mediates lysine63 linked polyubiquitin chain to facilitate activation of TAK1 upon a series of stimulations. In over-expression studies, TAB2 and TAB3 are expressed at very low levels and require co-expression with TAK1 to enhance their expression. The NZF and CUE domains are ubiquitin binding domain on TAB3 and TAB2. Since TAK1 is bound to TAB1 and TAB2/TAB3 *in vivo*, immunoprecipitation of any component of TAK1 complex will purify the whole complex together. Therefore, we enquired whether the interaction between TAK1 and TSC2 was direct or mediated by other components in TAK1 complex such as TAB1, TAB2 or TAB3. To answer this question, Flag-TSC2 was over expressed with GST-TAB1 or GST-TAK1 alone, or GST-TAK1 with HA-TAB2/TAB3, HA-TAB3(NZF) or HA-TAB3(CUE/NZF). The HA-TAB3(NZF) truncate is missing the NZF domain in its amino terminus while HA-TAB3(NZF/CUE) TAB3 truncate is missing both NZF domain in amino terminus and CUE domain in carboxylic terminus. Since the TAB2 and TAB3 do no express well in the absence of TAK1, they were over expressed with TAK1 to enhance their expression. As shown in Figure 2.2 A. GST-TAB1 alone did not bind to TSC2. Additional TAB2, TAB3 did not significantly change the amount of TSC2 in the pull down complex. Neither did TAB3 truncates reduce the binding of TSC2 to TAK1. These data indicated that TAK1 was the direct binding partner

of TSC2 in the TAK1 complex and that the presence of TAB1, TAB2 and TAB3 in TAK1 complex did not affect interaction between TSC2 and TAK1. In addition, binding of TSC2 and TAK1 was not affected by removal of the ubiquitin binding domains in TAB3.

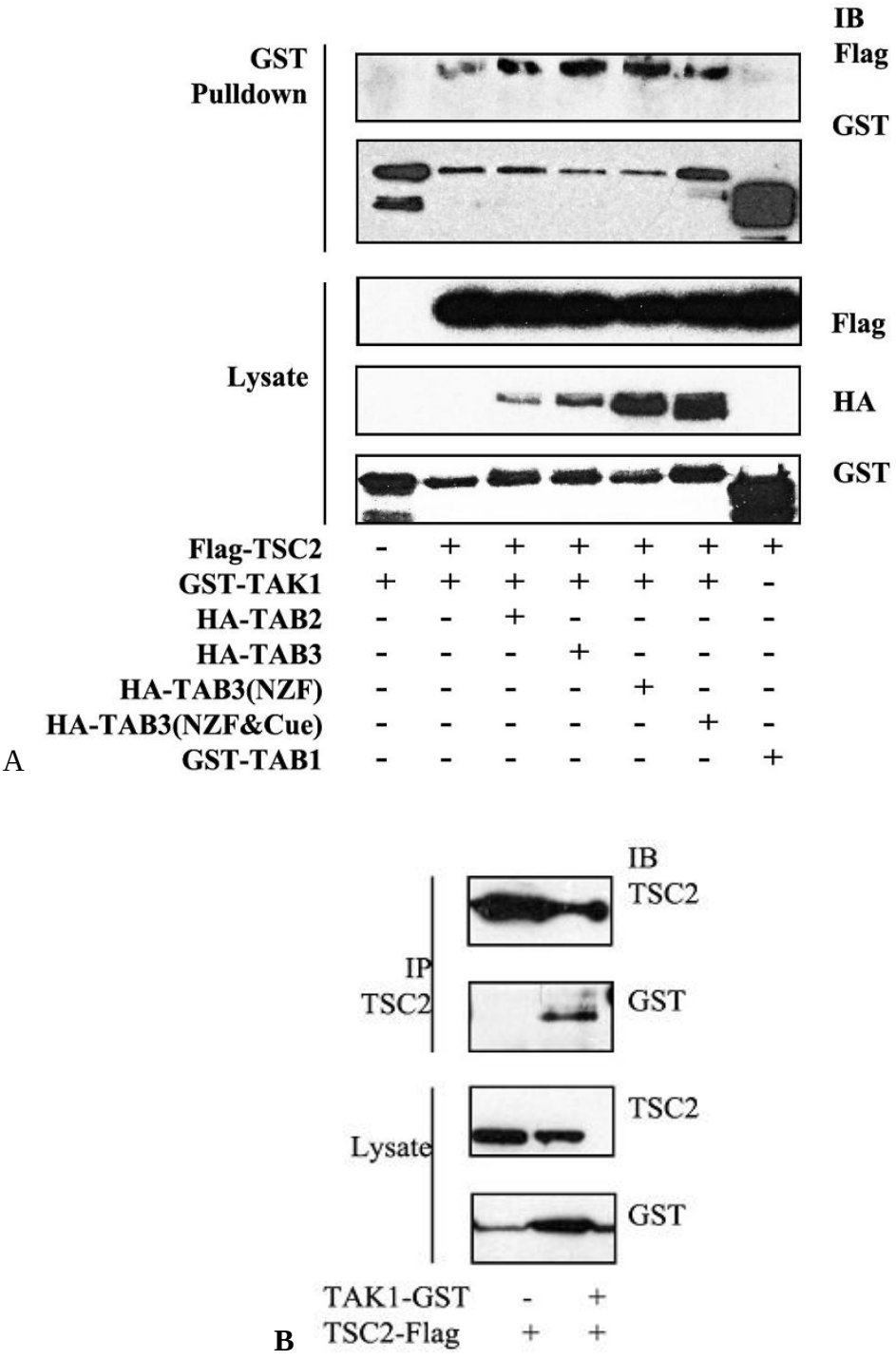


Figure 2.2 Over-expressed TSC2 is co-purified with TAK1

A. Flag-TSC2 is co-purified with TAK1. HEK293 cells were transfected with Flag-TSC2 without or with GST-TAK1 and HA-TAB2, HA-TAB3, HA-TAB3 (NZF) or HA-TAB3 (NZF&CUE). HEK293 cells were either transfected with GST-TAB1 and Flag-TSC2. Cell lysate was subjected to GST pulldown and the proteins separated on SDS-PAGE and immunoblotted with GST and Flag antibodies. The cell lysate was also probed with Flag, HA and GST antibodies to detect the expression of transfected proteins.

B. Flag-TSC2 is co-purified with TAK1. Flag-TSC2 was transfected alone or together with GST-TAK1. Cell lysate was subjected to immunoprecipitation with TSC2 antibody, followed by SDS-PAGE and immunoblotting with TSC2 and GST antibodies. Cell lysate was also probed with TSC2 and GST antibodies to detect the expression of transfected proteins.

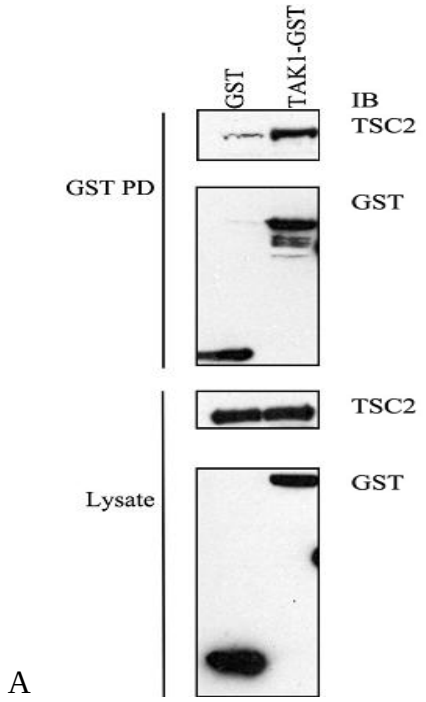
3.1.3 Endogenous TSC2 is co-purified with TAK1

Next, it is important to validate the interaction between endogenous TSC2 and TAK1 under the condition of discovering this interaction.

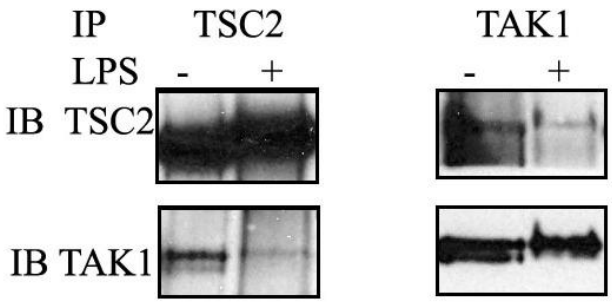
HEK293 cells were transfected with GST-TAK1 or GST vector and cell lysate was subjected to GST pulldown. Endogenous TSC2 was detected from GST-TAK1 pulldown complex while only a weak nonspecific signal appeared in GST alone pulldown complex, as shown in Figure 2.3A. This supported the LC-MS analysis that TSC2 bound to GST-TAK1 in HEK 293 cell lysate. As shown in Figure 2.3C, consistent with over expression studies, when GST-TAK1 was over-expressed with its binding partner TAB2/ TAB3 or TAB3 truncates, the interaction between endogenous TSC2 and TAK1 was not affected. Neither did GST-TAB1 bind to endogenous TSC2. These data further confirmed that TAK1 and TSC2 interacted directly and this association did not require the presence of other components in TAK1 complex.

Given that TAK1 is activated in RAW264.7 cells upon LPS stimulation, we enquired whether LPS stimulation can modulate the association of TAK1 and TSC2. As shown in Figure 2.3B, endogenous TAK1 was detected from TSC2 immunoprecipitation complex in both LPS stimulated and unstimulated cells. The endogenous TAK1 from LPS stimulated sample showed a mobility shift,

indicating that LPS stimulation resulted in the phosphorylation of TAK1 without affecting the binding between TSC2 and TAK1. When the same lysate was subjected to reciprocal immunoprecipitation with TAK1 antibody, TSC2 was detected from TAK1 immunoprecipitation complex in both LPS stimulated and unstimulated cells. TSC2 also exhibited a mobility shift in LPS stimulated sample, as shown in Figure 2.3 B.



A



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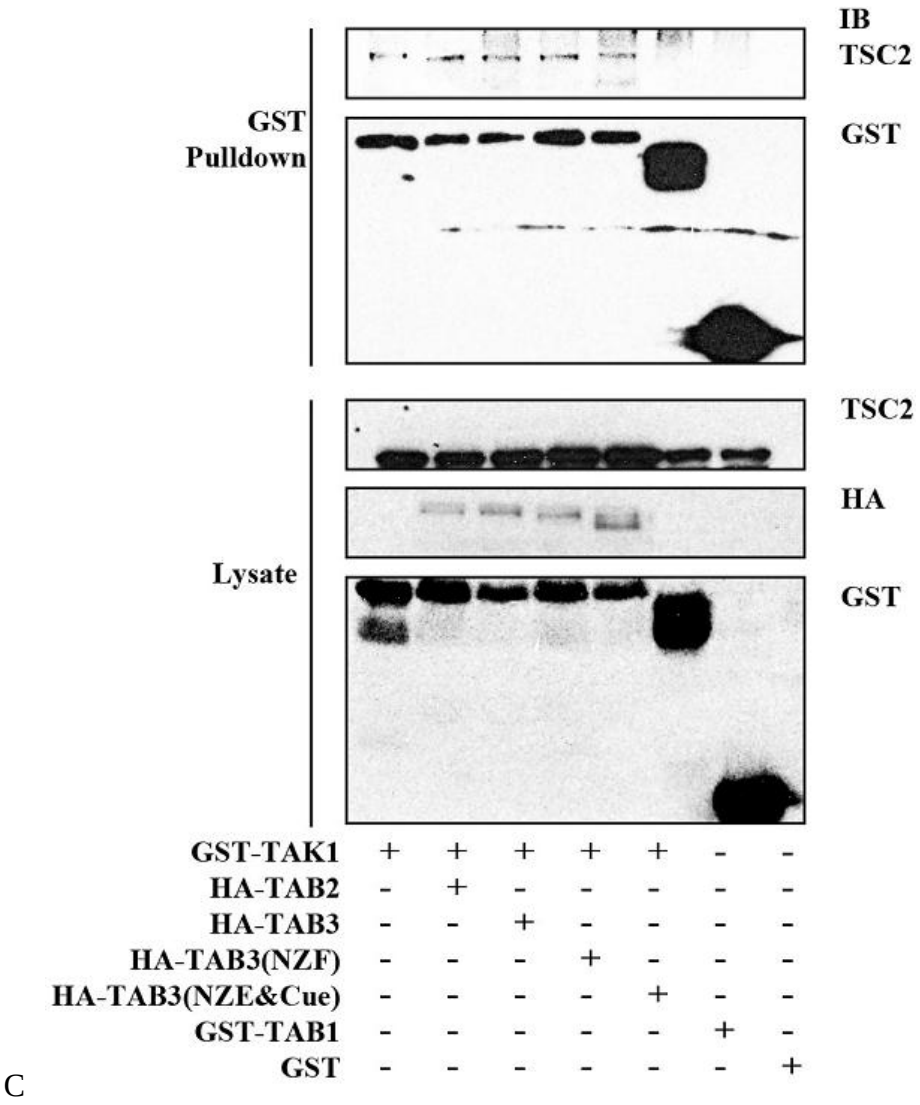


C

Figure 2.3 Endogenous TSC2 is co-purified with TAK1

A Endogenous TSC2 is co-purified with GST-TAK1; HEK293 cells were transfected with GST, GST-TAK1. Cell lysate was subjected to GST pulldown, followed by SDS-PAGE and immunobloting with TSC2 and GST antibodies. The lysate was also probed with TSC2 and GST antibodies;

B Endogenous TSC2 is co-purified with endogenous TAK1; RAW 264.7 cell lysate with or without LPS stimulation was subjected to IP with TAK1 antibody and IB with TAK1 antibodies and TSC2 antibodies, or IP with TSC2 antibody and IB with TSC2 and TAK1 antibodies;

C Endogenous TSC2 is co-purified with GST-TAK1; HEK293 cells were transfected with GST, GST-TAB1 or GST-TAK1 alone or together with HA-TAB2, HA-TAB3, HA-TAB3 (NZF) or HA-TAB3 (NZF&CUE) as indicated at the bottom. Cell lysate was subjected to GST pulldown, followed by SDS-PAGE and immunoblotting with TSC2 and GST antibodies. The lysate was also probed with TSC2 and GST antibodies.

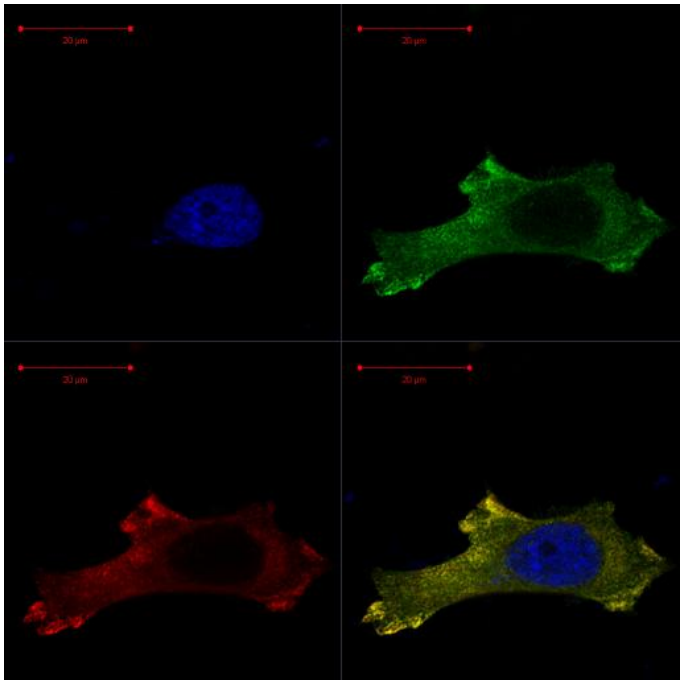
3.1.4 TSC2 and TAK1 are localized together

It has been reported that TSC2 is mainly located at the membrane, where it exerts its GTPase-activating protein activity to repress Rheb signalling [146]. However, in response to certain stimulation, TSC2 is phosphorylated and relocated to the cytoplasm so that the repression of Rheb is relieved. It is also a well-established model that TAK1 stays in cytoplasm to dock with the IRAK1/IRAK4/TRAF6/Ubc13/Uev1A complex, which leads to TAK1 full activation and switches on pathways downstream of TAK1 [147]. This suggests the possibility of that TAK1 and TSC2 encounter each other in cytoplasm. To verify the subcellular location of TSC2 and TAK1, MCF7 cells were transiently transfected with HA-TAK1 and Flag-TSC2 and the locations of the proteins were tracked by immunostaining 48 hours after transfection. As showed in Figure 2.4 A, both HA-TAK1 (labelled in red) and Flag-TSC2 (labelled in green) were mainly found in the cytoplasm. As indicated by the merged effect of two colours, HA-TAK1 and Flag-TSC2 were co-localized together.

The endogenous TAK1 and TSC2 in RAW 264.7 cells were also tracked for their association. Compared with over expressed TAK1 and TSC2, detection of endogenous proteins was relatively weak. Nevertheless, it was clear that endogenous TAK1 and TSC2 were both mainly located at the cytoplasm, as shown in Figure 2.4 B.

Taken together, these data supported the physical interaction between TAK1 and TSC2.

A



B

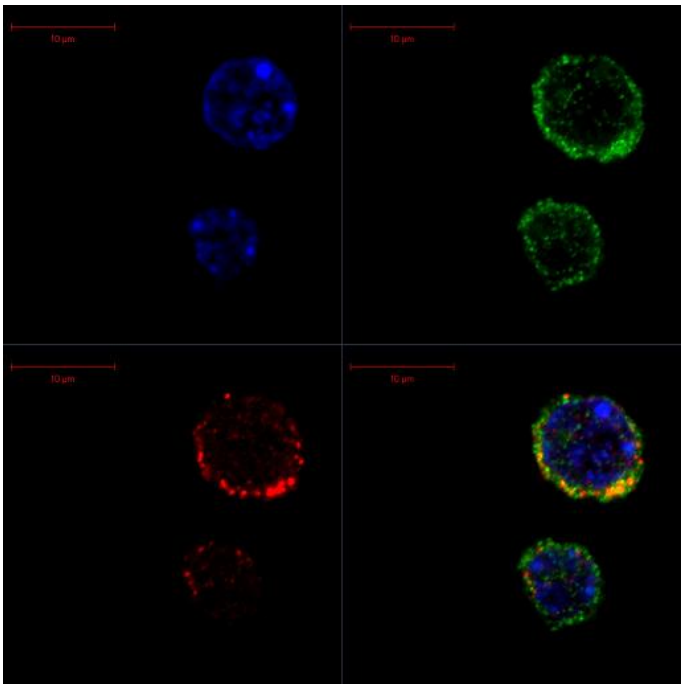


Figure 2.4 Subcellular localization of TSC2 and TAK1

A Subcellular location of over expressed TAK1 and TSC2 in MCF7 cells; MCF7 cells were placed on the glass cover slip, and transfected with HA-TAK1 and Flag-TSC2 via fugene transfection kit; 48 hours after transfection, the cells were fixed and processed for immunostaining. HA-TAK1was labelled in Red while Flag-TSC2 was labelled in Green, sample was visualized under Zeiss LSM710 system;

B Subcellular location of endogenous TAK1 and TSC2 in RAW264.7 cells; cells were placed on the cover slip, 24 hours later, cells were processed for immunostaining, TAK1 was labelled in Green while TSC2 was labelled in Red, sample was visualized under Zeiss LSM710 system.

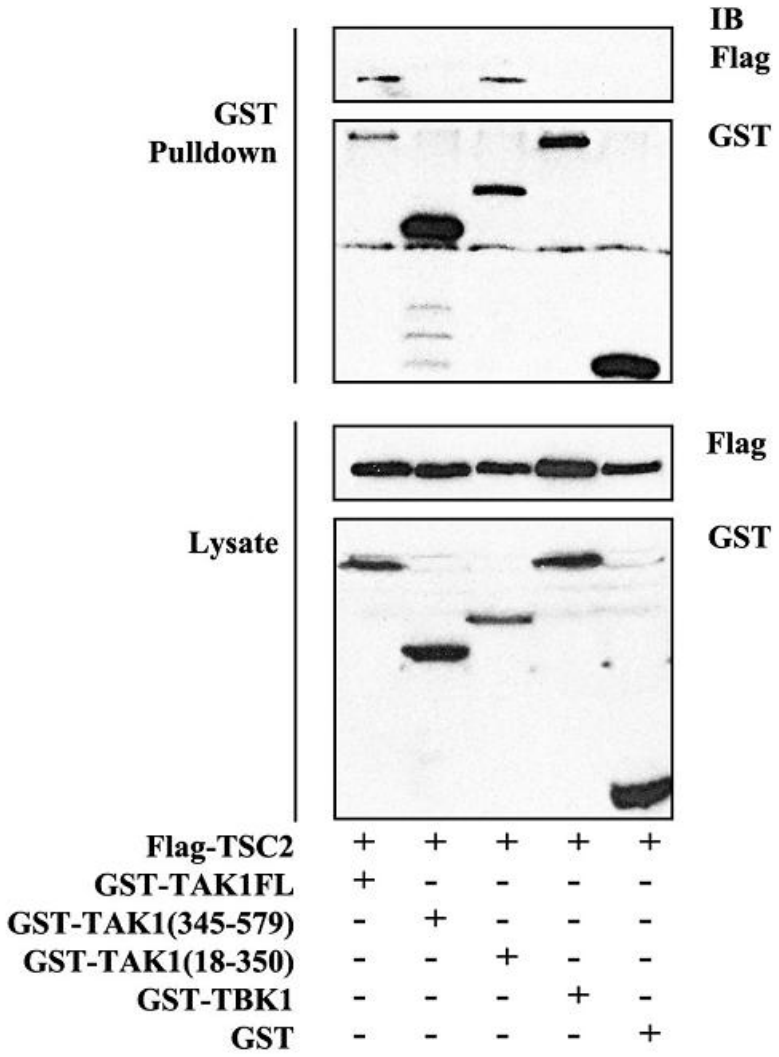
3.1.5 N terminal TAK1is mapped as binding domain for TSC2

TAK1 is a kinase with multiple upstream regulation signals and downstream targets. It interacts with most of its substrate through its kinase domain at amino terminus while it receives the activation signals from polyubiquitin linked TAB2 and TAB3 by its carboxylic terminus. Following the confirmation of interaction between TAK1 and TSC2, we examined which domain in TAK1 was responsible for its binding with TSC2. GST tagged full length TAK1, TAK1 amino-terminus, TAK1 carboxylic-terminus as well as control protein TBK1 were transfected with Flag-TSC2 into HEK293 cells. The cell lysate was subjected to GST pulldown and immunoblotted with Flag antibody to detect the TSC2 binding. As shown in Figure 2.5A, with the comparable amount of input GST tagged proteins; Flag-TSC2 was only detected in GST-TAK1 full length and GST-TAK1 amino-terminus pulldown complex, indicating that the amino terminus of TAK1 was responsible for the interaction with TSC2.

In the parallel experiment, the interactions between GST tagged TAK1 truncates and endogenous TSC2 were tested. Consistent with over expression results, endogenous TSC2 was only detected from pulldown complex of full length TAK1 and amino-terminus of TAK1. No endogenous TSC2 was detected in the pulldown complex of carboxylic terminus of TAK1 as well as control proteins TBK1, IKKα, IKKβ and IKKε, as shown in Figure 2.5B.

Collectively, these data demonstrated that the amino-terminus of TAK1 which contains the domain of TAK1 bound to TSC2. This suggested that the interaction between TSC2 and TAK1 may be a substrate-kinase relationship.

A


IB
Flag
GST
Pulldown
GST
Flag
Lysate
GST
Flag-TSC2 + + + + +
GST-TAK1FL + - - - -
GST-TAK1(345-579) - + - - -
GST-TAK1(18-350) - - + - -
GST-TBK1 - - - + -
GST - - - - +

B

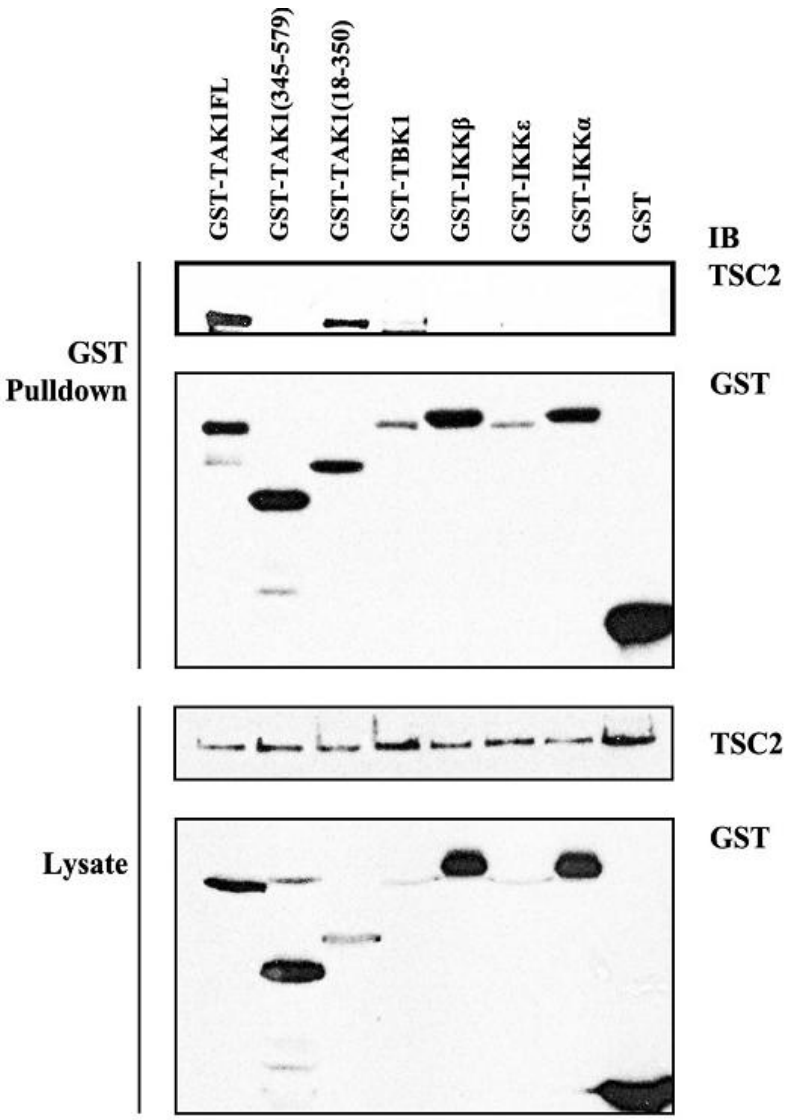


Figure 2.5 TSC2 interacts with the N-terminal TAK1.

A Over expressed TSC2 binds to the N-terminus of TAK1; HEK293 cells were transfected with Flag-TSC2 and GST-TAK full length, GST-TAK1 C-terminus(345-579), GST-TAK1 N-terminus(18-350), GST-TBK1 or GST. Cell lysate was subjected to GST pulldown and immunoblotted with Flag and GST antibodies. Cell lysate was also directly probed with Flag and GST antibodies to check the expression of transfected plasmids.

B Endogenous TSC2 binds to the N-terminus of TAK1; HEK293 cells were transfected with GST, GST-TAK1, GST-TAK1 C-terminus (345-579), GST-TAK1 N-terminus (18-350), GST-TBK1, GST-IKKβ, GST-IKKε, GST-IKKα respectively. Cell lysate was subjected to GST pull down and immunoblotted with TSC2 and GST antibodies. Cell lysate was also directly probed with TSC2 and GST antibodies.

3.2 Phosphorylation of TSC2 by TAK1 in response to LPS

The above studies have shown clearly that TAK1 directly interacted with mTOR pathway regulatory protein TSC2. Given that N-terminal TAK1, which contains the kinase domain, bound to TSC2, it is worth questioning the functional significance of the TSC2-TAK1 interaction.

3.2.1 LPS stimulation induces mobility change of TSC2

Previously in Figure 2.3C, the immunoprecipitated TSC2 from LPS stimulated RAW264.7 cell showed the slower mobility on western blot than the one from unstimulated RAW264.7 cell. The mobility change is a reflection of molecular weight change. The slower the protein moves on SDS-PAGE, the larger the molecular weight is. Thus, this mobility change of TSC2 upon LPS stimulation probably indicated the post-translational modification of TSC2. When TAK1 inhibitor (5Z-7-Oxozeaenol)[148] was applied in RAW264.7 cells prior to LPS treatment, the mobility shift of TSC2 was altered, shown in Figure 2.6A. This implied that TSC2 mobility was specifically changed upon LPS stimulation in a manner depending on TAK1 kinase activity.

3.2.2 λ phosphatase treatment suppresses LPS induced TSC2 mobility change

Given that TAK1 inhibitor altered the mobility shift of TSC2 upon LPS stimulation, it is reasonable to propose that the mobility shift of TSC2 was due to the modification of phosphorylation since TAK1 is a serine/threonine kinase and it is activated upon LPS. To test this idea, TSC2 immunopurified from LPS treated RAW264.7 cell was incubated with λ phosphatase or the control buffer

respectively. As shown in Figure 2.6B, λ phosphatase treatment totally altered the mobility shift of TSC2 upon LPS stimulation, indicating that mobility shift of TSC2 after LPS stimulation was due to phosphorylation modification.

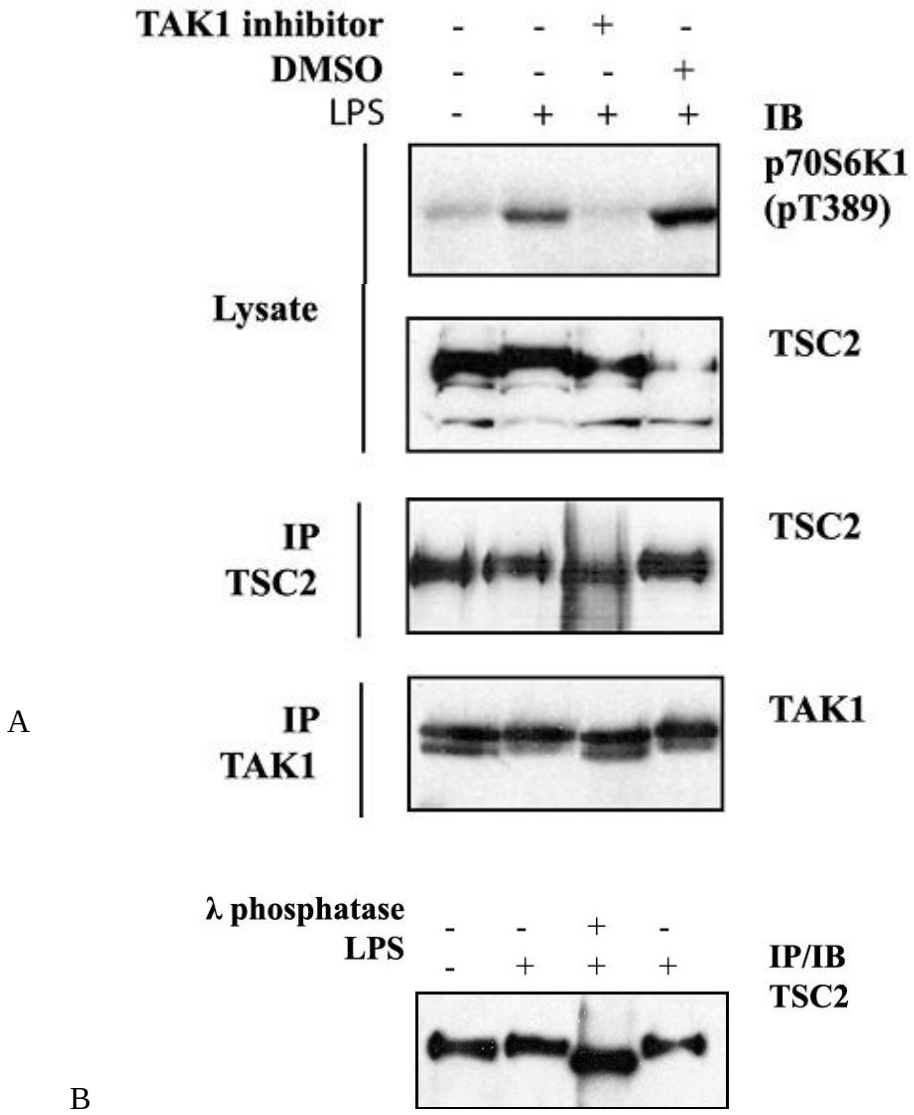


Figure 2.6 LPS stimulation results in TSC2 mobility shift

A RAW 264.7 cells were pre-incubated with 10 μM TAK1 inhibitor or equal volume of DMSO for 1h, followed by stimulation with 100 ng/ml LPS for 1h. Cell lysate was subjected to IP/ IB with TSC2 or TAK1antibodies respectively. Cell lysate was also probed with TSC2 and p70S6K1 (pT389) antibodies.

B LPS stimulated RAW264.7 cell lysate was subjected to IP with TSC2 antibody, followed by λ phosphatase treatment for 1h. Samples were then processed for western blot and probed with TSC2 antibody.

3.2.3 TSC2 is phosphorylated on Thr1462 and Ser939 upon IL-1β, TNFα and LPS stimulations

In addition to LPS stimulation, TNF α and IL-1 β can also induce the activation of TAK1. Thus, it is reasonable to examine the phosphorylation status of TSC2 upon IL-1β and TNFα stimulations.

The previous date using TAK1 inhibitor implied the impact of TAK1 kinase activity on TSC2 phosphorylation. Therefore, the wild type and TAK1 deficient mouse embryonic fibroblast cells (MEFs) were utilised to confirm whether TAK1 mediates phosphorylation of TSC2 upon LPS, TNFα and IL-1β stimulation. As introduced in 2006[149], in Map3K7 $^{flox/flox}$ mice, the floxed genomic fragment was excised by retroviral expression of Cre protein together with green fluorescent protein (GFP). A series of experiments, including southern blotting, RT-PCR and western blotting, demonstrated that endogenous TAK1 gene in this TAK1 deficient cell line was total converted into a deleted version of TAK1, which encoded a TAK1 protein lack of ATP binding domain from residue 40 to 70. The original data also supported that this TAK1 deficient cell line fails to activate NF-κB and AP-1 in response to IL-1β and TNFα [149, 150].

The wild type and TAK1 deficient MEF cells were stimulated with LPS, TNFα and IL-1β, respectively. The cell lysates were resolved on 3~8% pre-cast gradient gel for the better separation of TSC2's mobility. As shown in Figure 2.7A and B, at each stimulation time point, TSC2 from wild type MEFs moved faster in gradient gel than TSC2 from TAK1 deficient MEFs. For LPS stimulation, the mobility shift of TSC2 between TAK1 wild type and deficient

MEFs was clear after 30 minutes stimulation, as shown in Figure 2.7A. For IL-1β stimulation, the mobility shift of TSC2 between two cell lines was obvious at 30 minute and 120 minute stimulation (Figure 2.7B). For TNFα stimulation, the mobility shift of TSC2 was weakly separated after 60 minutes stimulation (Figure 2.7B). These data suggested that LPS, TNFα and IL-1β all induced mobility shift of TSC2 in wild type MEFs while the mobility change of TSC2 was attenuated in TAK1 deficient cells.

The phosphorylation of TSC2 has been intensively studied in past few years. The reported phosphorylation sites on TSC2 are listed in Table4 as reference. Four commercial phosphor-antibodies for TSC2 are available (Cell Signaling Technologies, USA), recognizing the phosphorylation of TSC2 on Ser939, Ser1254, Thr1462 and Tyr1571, respectively. These four antibodies were used to recognize the phosphorylation status of TSC2 after cells were stimulated with LPS, TNFα and IL-1β as well as IGF1, which was used as a positive control for the recognition of phosphorylation on TSC2 Ser939 and Thr1462 according to previous reports.

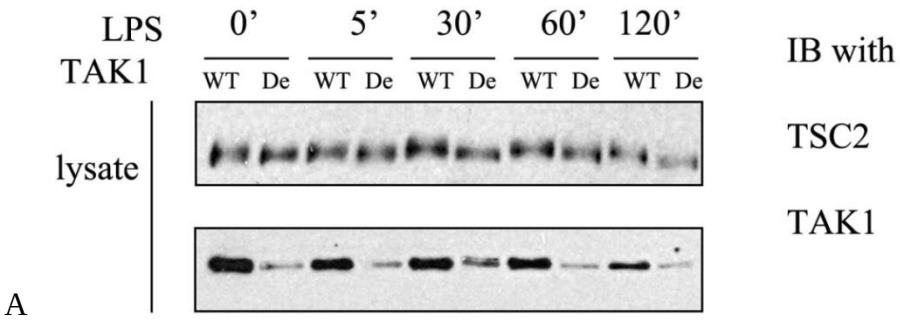
As shown in Figure 2.7F, phospho-antibodies against TSC2 phosphorylation on Ser1254 and Tyr1571 barely recognized the signal from LPS treated wild type or TAK1 deficient MEFs, while phospho-antibodies against TSC2 phosphorylation on Ser939 and Thr1462 recognized the TSC2 in both wild type and TAK1 deficient MEFs upon stimulation with LPS. Importantly, the phosphorylation of TSC2 on Thr1462 was stronger in wild type MEFs than that in TAK1 deficient MEF cells. However, the phosphorylation of TSC2 on Ser 939 was at similar level between wild type and TAK1 deficient MEF cells upon LPS stimulation. Since the phosphorylation of TSC2 on Thr1462 and Ser

939 was previously reported to be the phosphorylation sites of Akt/PKB, the phosphorylation status of Akt/PKB in wild type and TAK1 deficient MEFs was also examined. As shown in Figure 2.7F, Akt was phosphorylated on both Ser308 and Thr473 upon LPS stimulation, while, the activity of Akt was not affected in TAK1 deficient cells as suggested by phosphorylation of Akt on Ser308. These results implied that TAK1 deficient cells exhibited attenuated phosphorylation of TSC2 on Thr1642 upon LPS stimulation, without affecting PI3K-Akt pathway.

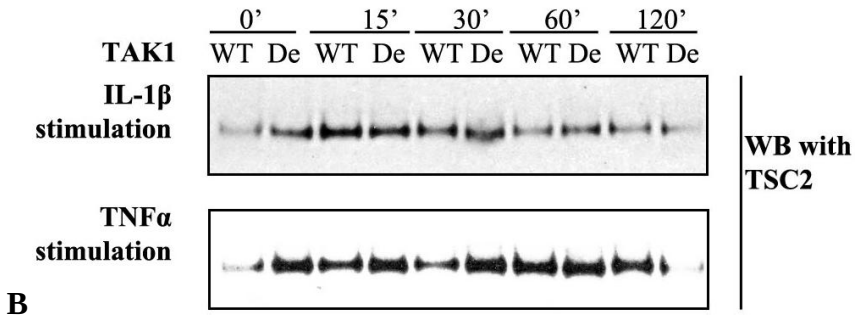
We also observed the reduced phosphorylation of TSC2 on Thr1462 in TAK1 deficient cells upon TNFα and IL-1β stimulations, as shown in Figure 2.7 C and D. In these conditions, the phosphorylation of Akt/PKB on Ser308 and Thr473 was not affected.

However, there was no difference of TSC2 phosphorylation signals between wild type and TAK1 deficient MEF cells upon IGF stimulation, as shown in Figure 2.7E. There was no difference of Akt phosphorylation between wild type and TAK1 deficient MEF cells.

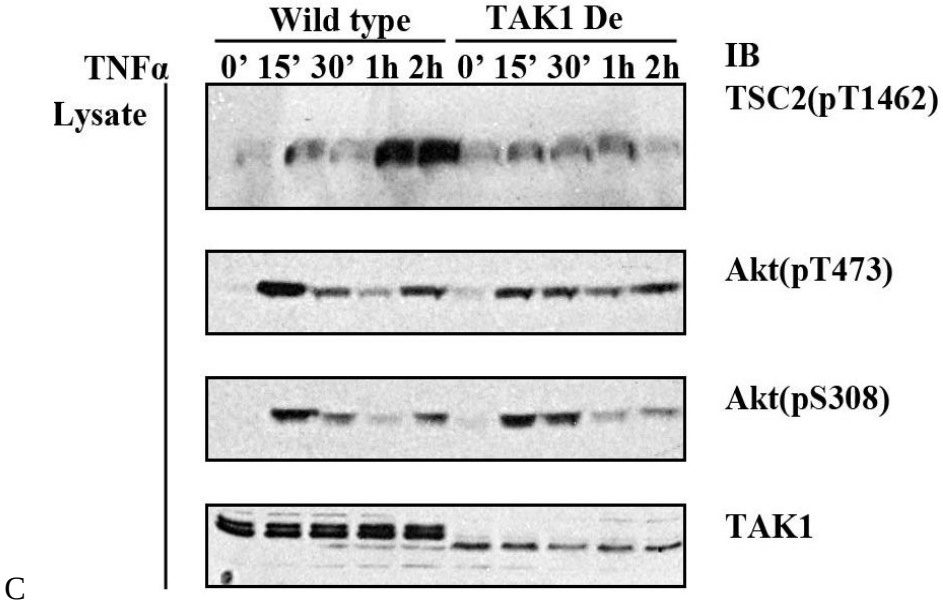
Collectively, these data implied that TAK1 was necessary for phosphorylation of TSC2, especially on Thr1462, in response to LPS, TNFα and IL-1β but not IGF stimulation.


LPS
TAK1
0’
5’
30’
60’
120’
WT De WT De WT De WT De WT De
lysate
IB with
TSC2
TAK1

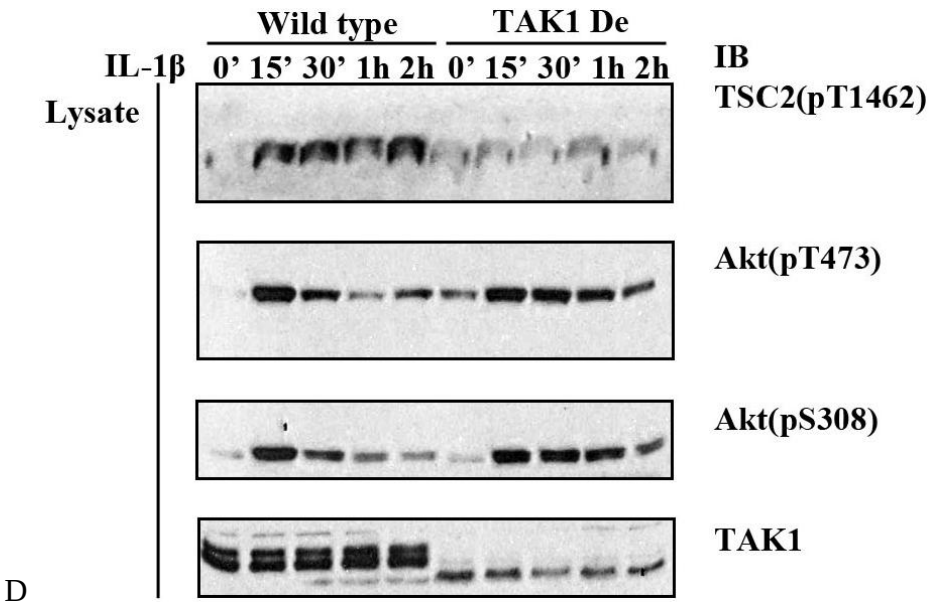
A


0’
15’
30’
60’
120’
TAK1
WT De WT De WT De WT De WT De
IL-1β
stimulation
TNFα
stimulation
WB with
TSC2

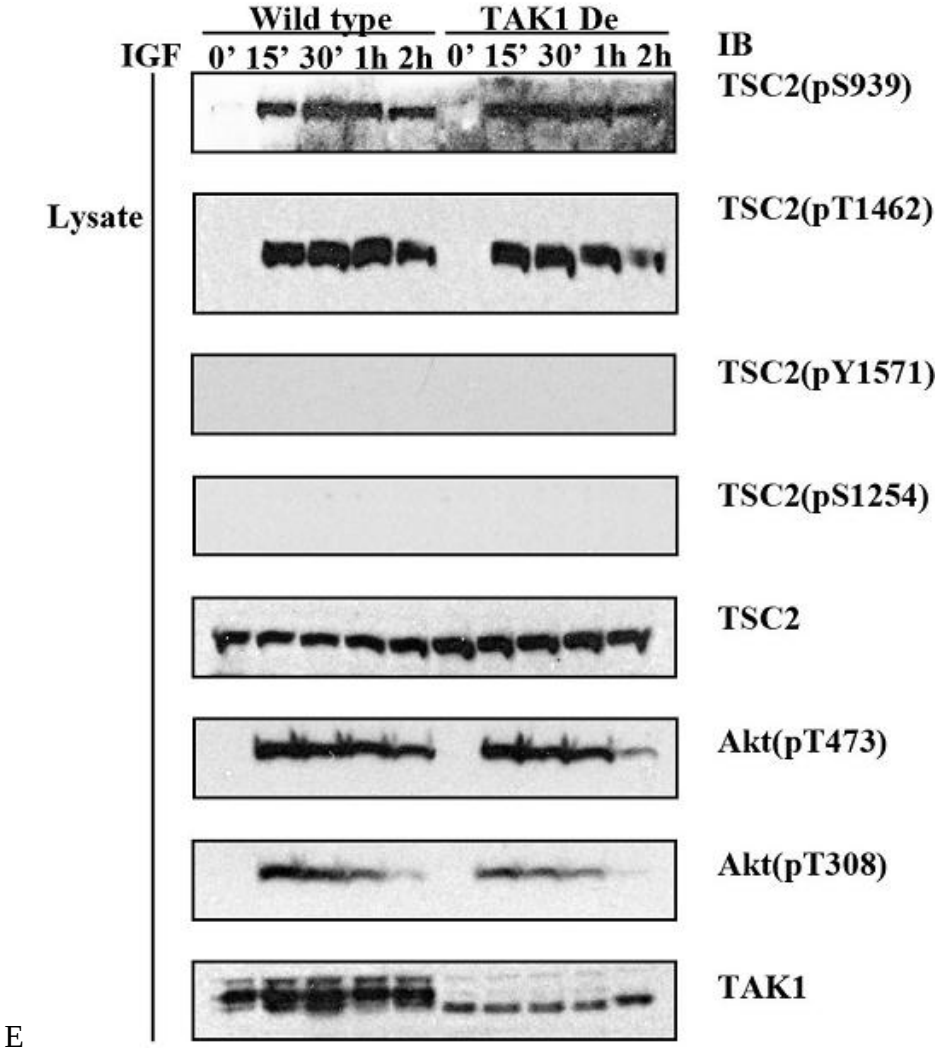
B


Wild type
TAK1 De
TNFα
0’ 15’ 30’ 1h 2h 0’ 15’ 30’ 1h 2h
Lysate
IB
TSC2(pT1462)
Akt(pT473)
Akt(pS308)
TAK1

C


Wild type
TAK1 De
IL-1β
0' 15' 30' 1h 2h 0' 15' 30' 1h 2h
IB
Lysate
TSC2(pT1462)
Akt(pT473)
Akt(pS308)
TAK1

D


Wild type
TAK1 De
IGF
0' 15' 30' 1h 2h 0' 15' 30' 1h 2h
IB
Lysate
TSC2(pS939)
TSC2(pT1462)
TSC2(pY1571)
TSC2(pS1254)
TSC2
Akt(pT473)
Akt(pT308)
TAK1

E

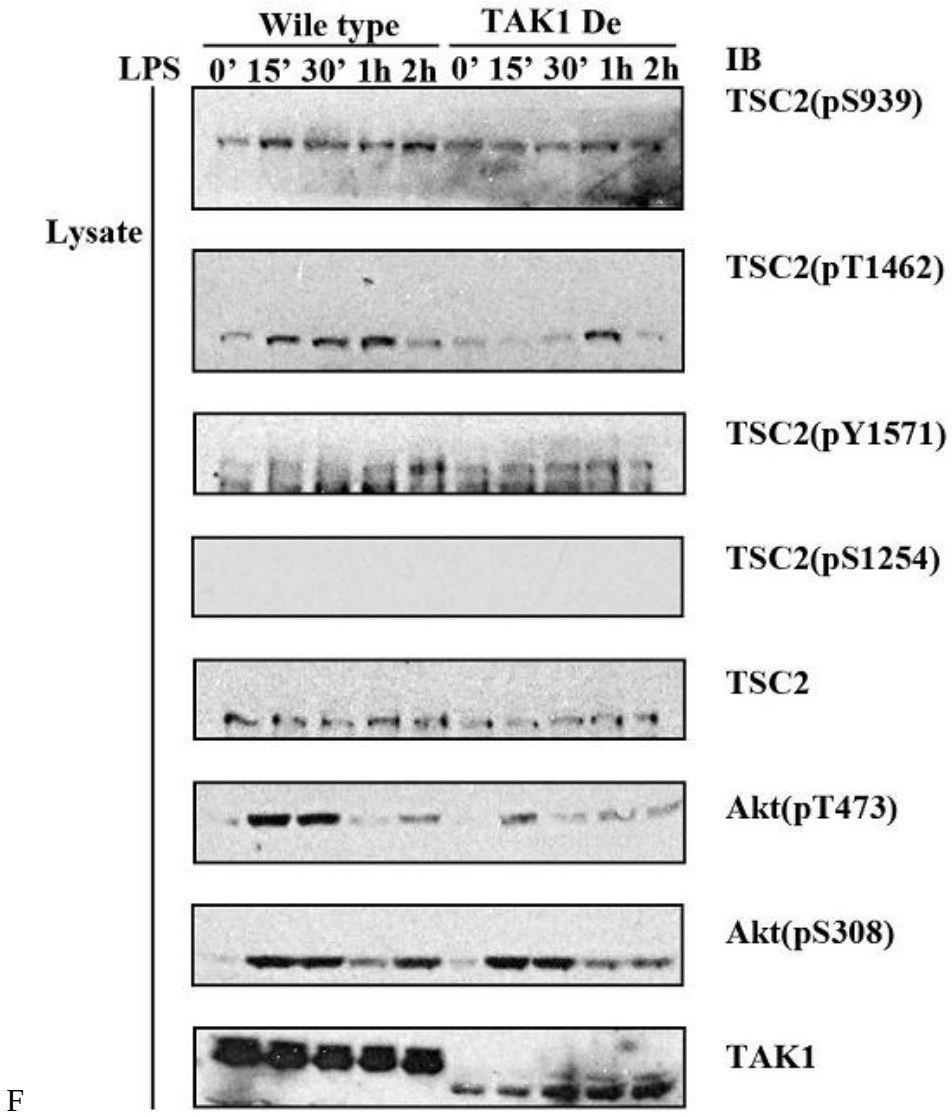


Figure 2.7 TSC2 is phosphorylated upon IL-1β, TNFα and LPS stimulations.

A and B The mobility of TSC2 in wild type and TAK1 deficient MEFs is different upon various stimulations. Wild type and TAK1 deficient cells were stimulated with 200 ng/ml LPS, 100 ng/ml TNFα or 100 ng/ml IL-1β for the indicated time. Cell lysate was processed for western blot and probed with TSC2 antibody.

C and D The phosphorylation of TSC2 upon TNFα or IL-1β stimulations is different in wild type and TAK1 deficient MEFs. Wild type and TAK1 deficient MEFs were stimulated with 100 ng/ml TNFα or IL-1β for the indicated time. Cell lysate was then processed for western blot and probed with TSC2(pT1462), Akt (pT473), Akt (pS308) and TAK1 antibodies.

E The phosphorylation of TSC2 upon IGF stimulation is different in wild type and TAK1 deficient MEFs. Wild type and TAK1 deficient cells were stimulated with 25 ng/ml IGF for the indicated time. Cell lysate was then processed for western blot and probed with TSC2(pS939), TSC2(pT1462), TSC2(pS1254), TSC2(pY1571), TSC2 and TAK1 antibodies.

F The phosphorylation of TSC2 upon LPS stimulation is different in wild type and TAK1 deficient MEFs. Wild type and TAK1 deficient MEFs were stimulated with 200 ng/ml LPS for the indicated time. Cell lysate was then processed for western blot and probed with TSC2(pS939), TSC2(pT1462), TSC2(pS1254), TSC2(pY1571), TSC2, Akt(pT473) and Akt(pS308) and TAK1 antibodies.

Table4 List of phosphorylation sites on TSC2

phosphorylation site	responsible kinase	Stimulation event
pS664 /pS540	ERK	PMA
pS939	Akt/PKB	Insulin
pS960	not identified	PMA stimulation
pS981	Akt/PKB	Insulin
pS1155	not identified	PMA stimulation
pS1254	MK2	serum stimulation
pS1337/pS1338/pS1341	not identified	cell growth during G1 and M phase
pS1364	ERK	PMA stimulation
pS1448	not identified	cell growth during G1 and M phase
pT1462	Akt/PKB	Insulin
pY1571	Not identified	pervanadate[151]

3.2.4 TSC2 is phosphorylated when it is co-expressed with TAK1

The data above indicated that TAK1 was necessary for the phosphorylation of TSC2 in response of LPS, TNFα and IL-1β. However, whether TAK1 is sufficient to directly phosphorylate TSC2 is still unclear.

In HEK293 cells when TSC2 was over expressed with TAK1 complex, which presented the active form of TAK1, the mobility of TSC2 became slower, as shown in Figure 2.8. This phenomenon suggested that the active form of TAK1 was sufficient to phosphorylate TSC2 in over expressed study. Moreover, when the transfected cells were treated with TAK1 inhibitor for 1 hour before harvested, the mobility shift of TSC2 was altered compared with TSC2 from DMSO or IKKβ inhibitor treated cells, indicating that the mobility shift of

TSC2 specifically depended on kinase activity of TAK1 but not IKKβ in this context.

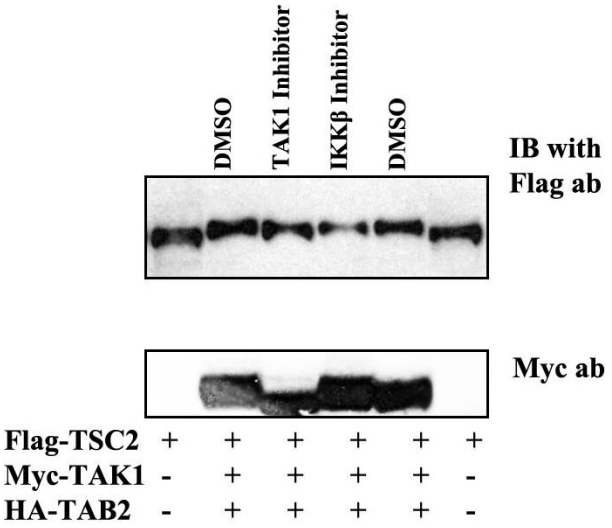


Figure 2.8 TSC2 is phosphorylated when it is co-expressed with TAK1

HEK293 cells were transfected with Flag-TSC2 alone or together with Myc-TAK1 and HA-TAB2. Flag-TSC2 and TAK1 complex co-transfected cells were then incubated with DMSO, 10 μM TAK1inhibitor or IKKβ inhibitor respectively. Cell lysate was processed for western blot and probed with Flag and Myc antibodies.

3.2.5 TAK1 does not affect the association of TSC complex

TSC2 is considered as molecular switch of mTOR pathway. In previous study, there are three proposed mechanisms by which TSC2 mediates mTOR pathway regulation [152]. Firstly, upon the stimulations, TSC2 is phosphorylated and subsequently subjected to ubiquitin mediated degradation to release GTPase Rheb and elevate mTOR activity. Secondly, the stimulation leads to the disassociation of TSC2 with its binding partner TSC1, then the separated TSC complex loses the integrity to function as the GTPase activating protein and mTOR pathway is activated. Thirdly, stimulation alters subcellular localization of TSC2 from membrane to cytoplasm and isolates TSC2 so that it is unable to inhibit Rheb, allowing mTOR pathway to be activated.

The common point of these proposals is that phosphorylation of TSC2 upon stimulation is required for the following processing of TSC2, while the diverse consequences of the phosphorylation are the main difference among these proposals. Given the physical interaction of TAK1-TSC2 and phosphorylation of TSC2 mediated by TAK1, it is important to test whether previous proposed TSC2 regulation mechanisms are applicable in TAK1-TSC2 connection.

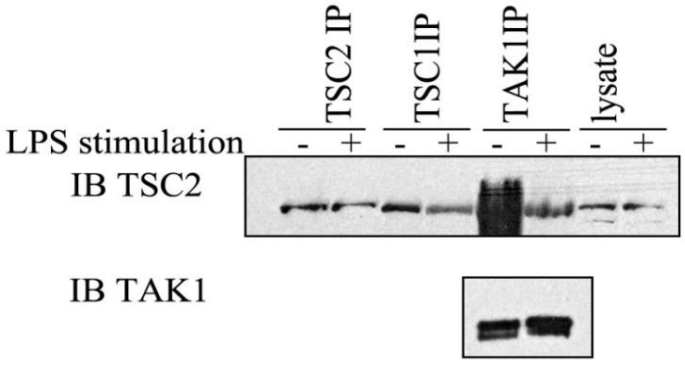
Firstly, ubiquitin-mediated TSC2 degradation was examined following phosphorylation of TSC2. There was no clue about TSC2 degradation upon stimulation with LPS, TNFα and IL-1β so far, as suggested by all the figures above. When TSC2 was co-over expressed with TAK1 in addition to ubiquitin, TSC2 was still stable compared with TSC2 expressed alone or TSC2 with TAK1. However, TAK1 introduced more ubiquitin binding to TSC2 as shown in Figure 2.9B. Given the idea that lysine 63-linked poly-ubiquitin chain would lead to activation of targeted kinase and that activation of TAK1 was associated with non-covalent binding of poly-ubiquitin chain, it was possible that interaction of TAK1 and TSC2 introduced the poly-ubiquitin chain binding to TSC2. However, without endogenous data, ubiquitination of TSC2 in physiological situation is still unclear. More efforts would be made to address this point.

Secondly, the association of TSC2 and TSC1 was examined as the consequence of TSC2 phosphorylation. TSC1 and TSC2 are considered as an integral element to regulate the GTPase Rheb. Thus, the dissociation of TSC1 and TSC2 means the loss of TSC2 function as a GTPase activating protein. Figure 2.9A showed the amount of TSC2 binding to TSC1 immunoprecipitated from LPS treated or untreated RAW264.7 cell lysate. There was a slight decrease of TSC2

binding to TSC1 from LPS treated cells, suggesting that the association of TSC complex was slightly affected by LPS stimulation. When TSC2 and TSC1 were over expressed with or without TAK1 in HEK293 cells, however, the association of TSC1 and TSC2 was not affected by additional TAK1, as shown in Figure 2.9C.

The subcellular localization change of TSC2 was one of the possibilities as the consequences of TSC2 phosphorylation. However, we were unable to test the impact of LPS stimulation on TSC2 subcellar localization due to the unsuccessful fractions of cell lysate. It still leaves as a question so far.

Collectively, the experiments above elucidated that LPS did not affect the stability of TSC2 and LPS stimulation resulted in the reduced association between TSC2 and TSC1, while was independent of TAK1 presence.



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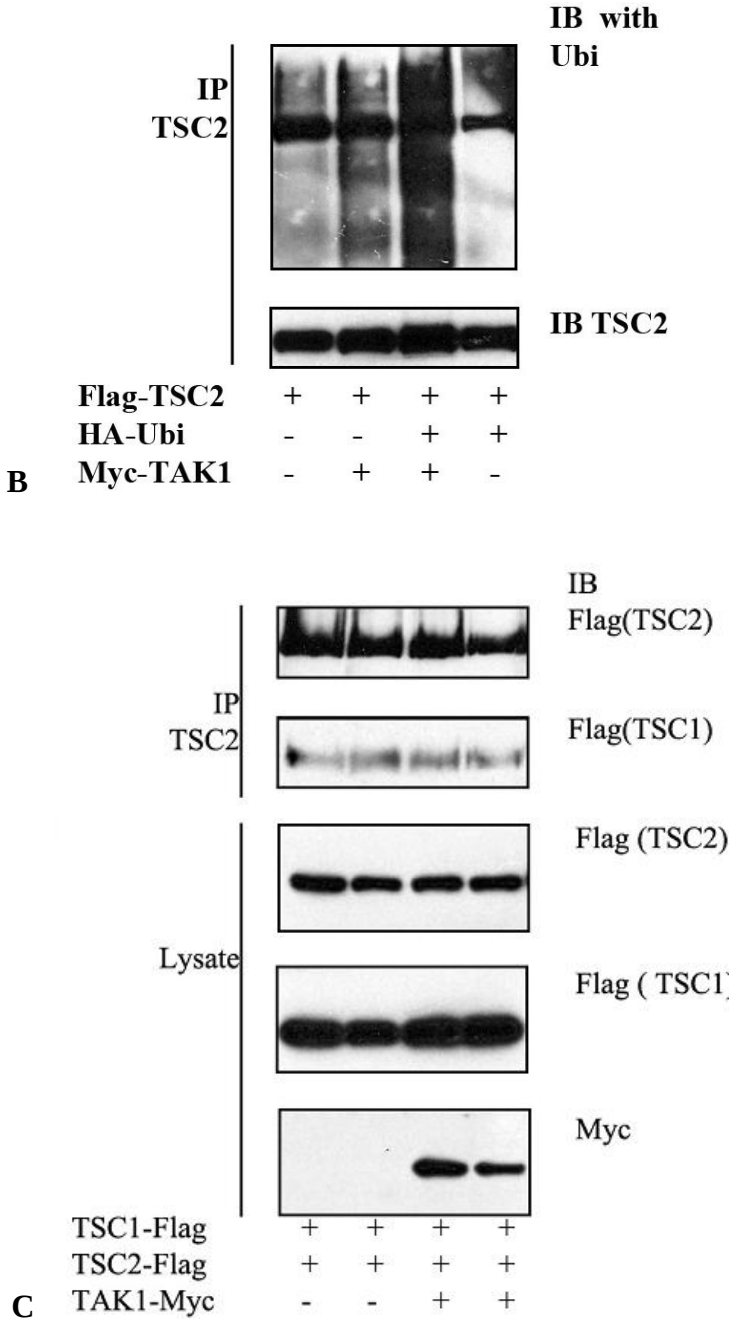


Figure 2.9 TAK1 does not affect the stability of TSC2 and association of TSC complex.

A Endogenous TSC2 is purified with TSC1 or TAK1; RAW 264.7 cell lysate with or without LPS stimulation was subjected to IP with TSC2, TSC1 or TAK1 antibodies and IB with TSC2 antibody or TAK1 antibody.

B TAK1 enhances the ubiquitin binding of TSC2; HEK293 cells were transfected with Flag-TSC2, Myc-TAK1 and HA-ubiquitin as indicated below. Cell lysate was subjected to immunoprecipitation with TSC2 and immunoblotted with ubiquitin and Flag antibodies.

C TAK1 does not affect the association of TSC complex; HEK293 cells were transfected with Flag-TSC1, Flag-TSC2 and Myc-TAK1 as indicated. Cell lysate was subjected to immunoprecipitated with TSC2 antibody, followed by immunoblotting with Flag antibody. Cell lysate was also probed with Flag and Myc antibodies.

3.3 **Identification of novel phosphorylation sites on TSC2**

Although there were four phospho-antibodies for TSC2 commercially available, only two of them (pS939 and pT1462) recognized the phosphorylated TSC2 after LPS stimulation, as mentioned above. On the other side, LPS induced obvious mobility shift of TSC2 suggested multi-sites phosphorylation on TSC2. Therefore, it is important to identify the novel phosphorylation sites on TSC2 mediated by TAK1. To this end, both *in vivo* and *in vitro* approaches were applied.

Firstly, TSC2 was over-expressed with or without TAK1 complex in HEK293 cells. As mentioned in part 3.2.4, when TSC2 was over-expressed with TAK1 complex in HEK293 cells, TSC2 was phosphorylated. The TSC2 co-expressed with TAK1 complex and expressed alone were then purified and analyzed with Mass Spectrometry to identify the phosphorylation sites. Given the consideration that other active kinases in cells might phosphorylate over-expressed TSC2, TAK1 inhibitor was used in one sample to tell the phosphorylation sites bringing in by other kinases. As shown in Table 5, the phosphorylation sites on TSC2 in different conditions were listed.

Table 5 The phosphorylation sites identified on TSC2

Site	Abundance in 1#	Abundance in 2#	Abundance in 3#	Abundance in 4#
S660	**1/23**	**1/17,2/23**	**1/1**	
S664	**1/9, 2/23**	**3/17, 10/23**	**2/6, 4 /25**	**3/16**
T667			**1/1**	**1/16**
S939	**1/1**	**1/1**	**1/1**	**1/1**
S960		**2/5 , 2/3**		
S981	**2/8**	**2/8**	**2/7**	**2/7**
S1072				**1/68**
T1077				**1/68**
S1152		**1/18**		
S1155		**2/18**	**3/17**	**1/14**
S1254	**1/22**	**1/15**		
S1336			**1/2**	
S1337		**1/3**		
S1338	**1/3**	**1/3**	**1/2**	
S1341		**1/3**		
S1364	**1/18**	**2/20**		
S1365			**1/16**	
S1411	**2/2**	**2/2**	**2/2**	**2/2**
S1448		**2/2**	**1/1**	
S1449	**1/10**	**1/8 ,1/8**	**1/5**	**1/6, 1/6**
T1462	**1/11**			

Note: Abundance = number of phosphorylated peptide/ total number of peptide sharing the same sequence (if the same site was found in two different sequences, the site abundances are counted separately).

1# is immunoprecipitation complex from cells expressing TSC2 alone (sequence coverage= 47%);

2# is immunoprecipitation complex from cells expressing TSC2with TAK1 complex (GST-TAK1+Flag-TAB1+HA-TAB2) (53%);

3# is immunoprecipitation complex from cell expressing TSC2 with GST-TAK1 (sequence coverage= 60%);

4# is immunoprecipitation complex form cell expressing TSC2 with GST-TAK1, cells were treated with TAK1 inhibitor for 1 hour before harvesting (sites appearing in this sample would be considered as false positive) (sequence coverage= 52%).

In this table, there were total 21 sites identified with phospho-group labelling on TSC2 in all these conditions. There were 10 sites which were labelled with phospho-group when TSC2 was over expressed alone. There were 6 extra sites (S960, S1152, S1155, S1337, S1341, S1448) labelled with phospho-group and

one site missing (T1462) when TSC2 was over expressed with TAK1 complex, compared with sites identified on TSC2 expressed alone. There were 5 extra sites (T667, S1155, S1336, S1365, S1448) labelled with phospho-group and two sites missing (S1254, T1462) when TSC2 was over-expressed with GST-TAK, compared with sites identified on TSC2 expressed alone. When TAK1 inhibitor was used, there were 12 sites missing among total 19 sites identified on TSC2 which was expressed alone, co-expressed with either TAK1 or TAK1 complex; there were seven sites (S664, T667, S939, S981, S1155, S1411, S1449) remaining and two new sites from the sample treated with TAK1 inhibitor.

The TAK1 inhibitor 5Z-7-Oxozeaenol is a compound to specifically inhibit TAK1 kinase activity, so the phosphorylation sites remaining in TAK1 inhibitor treated sample were considered as non-specific sites for TAK1 mediated TSC2 phosphorylation. Without over-expressed TAK1, the phosphorylation sites appearing in TSC2 single expressed sample may be due to the post translation modification and auto phosphorylation of over-expressed protein, which might not be necessary for the activation of mTOR mediated by TAK1. Only the sites specifically appearing on TSC2 co-expressed with either TAK1 or TAK1 complex should be considered as the potential phosphorylation sites by TAK1. There were totally seven such sites as potential targets: S960, S1152, S1336, S1337, S1365, S1441, S1448, none of which can be confirmed with phospho-antibody.

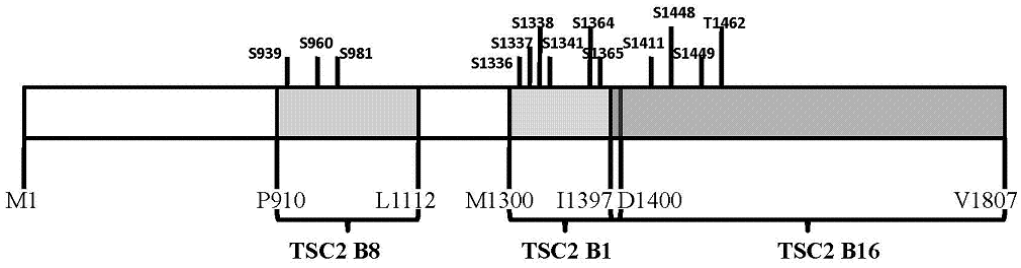
To verify the phosphorylation sites mediated by TAK1 complex, *in vitro* kinase assay was carried out. TSC2 is a large protein with totally 1807 residues, which makes it hard to express the full length version TSC2 in the bacterial system.

According to the phosphorylation sites found on full-length TSC2 from mammalian cell HEK293, three TSC2 truncates with possible candidate sites were cloned in to GST fused expression vector. These three truncates covered the sequences of TSC2 1300-1400 [B1], 910-1112[B8] and 1139-1807[B16], respectively. The schematic map of TSC2 truncates was shown in Figure 2.10A. TSC2 truncates were expressed in *E.coli* and purified by GST pull down. The purified proteins were incubated with TAK1 complex, expressed in mammalian cells, *in vitro* system in the presence of [^{32}Pγ]-ATP. After auto exposure, the [^{32}Pγ]–ATP labelled substrates were shown in Figure 2.10B.

GST-TSC2 B1 [1300-1400] was clearly labelled by [^{32}Pγ]–ATP as substrate of TAK1 complex. This labelled GST-TSC2 B1 truncate was then digested into peptides with trypsin and analyzed by HPLC. As shown in Figure 2.10C, when peptides were eluted out from HPLC, the radioactivity of each fraction was also monitored and recorded. Shown as a sharp peak, the intensive radioactivity was detected from fraction 63 to 66, indicating that the peptides eluted in these fractions were labelled with phospho-group. Meantime, the GST-TSC2 B1 was also processed in the exactly the same way but with nonradioactive ATP. When this nonradioactive phospho-group labelled truncate passed through HPLC, the same fractions (fraction 63 to 66) were collected, pulled together and send for MALDI-MS/MS to analyze the sequence of highly phosphorylated peak. The phosphorylated sequence was identified as a peptide containing residues from 1362 to 1384, which was VVSSEGGRPSVDLSFQPSQPLSK. Unfortunately, the MALDI-MS/MS result did not indicate the precise phosphorylation site among six serine residues present in this sequence, shown in Figure 2. 10D.

Compared with the potential phosphorylation sites discovered on TSC2 from mammalian expression system, it was serine 1365 that existed in both results. Thus, Ser 1365 was considered with highest potential as TAK1 mediated phosphorylation site on TSC2.

A



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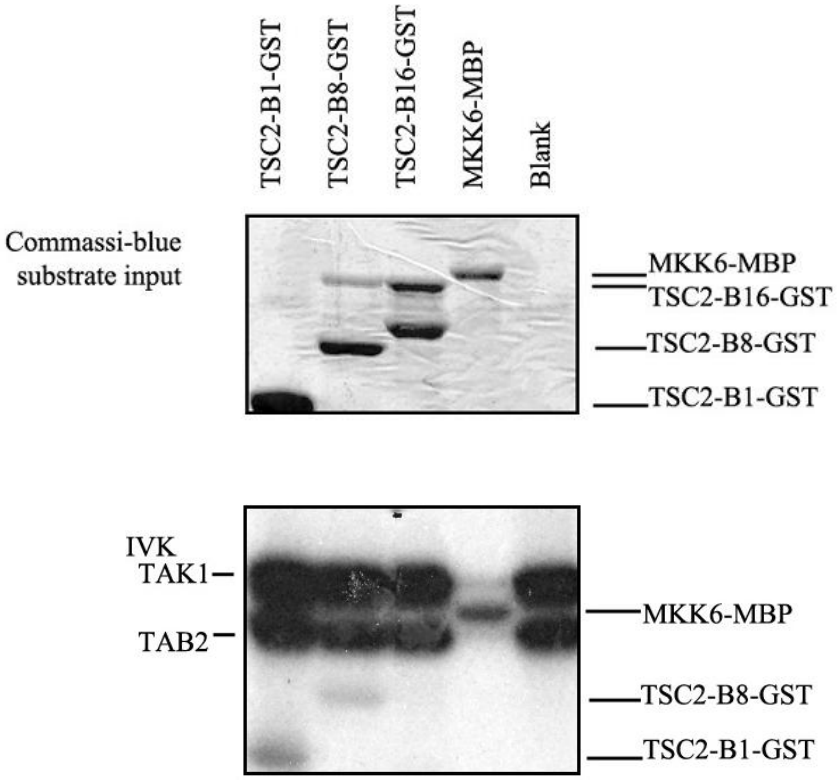

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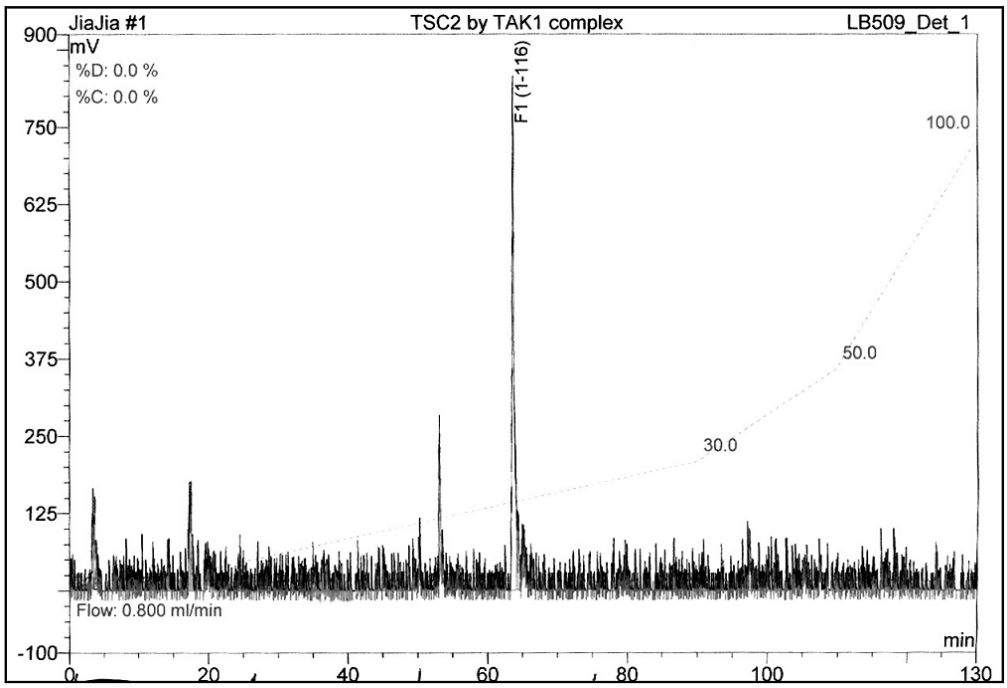

D

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. %	Modification	Rank	Result Type
838.576	838.5918	0.0158	19	822	829	ALPVLVVK					Mascot
979.4972	979.585	0.0878	90	1410	1417	LSPEVKAR			Phospho (STY)[2]		Mascot
979.4972	979.585	0.0878	90	1410	1417	LSPEVKAR			Phospho (ST)[2]		Mascot
1051.5782	1051.7705	0.1923	183	8	16	DSGLKEKFK					Mascot
1110.5902	1110.651	0.0608	55	1346	1355	SLHAEELVGR					Mascot
1126.5657	1126.6329	0.0672	60	1057	1065	TKTWLVGNK			Phospho (ST)[1]		Mascot
1182.599	1182.7202	0.1212	102	455	464	SESRGAVRIK			Phospho (ST)[1]		Mascot
1194.4552	1194.6244	0.1692	142	1482	1491	SRATASNAEK			Phospho (ST)[1], Phospho (ST)[4]		Mascot
1201.5137	1201.6931	0.1794	149	1730	1739	SNPTDIYPSK			Phospho (STY)[1]		Mascot
1201.5137	1201.6931	0.1794	149	1730	1739	SNPTDIYPSK			Phospho (Y)[7]		Mascot
1251.7096	1251.6375	-0.0721	-58	681	691	LGSVPYSLLFR					Mascot
1283.5797	1283.6848	0.1051	82	968	978	ESSAAEAFRCR			Carbamidomethyl (C)[10]		Mascot
1333.6035	1333.696	0.0925	69	538	548	SLSPPPELEER			Phospho (ST)[1]		Mascot
1500.7974	1500.7692	-0.0282	-19	906	917	KDFVPFITKGLR			Phospho (ST)[8]		Mascot
1521.7012	1521.791	0.0898	59	251	261	ELCEPCWKLMR			Carbamidomethyl (C)[3,6]		Mascot
1701.7173	1701.8199	0.1026	60	1795	1807	RLISSVEDFTEFV			Phospho (ST)[4], Phospho (ST)[10]		Mascot
1749.8458	1749.8292	-0.0166	-9	1794	1807	KRLISSVEDFTEFV			Phospho (ST)[11]		Mascot
1794.8058	1794.8818	0.076	42	126	139	VIKDYPSNEDLHER			Phospho (Y)[5]		Mascot
2153.1099	2153.2104	0.1005	47	1776	1795	APAQTPAEPTPGYEVGQ RKR					Mascot
2210.1206	2210.22	0.0994	45	937	954	STSLNERPKSLRIARPPK			Phospho (ST)[1], Phospho (ST)[2]		Mascot
2399.2693	2399.2891	0.0198	8	1057	1078	TKTWLVGNKLVTVTTSV GTGTR			Phospho (ST)[1]		Mascot
2401.2358	2401.3867	0.1509	63	1362	1384	VVSSEGGRPSVDLSFQP SQPLSK	146	100			Mascot
2401.2358	2401.3867	0.1509	63	1362	1384	VVSSEGGRPSVDLSFQP SQPLSK					Mascot

Figure 2.10 Attempt to identifying the phosphorylation sites of TSC2 mediated by TAK1

A The schematic map of TSC2, TSC2 truncates were coloured in gray;

B TSC2 B1 truncate and B8 truncate were labelled in *in vitro* kinase assay. E.coli expressed TSC2 truncates were purified, and incubated with TAK1 complex expressed by mammalian cells, [γ^{32}P]-ATP, $MgCl_2$, 0.1% β-ME and Tris-HCl at 30°C for 40minutes. Samples were then separated on SDS-PAGE and stained with Coomassie Blue. The phospho-signals were captured via autoradiography.

C The Radioactivity chart of different fractions of TSC2 B1 truncates. The radioactive labelled TSC2 B1 truncate was cut from gel and digested with trypsin. The digested peptides were extracted and analyze by HPLC, at the same time, the radioactivity of different fractions were monitored and record.

D The sequence analyzing of labeled peptide: fractions relative to the highest peak on the part C from the unlabeled sample were pulled together and analyzed by MALDI-MS/MS for the sequence, the results were listed here.

3.4 Activation of mTOR signalling pathway upon LPS, TNFα and IL-1β

3.4.1 LPS induces phosphorylation of p70S6K1 and 4EBP1

The TSC2 is a critical regulator of mTOR pathway so that phosphorylation of TSC2 mediated by TAK1 probably affects mTOR pathway activity. Given the interaction between TAK1 and TSC2 and phosphorylation of TSC2, it is reasonable to examine the activation status of mTOR pathway upon TAK1 active conditions, such as LPS stimulation.

As shown in figure 2.11A, LPS stimulation led to rapid, intensive and sustainable phosphorylation of p70S6K1 on Thr389. Phosphorylation p38α and JNK1/2 were also showed here as the indicator of LPS stimulation.

As discussed in introduction: phosphorylation of p70S6K1 could reflect either mTOR activation or Akt/PKB activation while the full mTOR activation includes both phosphorylation of p70S6K1 on Thr389 and 4EBP1 on Thr70. We thus examined phosphorylation status of 4EBP1 upon LPS stimulation.

4EBP1 was phosphorylated on Thr70 in response to LPS stimulation, as shown in Figure 2.11A, implying the activation of mTOR.

Meanwhile, LPS induced phoshorylation of p70S6K1 and 4EBP1 was sensitive to rapamycin--- the specific mTOR inhibitor which suppresses mTORC1 activity selectively, as shown in Figure 2.11B, indicating that LPS induced phosphorylation of p70S6K1 and 4EBP1 was mediated by mTOR.

Collectively, these data suggested that LPS stimulation on RAW264.7 cell led to full activity of mTOR, including phosphorylation of p70S6K1 on Thr389 and 4EBP1 on Thr70.

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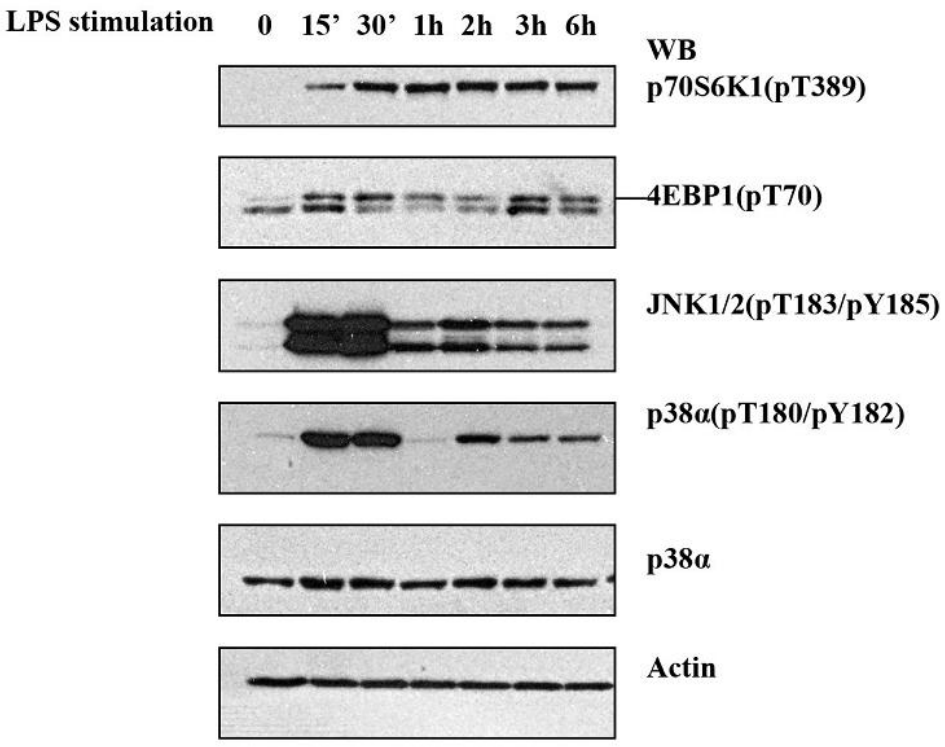

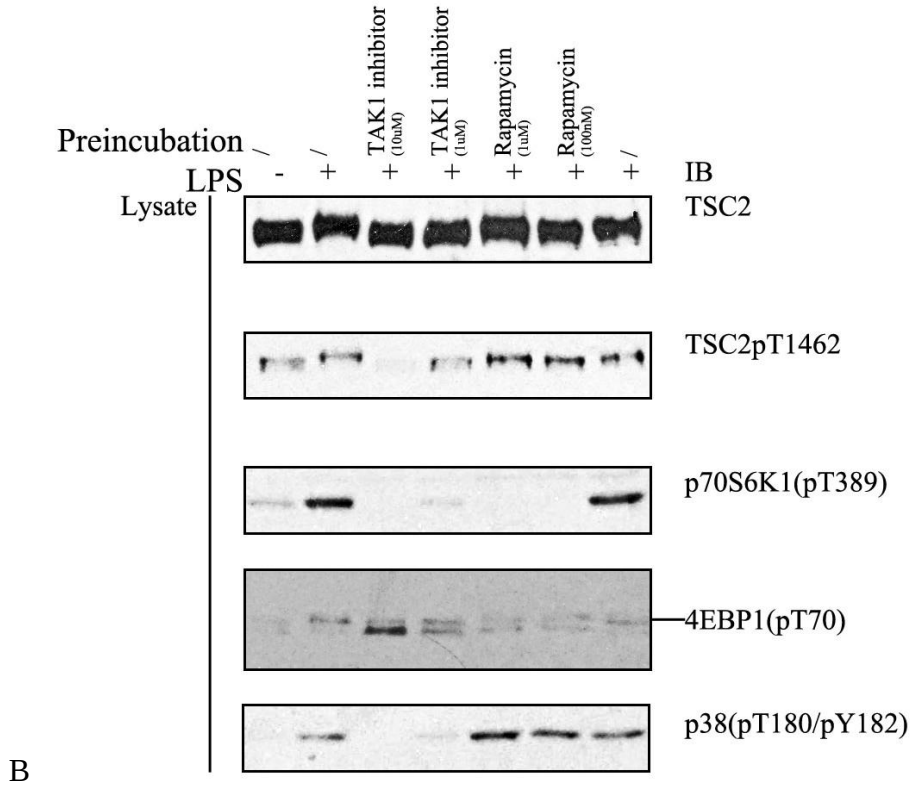

Figure2.11 LPS induces the phosphorylation of p70S6K1 and 4EBP1.

A LPS induces phosphorylation of both p70S6K1 and 4EBP1. RAW264.7 cells were stimulated with LPS for the indicated time, cell lysate was probed with p70S6K1 (pT389), 4EBP1(pT70), JNK1/2 (pT183/pY185), p38α (pT180/pY182), p38α and Actin antibodies.

B Rapamycin suppresses LPS induced phosphorylation of both p70S6K1 and 4EBP1. RAW 264.7 cells were pre-incubated with 5μM TAK1 inhibitor, 1μM TAK1 inhibitor, 1μM rapamycin or 100nM rapamycin respectively. RAW264.7 cells were then treated with 100ng/ml LPS treatment for 1hour. Cell lysate was incubated with TSC2, TSC2 (pT1462), p70S6K1 (pT389), 4EBP1 (pT70) and p38α (pT180/pY182) antibodies.

3.4.2 TNFα and IL-1β activate p70S6K1

Lipopolysaccharide is a ligand for TLR4 receptor. It shares similar downstream signalling cascades with pro-inflammatory cytokine TNFα and IL-1β. The fact that LPS can activate mTOR pathway brings up the question whether TNFα and IL-1β can also activate the mTOR pathway with the similar mechanisms. To test this possibility, Mouse Embryonic Fibroblast (MEF) cells were stimulated with TNFα and IL-1β and downstream events of mTOR were monitored. As

shown in Figure 2.12A, both TNFα and IL-1β stimulations effectively activated phosphorylation of p70S6K1 on Thr389. TNFα and IL-1β stimulations also led to phosphorylation of JNK1/2, which is an established readout of IL-1 and TNFα stimulations. IGF1 stimulation activated mTOR pathway as reported while it did not affect MAPK like JNK1/2. The efficient activation of mTOR pathway in response to TNFα or IL-1β treatment not only happened to MEF cells. Among the cell lines tested, TNFα stimulation activated mTOR pathway in HEK293 cells, MEFs, 3T3-L1 cells and MCF7 cells. IL-1β stimulation activated mTOR pathway in 3T3-L1 cells, MEFs and MCF7 cells. Besides, LPS activated mTOR pathway in RAW264.7 cells and MEFs. Figure 2.12B, C and D exhibited the activation of mTOR pathway in all these cell lines.

Collectively, these data suggested that mTOR pathway was activated by TNFα and IL-1β stimulations in different cell lines, indicating mTOR signalling was modified upon TNFα and IL-1β stimulations, as well as LPS stimulation.

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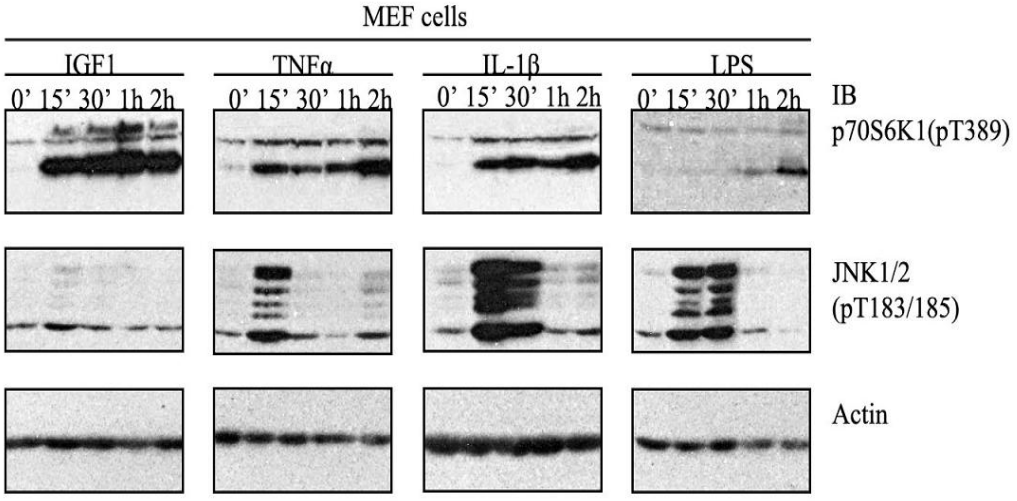

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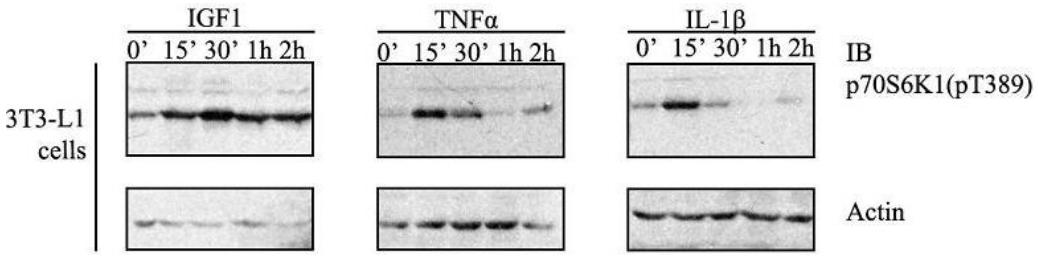

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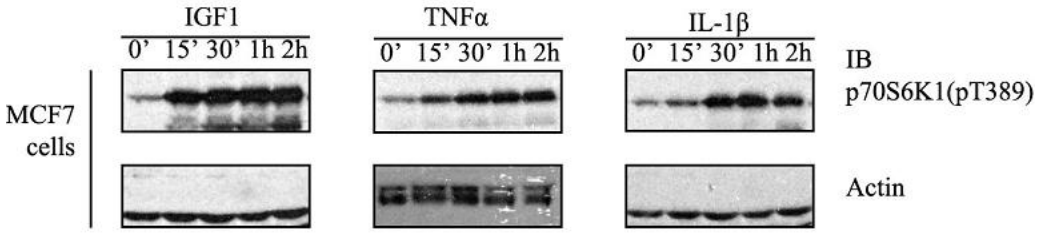

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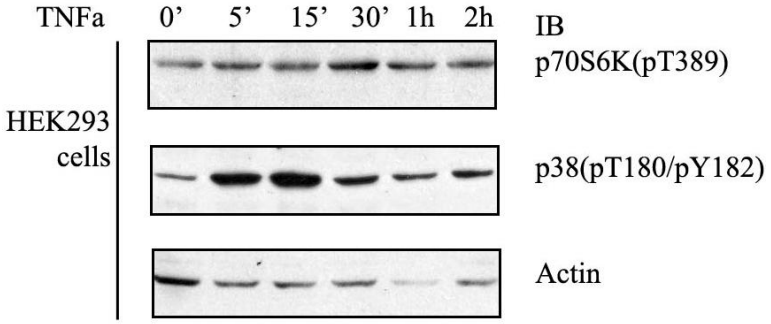


Figure 2.12 TNFα and IL-1β activate p70S6K1

A MEF cells respond to IGF1, TNFα IL-1β and LPS. Mouse Embryonic Fibroblast cells were treated with 25 ng/ml IGF1, 100 ng/ml TNFα, 100 ng/ml IL-1β or 200 ng/ml LPS for the indicated time. Cell lysate was probed with p70S6K1(pT389), JNK1/2(pT183/pY185) and Actin antibodies.

B, C and D, 3T3IL-1 cells, MCF7 cells and HEK293 cells were treated as A. Cell lysate was probed with the antibodies indicated.

3.4.3 LPS induced mTOR activation is mediated by Akt/PKB, IKKβ and TAK1

mTOR is activated by various growth signals, such as insulin, growth factors IGF1 and EGF, energy availability and mitogen, which may go through kinases like PI3K/PDK, MAPK p38α, AMPK or ERK respectively. The details on this part have been described in introduction. LPS induced mTOR activation was only recently discovered. The kinase involved in this event and the delicate mechanism of this signalling cascade were not clear yet.

To better define the kinases which mediate LPS induced mTOR activation, inhibitors against various kinases were used and their impacts on mTOR activation were observed, as shown in Figure 2.13 A,B and C.

AMPK plays an important role in mTOR regulation. When AMPK inhibitor was used in addition to LPS treatment, phosphorylation of p70S6K1 was even stronger than the signal induced by LPS treatment alone, as shown in Figure 2.13A. Since basic AMPK activation suppressed mTOR activation, it was reasonable that inhibition of AMPK enhanced the phosphorylation of p70S6K1 upon LPS stimulation. This result also suggested that AMPK did not conduct mTOR activation upon LPS stimulation.

As shown in Figure 2.13B, besides the phosphorylation of p70S6K1, LPS stimulation induced Akt/PKB phosphorylation on Ser308 (by PDK1) and Thr473 (by mTORC2), which were indicators of full Akt/PKB activity. Thus, Akt was also activated upon LPS stimulation.

Secondly, as we mentioned in paragraph 3.4.1, LPS induced phosphorylation of p70S6K1 was blocked by mTORC1 inhibitor rapamycin, which did not affect the phosphorylation status of PDK, Akt/PKB or MAPK JNK1/2 and p38α, as shown in Figure 2.13B. This confirmed that LPS induced phosphorylation of p70S6K1 via mTOR.

Thirdly, in Figure 2.13B, PI3K inhibitor wortmannin efficiently blocked Akt/PKB phosphorylation on Ser308 and Thr473. Wortmannin also totally swept LPS induced p70S6K1 phosphorylation, indicating that PI3K itself or its downstream was involved in LPS induced mTOR activation. However, a recent report revealed that wortmannin also blocked the classII PI3K activity and affected the basic mTOR activity by interrupting the cells' sense to amino acids [65]. There are also reports showing that wortmannin can directly inhibit mTOR since mTOR is a member of the PI3K family. These facts compromised the specificity of wortmannin.

Fourthly, in Figure 2.13B, PDK1 inhibitor BX759 efficiently blocked Akt/PKB phosphorylation on Ser308 (by PDK1) but had little effects on Akt/PKB phosphorylation on Thr473 (by mTORC2). However, BX759 did not affect LPS induced p70S6K1 phosphorylation, indicating that PDK1 was not directly involved in LPS induced mTOR activation. Since PDK1 is downstream of PI3K, results from PDK1 inhibitor also excluded the possibility that LPS induced mTOR activation depended on PI3K.

Fifthly, in Figure 2.13B, MEK1 inhibitor PD98059 showed no effect on LPS induced Akt/PKB phosphorylation and p70S6K1 phosphorylation, implying that MEK1-ERK pathway was dispensable for LPS induced mTOR activation.

Sixthly, in Figure 2.13B, p38α inhibitor SB203580 showed impact in increasing LPS induced JNK phosphorylation since p38α was reported to mediate the negative feedback loop on TAK1 kinase. However, p38α inhibitor did not affect LPS induced phosphorylation of p70S6K1, implying that p38α and its downstream MK2 were dispensable for LPS induced mTOR activation.

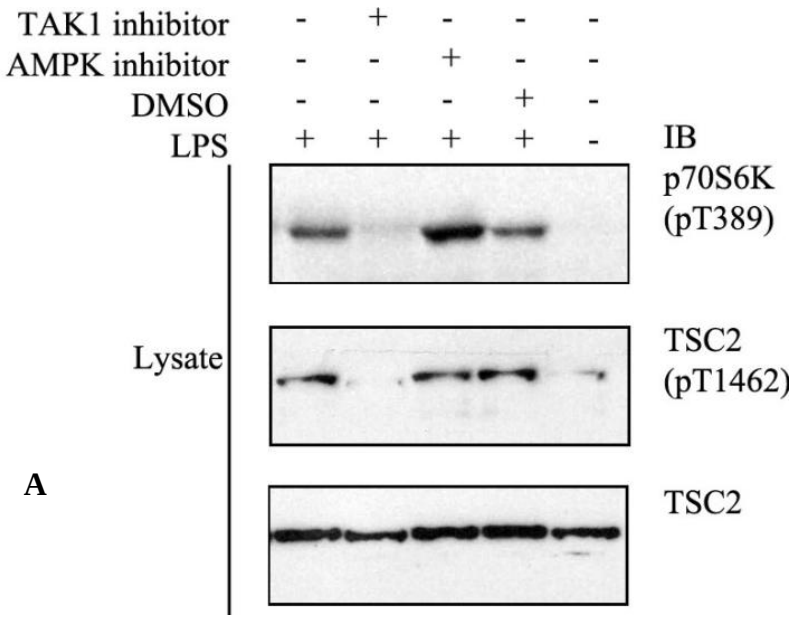
Seventh, in Figure 2.13B, TAK1 inhibitor did not affect either PDK1 or Akt/PKB phosphorylation, indicating that it had no impact on classic mTOR activation pathway. However, TAK1 inhibitor did suppress the phosphorylation of p70S6K1, as well as p38α and JNK, implying that TAK1 was required for LPS induced mTOR activation.

Lastly, it is the involvement of IKKβ in LPS induced mTOR activation that is difficult to elucidate. It was reported that IKKβ mediated TNFα induced mTOR activation. However, upon TNFα, LPS and IL-1β stimulation, both IKKβ and TAK1 are activated and TAK1 was upstream of IKKβ. This brings up the question that TAK1 may activate mTOR pathway through IKKβ.

To answer this question, inhibitors against IKKβ (BAY11-7082) or TAK1 (5Z-7-Oxozeaenol) were applied. As shown in Figure 2.13C, IKKβ inhibitor (BAY11-7082) and TAK1 inhibitor (5Z-7-Oxozeaenol) both suppressed the phosphorylation of p70S6K1. As previously reported, IKKβ inhibitor also suppressed the phosphorylation of TSC1, as indicated by the mobility shift (lacking of commercial TSC1 phospho-antibody). However, IKKβ inhibitor did not affect the mobility shift and phosphorylation status of TSC2. TAK1 inhibitor altered the electrophoretic mobility shift of TSC2 and phosphorylation of TSC2 on Thr1462. TAK1 inhibitor also suppressed the mobility shift of

TSC1, which is probably through suppressing the activity of IKKβ. These data suggested that TAK1 and IKKβ mediated mTOR activation upon LPS stimulation in parallel manners and by targeting on TSC2 and TSC1 respectively.

Collectively, these data showed that Akt/PKB, IKKβ and TAK1 were involved in LPS induced mTOR activation. So for, the mechanism of Akt/PKB activation upon LPS stimulation is not clear yet. And the possibility that other kinase may involve in LPS induced mTOR activation still remains.



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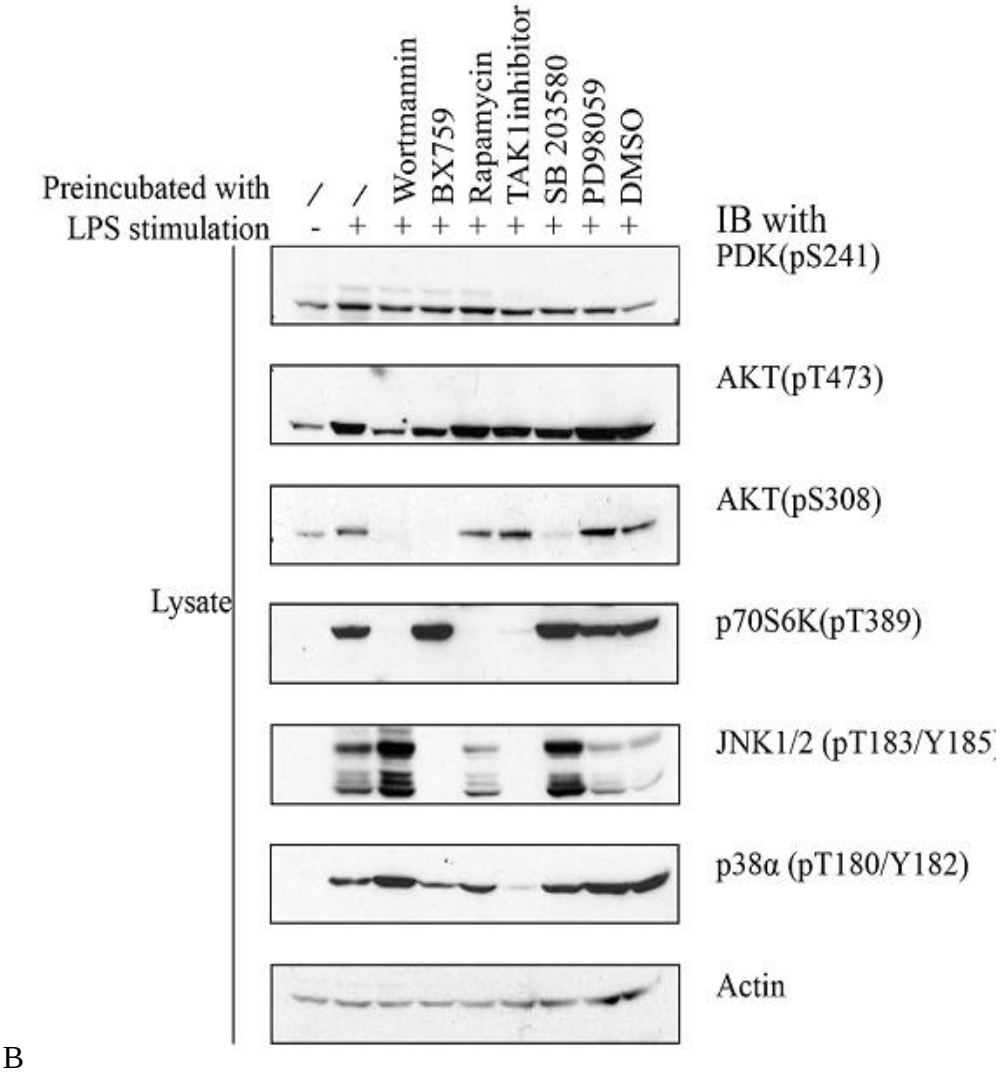
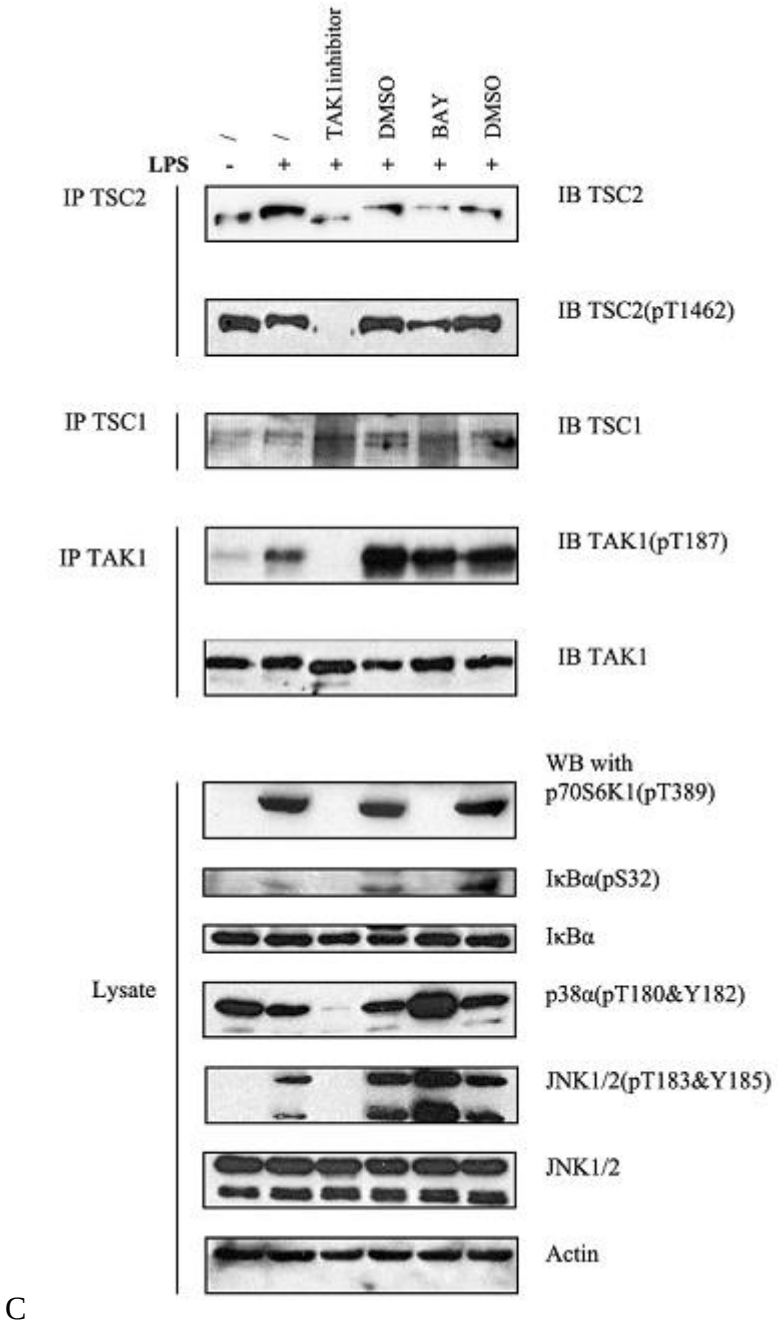

Preincubated with
LPS stimulation
/ / Wortmannin BX759 Rapamycin TAK1inhibitor SB 203580 PD98059 DMSO
- + + + + + + + +
IB with
PDK(pS241)
AKT(pT473)
AKT(pS308)
Lysate
p70S6K(pT389)
JNK1/2 (pT183/Y185
p38α (pT180/Y182)
Actin

B



C

Figure 2.13 The kinases involved in LPS induced mTOR activation

A AMPK is not involved in LPS induced mTOR activation. RAW264.7 cells was treated with 10 μM TAK1 inhibitor, 10 mM AMPK inhibitor or DMSO for 1hour, followed with 1 hour 100 ng/ml LPS treatment. Cell lysate was probed with p70S6K1 (pT389), TSC2 (pT1462) and TSC2 antibodies.

B Akt and TAK1 might be involved LPS induced mTOR activation. RAW 264.7 cells was treated with 1 μM Wortmannin, 10 μM BX759, 1μM rapamycin, 10 μM TAK1 inhibitor, 10 μM SB303580, 10 μM PD98059 or equal volume of DMSO for 1 hour respectively, followed by 1 hour 100 ng/ml LPS treatment. Cell lysate was probed with PDK1 (pS241), Akt (pT473), Akt (pS308), p70S6K1 (pT389), JNK1/2(pT183/pY185), p38α (pT180/pY182) and Actin antibodies.

C IKKβ inhibitor suppresses LPS induced mTOR activation without affecting TSC2 phosphorylation. RAW264.7 cells was treated with 10 μM TAK1 inhibitor, 40 μM IKKβ

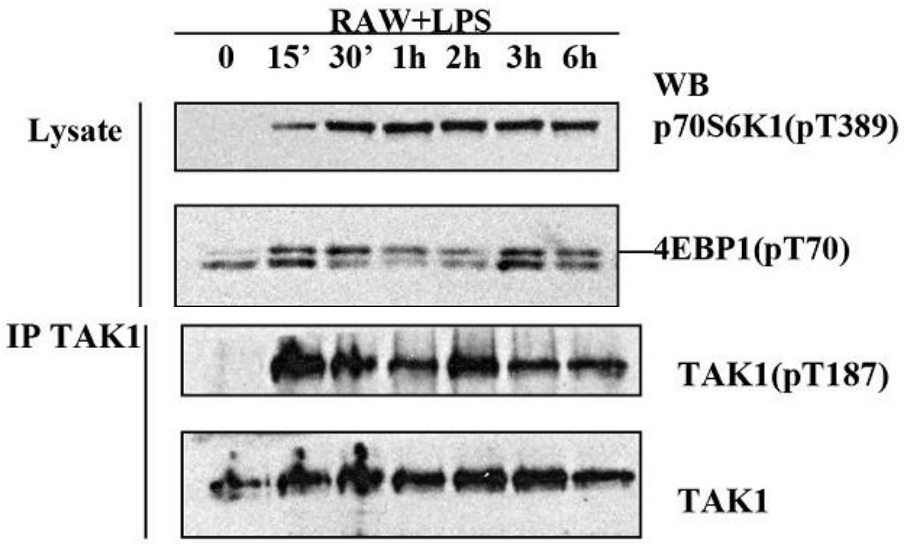
inhibitor or DMSO for 1hour, followed with 1 hour 100 ng/ml LPS treatment. Cell lysate was probed with p70S6K1(pT389), IκB(pS32), IκB, JNK1/2, JNK1/2(pT183/pY185), p38α (pT180/pY182), p38α and Actin antibodies. Cell lysate was also subjected to IP with TSC2 antibody and IB with TSC2 (pT1462) and TSC2 antibodies, or IP/IB with TSC1 antibody, or IP with TAK1 antibody and IB with TAK1(pT187) and TAK1 antibodies.

3.5 TAK1 is involved in LPS induced mTOR activation

3.5.1 TAK1 kinase activity is associated with mTOR activation upon IL-1β, TNFα and LPS stimulations

TAK1 is activated upon LPS, TNFα and IL-1β stimulations to regulate inflammatory response. TAK1 activation is reflected on phosphorylation of its Thr187. In this context, we noticed that phosphorylation of TAK1 on Thr 187 was associated with phosphorylation of p70S6K1 in response to IL-1β, TNFα and LPS up to six hours, as shown in Figure 2.14.

A



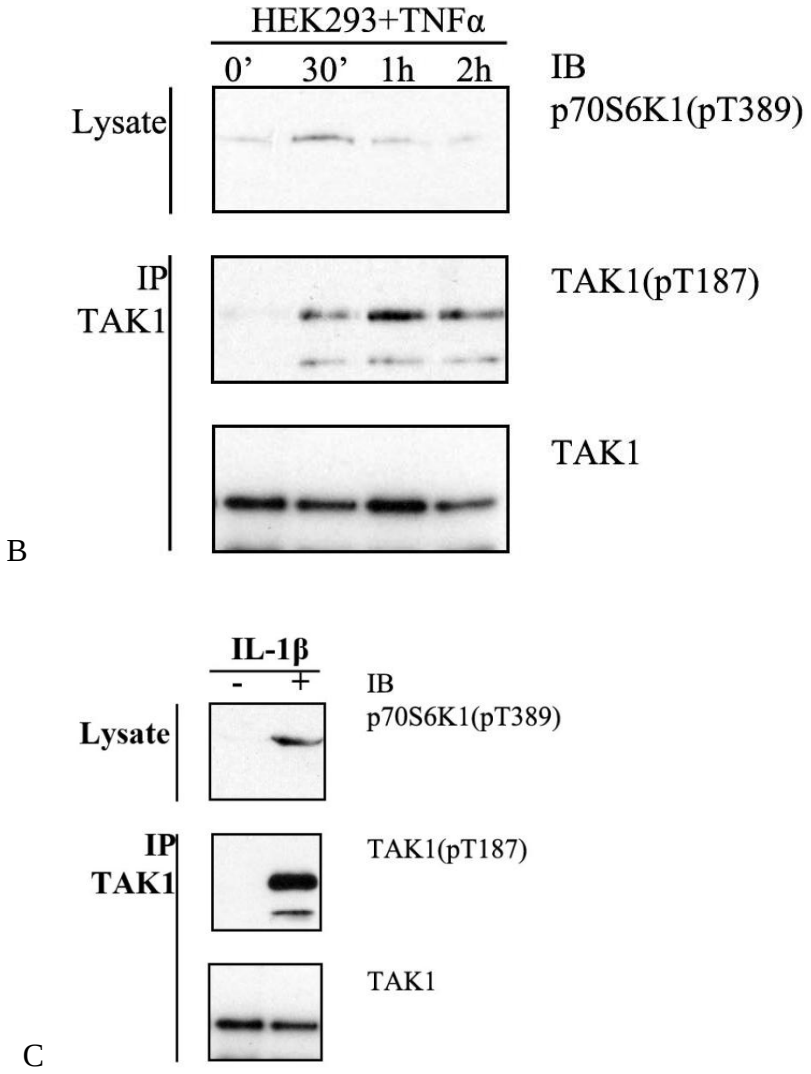


Figure 2.14 TAK1 activity associates mTOR activation upon LPS, IL-1β and TNFα stimulations.

A TAK1 activity associates mTOR activation upon LPS stimulation. RAW264.7 cells were treated with 100 ng/ml LPS stimulation for the indicated time, cell lysate was probed with p70S6K1(pT389) and 4EBP1(pT70) antibodies. Cell lysate was also subjected to IP with TAK1 antibody, followed by IB with TAK1 (pT187) and TAK1 antibodies.

B TAK1 activity associate mTOR activation up TNFα stimulation. HEK293 cells were treated with 50 ng/ml TNFα for the indicated time. Cell lysate was probed with p70S6K1 (pT389) antibody. Cell lysate was also subjected to IP with TAK1 antibody, followed by IB with TAK1 (pT187) and TAK1 antibodies.

C TAK1 activity associates mTOR activation up TNFα stimulation. 293IL-1R cells were treated with 50 ng/ml IL-1β for 2 hours. Cell lysate was probed with p70S6K1(pT389) antibody. Cell lysate was also subjected to IP with TAK1 antibody, followed by IB with TAK1 (pT187) and TAK1 antibodies.

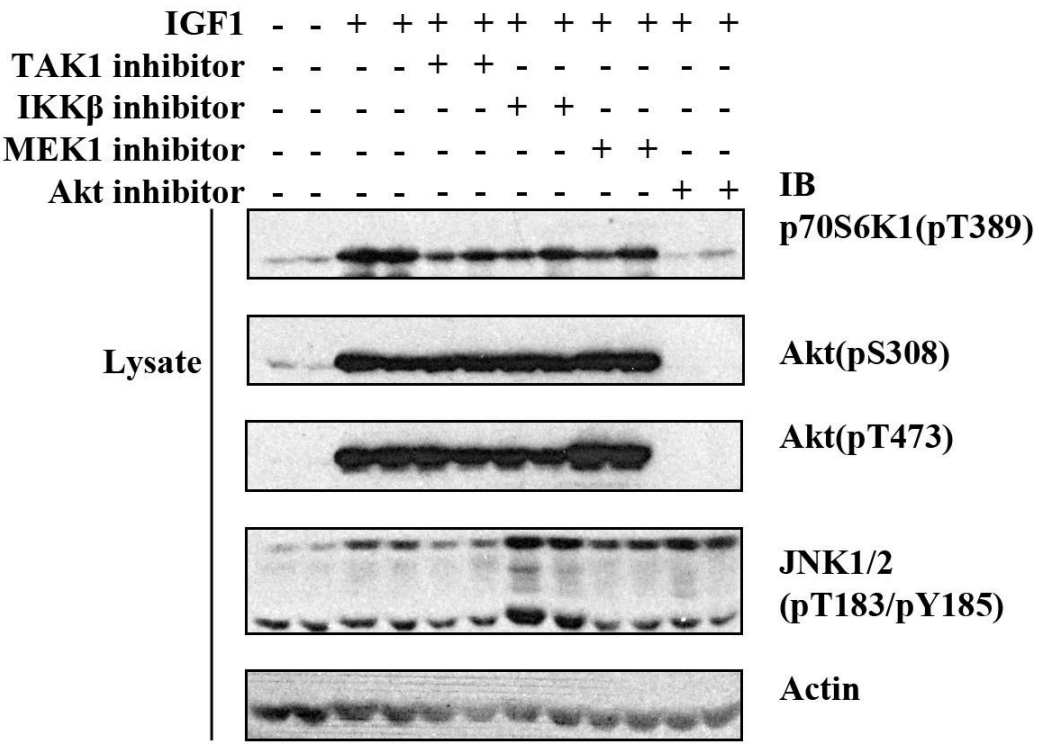
3.5.2 TAK1 inhibitor suppresses mTOR activation upon IL-1β, TNFα and LPS stimulations

Given the association of TAK1 activity with p70S6K1 phosphorylation upon IL-1β, TNFα and LPS stimulation, it is reasonable to link TAK1 activity and mTOR activation together. Therefore, it was tested whether inhibition of TAK1 activity affected mTOR activation. On MCF7 cells, inhibitor of TAK1 totally blocked LPS induced TAK1 activity, as indicated by phosphorylation of JNK1/2 (Figure 2.15D). At the same time, TAK1 inhibitor also blocked LPS induced p70S6K1 phosphorylation on Thr389. TAK1 inhibitor blocked TAK1 activation as well as phosphorylation p70S6K1 on Thr389 induced by TNFα and IL-1β stimulation, as shown in Figure 2.15B and C. As we concluded in paragraph 3.4.3, both Akt/PKB and IKKβ were required for LPS induced mTOR activity. Figure 2.15 B and C indicated that Akt/PKB and IKKβ were also required for mTOR activation induced by IL-1β and TNFα.

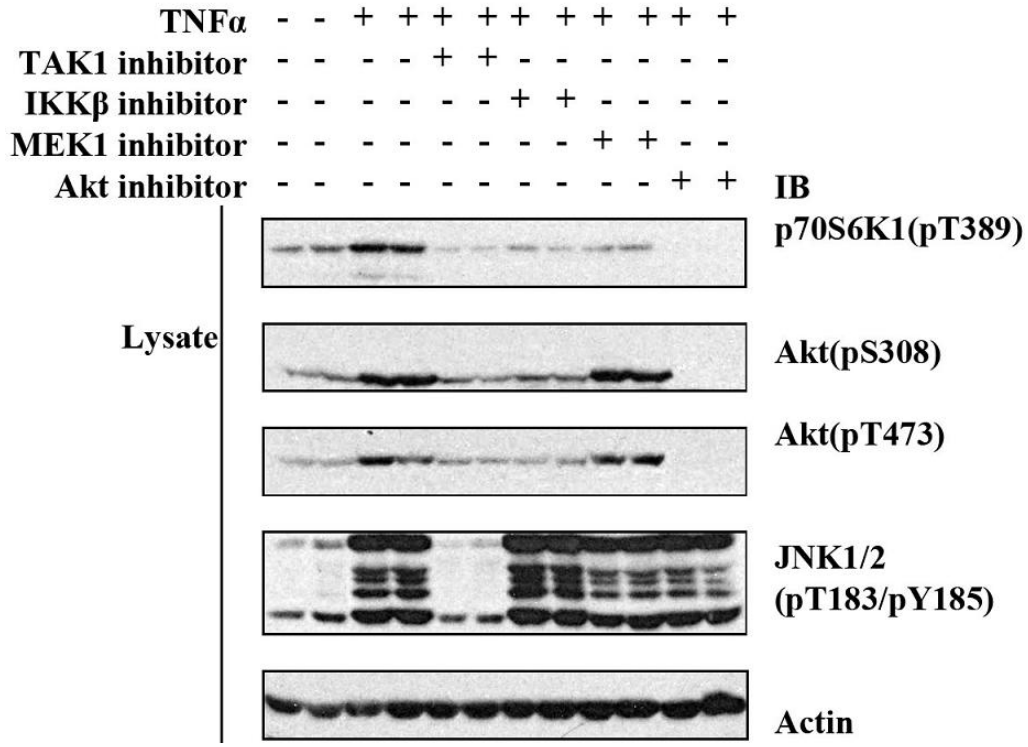
In contrast, IGF1 did not induce TAK1 activation, suggested by phosphorylation of JNK1/2 on Thr183 and Tyr185. TAK1 inhibitor also failed to regulate IGF1 induced phosphorylation of p70S6K1, shown in figure 2.15A. These data suggested that TAK1 was not required for IGF1 induced mTOR phosphorylation.

Collectively, the above results implied that TAK1 inhibitor specifically suppressed LPS, TNFα and IL-1β induced mTOR activation but not IGF induced mTOR activation.

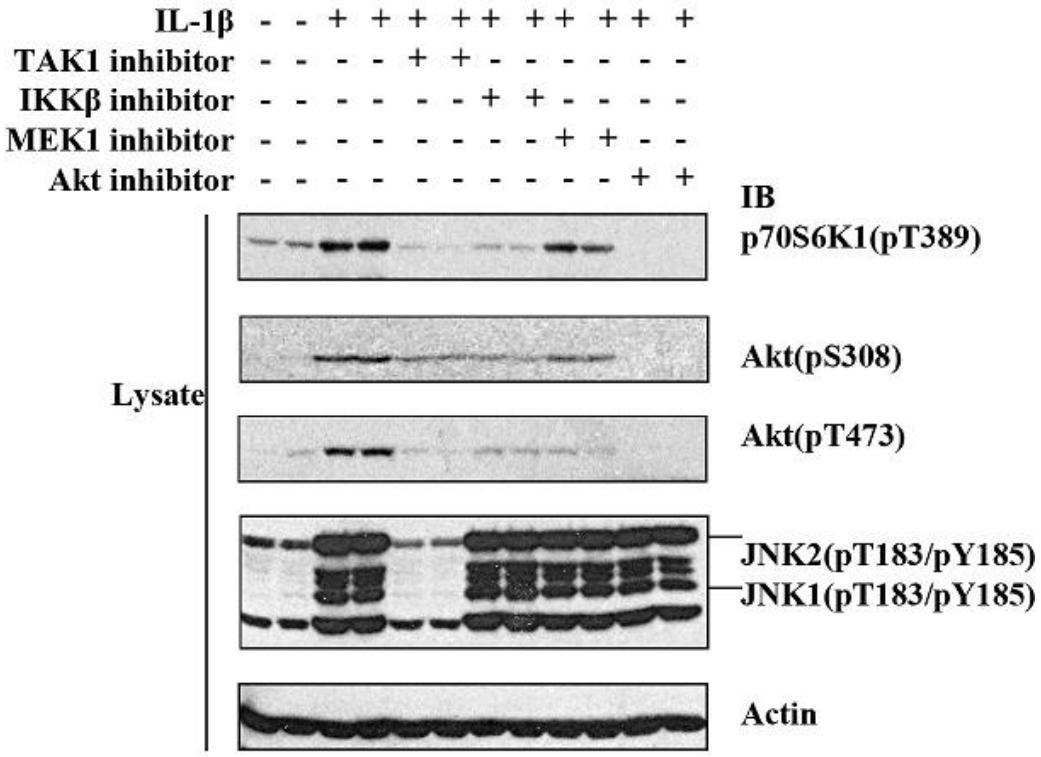
A


IGF1 - - + + + + + + + + + +
TAK1 inhibitor - - - - + + - - - - - -
IKKβ inhibitor - - - - - - + + - - - -
MEK1 inhibitor - - - - - - - - + + - -
Akt inhibitor - - - - - - - - - - + +
IB
p70S6K1(pT389)
Lysate
Akt(pS308)
Akt(pT473)
JNK1/2
(pT183/pY185)
Actin

B


TNFα - - + + + + + + + + + +
TAK1 inhibitor - - - - + + - - - - - -
IKKβ inhibitor - - - - - - + + - - - -
MEK1 inhibitor - - - - - - - - + + - -
Akt inhibitor - - - - - - - - - - + +
IB
p70S6K1(pT389)
Lysate
Akt(pS308)
Akt(pT473)
JNK1/2
(pT183/pY185)
Actin

C


IL-1β - - + + + + + + + + + +
TAK1 inhibitor - - - - + + - - - - - -
IKKβ inhibitor - - - - - - + + - - - -
MEK1 inhibitor - - - - - - - - + + - -
Akt inhibitor - - - - - - - - - - + +
IB
p70S6K1(pT389)
Akt(pS308)
Lysate
Akt(pT473)
JNK2(pT183/pY185)
JNK1(pT183/pY185)
Actin

D

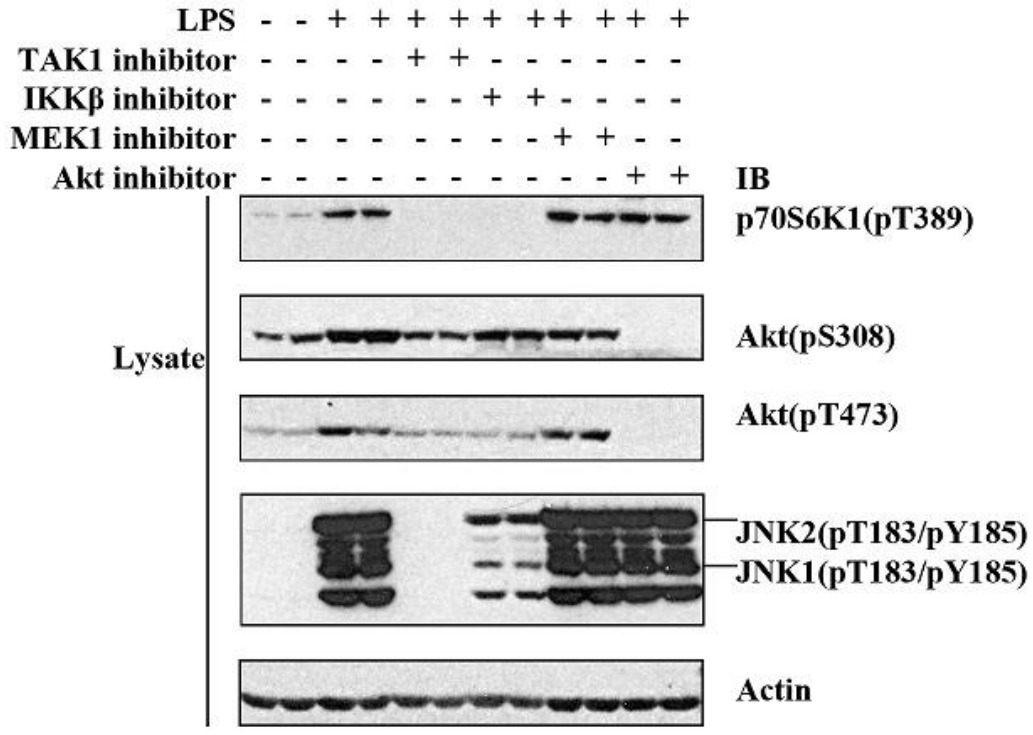

LPS - - + + + + + + + + + +
TAK1 inhibitor - - - - + + - - - - - -
IKKβ inhibitor - - - - - - + + - - - -
MEK1 inhibitor - - - - - - - - + + - -
Akt inhibitor - - - - - - - - - - + +
IB
p70S6K1(pT389)
Akt(pS308)
Lysate
Akt(pT473)
JNK2(pT183/pY185)
JNK1(pT183/pY185)
Actin

Figure 2.15 TAK1 inhibitor suppresses mTOR activation induced by IL-1β, TNFα and LPS.

A TAK1 inhibitor does not affect IGF1 induced mTOR activation. MCF7 cells were pre-incubated with 5 μM TAK1 inhibitor, 40 μM IKKβ inhibitor, 10 μM MEK1 inhibitor or 1 μM Akt inhibitor for 1hour, followed by 25 ng/ml of IGF1 treatment of 30 minutes. Cell lysate was probed with p70S6K1(pT389), Akt(pS308), Akt(pT473), JNK1/2(pT183/pY185) or Actin antibodies.

B TAK1 inhibitor blocks TNF α / IL-1β induced mTOR activation. MCF7 cells were treated as A, but with 100 ng/ml TNF α or IL-1β stimulation for 30 minutes.

C TAK1 inhibitor blocks IL-1β induced mTOR activation. MCF7 cells were treated as in A, but with 100 ng/ml IL-1β stimulation for 30 min.

D TAK1 inhibitor blocks LPS induced mTOR activation. RAW264.7 cells were treated as A, but with 100 ng/ml LPS stimulation for 30 minutes.

3.5.3 Over-expression of TAK1 leads to phosphorylation of p70S6K1

Since inhibition of TAK1 kinase activity suppressed mTOR activation, we tested whether introduction of exogenous TAK1 might lead to mTOR activation. To test this possibility, TAK1 plasmid was transfected into HEK293 cells. Meanwhile, IKKβ was used as a positive control because it was reported to activate mTOR pathway and lead to phosphorylation of p70S6K1. As shown in Figure 2.16, over-expressed TAK1 and IKKβ showed similar capacity to activate endogenous p70S6K1 but over-expressed MAPK p38α did not. Over-expression of TAK1 resulted in mTOR activation, indicating that TAK1 was sufficient to activate mTOR pathway.

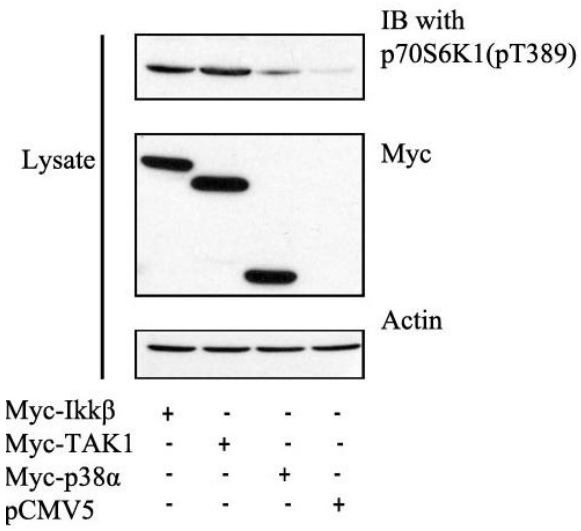


Figure 2.16 Over expression of TAK1 leads to mTOR activation

HEK293 cells were transfected with Myc tagged IKKβ, TAK1 or p38α or empty vector pCMV5. Cells were harvested 36 hours after transfection. Cell lysate was probe with p70S6K1 (pT389), Myc and Actin antibodies.

3.5.4 mTOR activation is attenuated in TAK1 knockdown cells

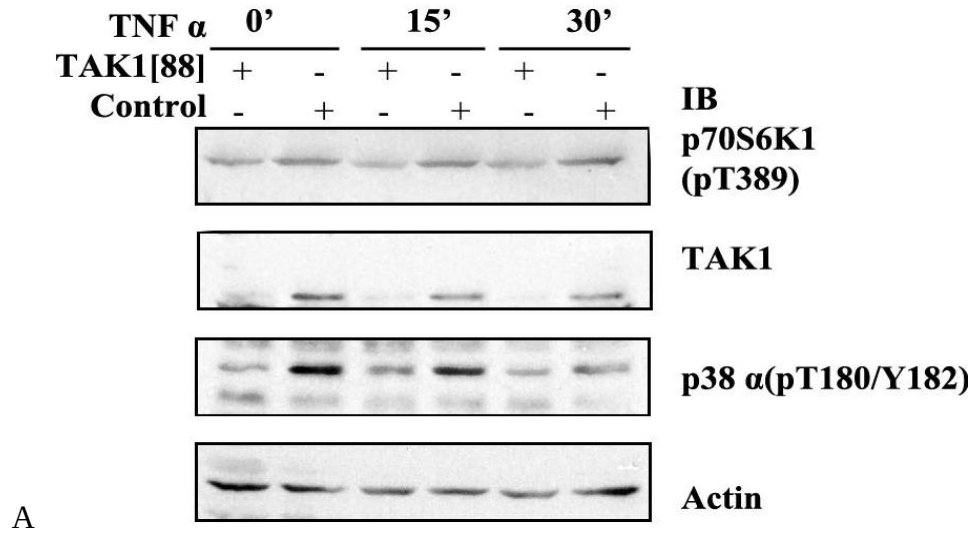
To address whether TAK1 is necessary for mTOR activation induced by LPS, and TNFα, shRNA approach was applied.

TAK1 shRNA(88) or control vector was transiently transfected in to HEK293 cells. 36 hours after transfection, cells were cultured in serum free medium for overnight and stimulation with TNFα. When cells were harvested, expression level of TAK1 was probed. As shown in Figure 2.17A, TAK1 expression was efficiently blocked in the TAK1 shRNA(88) transfected cells, in which phosphorylation of p38α upon TNFα stimulation was subsequently reduced. In unstimulated cells, the phosphorylation of p70S6K1 in TAK1 knockdown cells was slightly weaker than that in control cells; after stimulation, the phosphorylation of p70S6K1 between TAK1 knockdown cells and control cells was clearly different: it remained at the basic level in knockdown cells while it

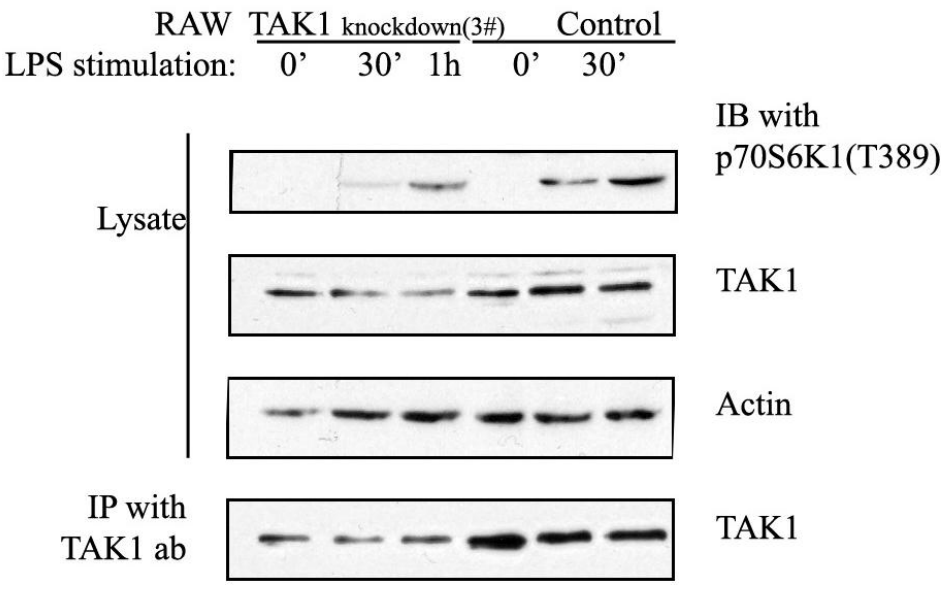
continuously increased in control cells. Transient knockdown of TAK1 in HEK293 cells attenuated the phosphorylation of p70S6K1 upon TNFα stimulation, indicating that TAK1 was necessary for TNFα induced phosphorylation of p70S6K1.

Next, we tested the impact of TAK1 in LPS induced phosphorylation of p70S6K1 in RAW264.7 cells since LPS induced mTOR activation was the starting point of this study. The TAK1 shRNA(88) was transfected into RAW264.7 cells via Fugene Transfection reagent. 18 hours after transfection, cells were cultured in selective medium for a further10 days. The remaining colonies which survived the selection were picked and transferred into 96-wells plate and continuously cultured in selective media for stable cell line. After one month selection and culture, the TAK1 expression level was quantified in the cell line (Figure 2.17 B); there was about 50% decrease of expression of TAK1 in knockdown cells compared with that in control cells. Phosphorylation of p70S6K1 upon LPS stimulation in TAK1 knockdown cells was weaker than that in control cells, indicating that TAK1 was indispensable for LPS induced mTOR activation.

Collectively, the TAK1 transient knockdown cells and TAK1 stable knockdown cell lines both supported that TAK1 was necessary for TNFα and LPS induced mTOR activation.



A



B

Figure 2.17 Suppression of TAK1 expression attenuates mTOR activation.

A Transient TAK1 knockdown attenuates TNFα induced mTOR activation. HEK293 cells were transfected with TAK1 shRNA(88) or control shRNA. 36h later, cells were cultured in the serum free medium for another 16hrs, followed by stimulation with 100 ng/ml TNFα stimulation for the indicated time. Cell lysate was probed with p70S6K1 (pT389), TAK1, p38 α (pT180/pY182) and Actin antibodies.

B Stable knock down of TAK1 attenuates TNFα induced mTOR activation. RAW264.7 cells were transfected with TAK1 shRNA(88) or control shRNA. 18h after transfection, cells were cultured in selective medium containing 500 μg/ml G418 for 10 days. The single cells colonies were chosen under microscope and transferred into 96-well plate. At the same time, the expression of TAK1 was monitored and the cells with the lowest TAK1 expression were propagated. These cells were stimulated with 50 ng/ml LPS for the indicated time. Cell lysate was probed by p70S6K1(pT389), TAK1 and Actin antibodies. Cell lysate was also subjected to immunoprecipitation with TAK1 antibody, followed by immunoblotting with TAK1 antibody.

3.5.5 mTOR response to IL-1β, TNFα and LPS is reduced in TAK1 deficient cells

The TAK1 deficient mouse embryonic fibroblast cells encode a TAK1 protein which lacks the ATP binding domain from residue 40 to 70. The resultant truncated TAK1 migrates faster as detected by western blotting but has no kinase activity. Thus, TAK1 deficient cell line fails to activate NF-κB and AP-1 in response to IL-1β and TNFα [149, 150]. Using TAK1 deficient cell, we tested whether the kinase activity of TAK1 was required for mTOR activation upon LPS, TNFα, IL-1β as well as IGF.

Figure 2.18A showed that mTOR responses to IGF in wild type and TAK1 deficient cell were not affected.

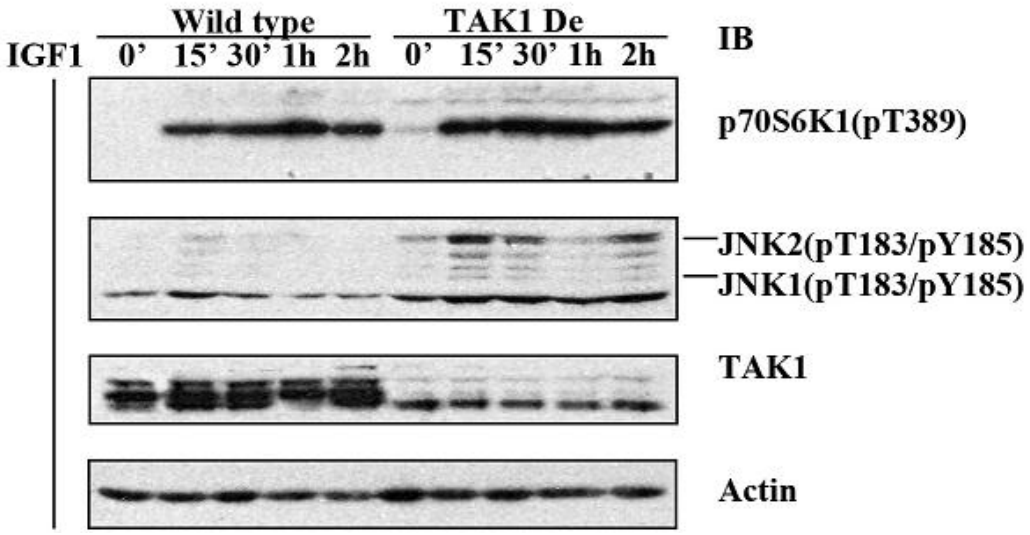
Figure 2.18 B showed that mTOR response, as represented by phosphorylation of p70S6K1, was no longer activated in TAK1 deficient cells upon LPS stimulation while it was in wild type cells. Meanwhile, the phosphorylation of JNK was not activated upon LPS stimulation in TAK1 deficient cells, either. This data indicated that mTOR activation as well as JNK activation upon LPS stimulation depended on TAK1 kinase activity.

Figure 2.18 C showed that mTOR response, represented by phosphorylation of p70S6K1, was not activated upon TNFα stimulation in TAK1 deficient cells as it was in wild type cells. This was companied with the reduced phosphorylation signal of JNK in TAK1 deficient cells. This data indicated that TAK1 kinase activity was indispensable for mTOR activation and JNK phosphorylation upon TNFα stimulation.

Figure 2.18 D showed that mTOR response, represented by phosphorylation of p70S6K1, was not induced upon IL-1β stimulation in TAK1 deficient cells while it was in wild type cells. There was also reduced phosphorylation signal of JNK from TAK1 deficient cells compared with the one from wild type cells. This data indicated that IL-1β induced mTOR activation and JNK phosphorylation required TAK1 kinase activity.

Collectively, these results from TAK1 deficient cell lines showed that TAK1 kinase activity was indispensible for mTOR activation in response to LPS, IL-1β and TNFα stimulations while it was not necessary for IGF induced mTOR activation.

A



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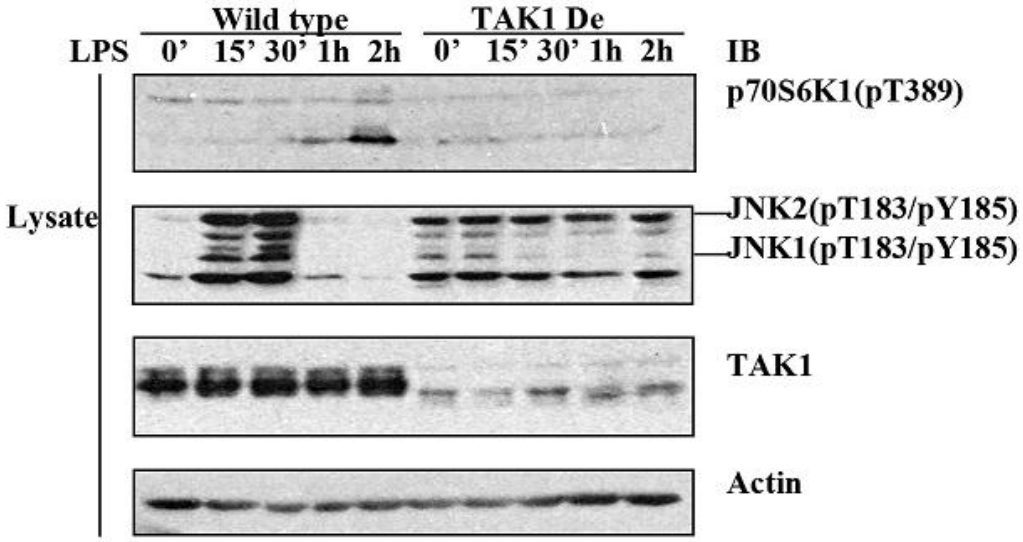
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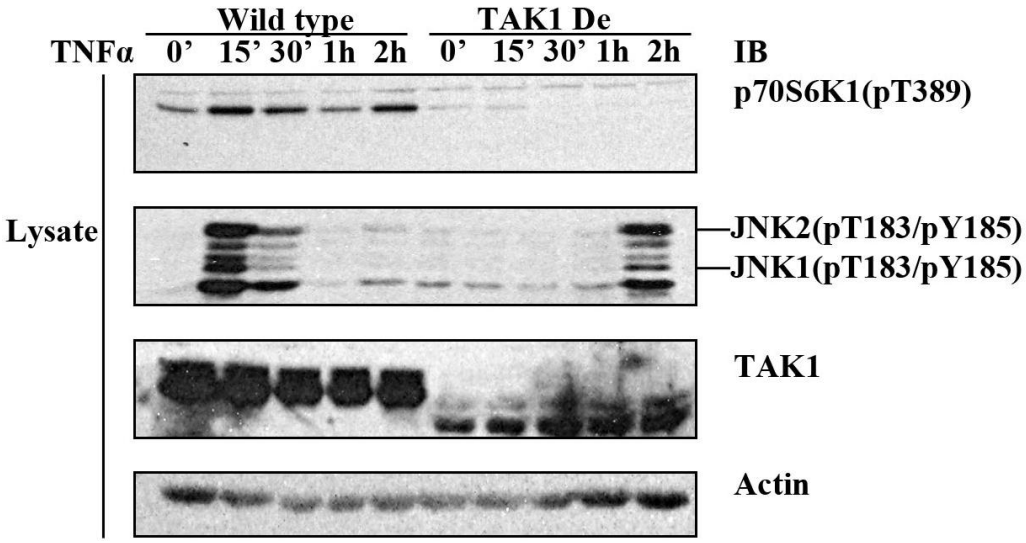
Lysate
JNK2(pT183/pY185)
JNK1(pT183/pY185)
TAK1
Actin

C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TNFα
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IB
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Lysate
JNK2(pT183/pY185)
JNK1(pT183/pY185)
TAK1
Actin

B

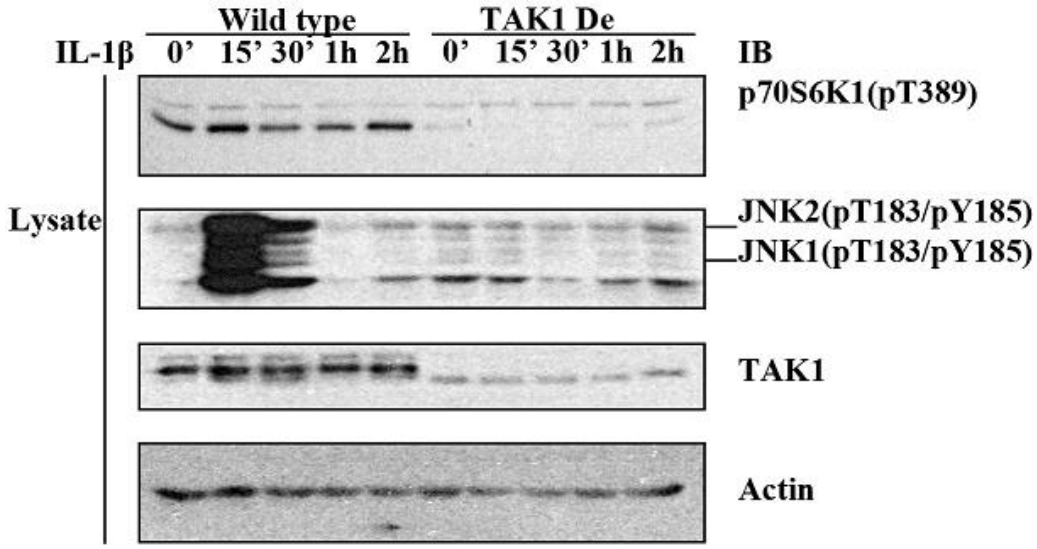


Figure 2.18 TAK1 deficient cells present weak

mTOR response to IL-1β, TNFα and LPS, but not to IGF.

A TAK1 deficient cells show reduced mTOR response to IGF. Wild type and TAK1 deficient MEF cells were starved for 16 hrs, followed by stimulation with 25 ng/ml IGF1 for the indicated time. Cell lysate was probed with p70S6K1(pT389), JNK1/2 (pT183/pY185), TAK1 and Actin antibodies.

B TAK1 deficient cells show reduced mTOR response to LPS. Wild type and TAK1 deficient MEF cells were stimulated by 200 ng/ml LPS for the indicated time. Cell lysate was probed with p70S6K1 (pT389), JNK1/2 (pT183/pY185), TAK1 and Actin antibodies.

C and D TAK1 deficient cells show reduced mTOR response to TNFα and IL-1β. Cells were treated as A, but with stimulation of 100 ng/ml TNFα or IL-1β.

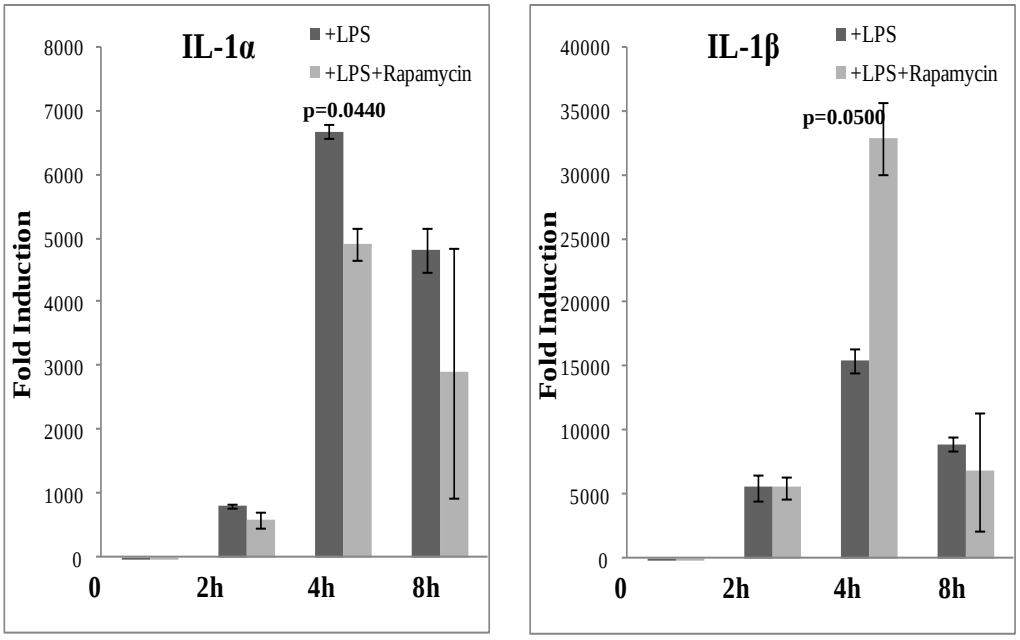
3.6 mTOR regulates mRNA synthesis of LPS induced cytokines

The end event of LPS, TNFα and IL-1β stimulations is induction of cytokine synthesis. Since LPS stimulation signal also activates mTOR pathway, it is important to find out the role which mTOR pathway plays in the regulation of cytokine synthesis. mTOR pathway regulates translation machinery assemble, which affects the global protein synthesis. Thus, it is difficult to gauge the specific effect of mTOR on LPS induced cytokine protein synthesis. Therefore,

we studied the mTOR pathway on LPS induced cytokine synthesis at mRNA level.

Real time PCR was used to monitor the mRNA synthesis of various cytokines in response to LPS treatment. The mTOR inhibitor rapamycin was added in one group of cells to block mTOR activation during LPS stimulation. Figure 2.19 A-K showed the synthesis of mRNA of indicated cytokines at different time points from LPS stimulated RAW264.7 cells with or without additional rapamycin treatment. The results are further summarized the Table.

A **B**



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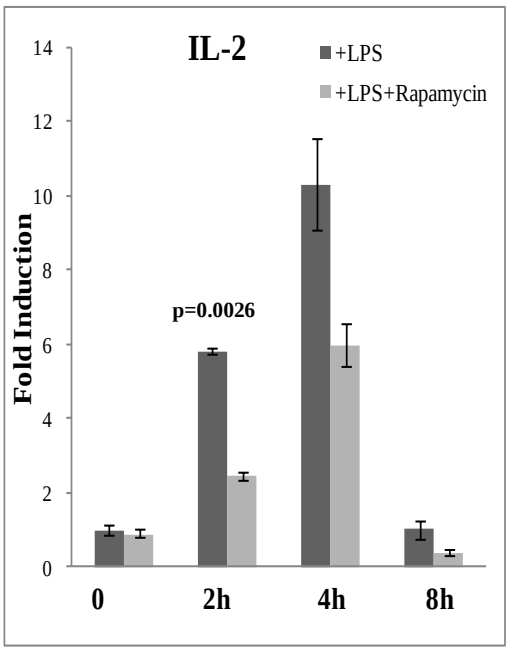
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+LPS+Rapamycin
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10
8
6
4
2
0
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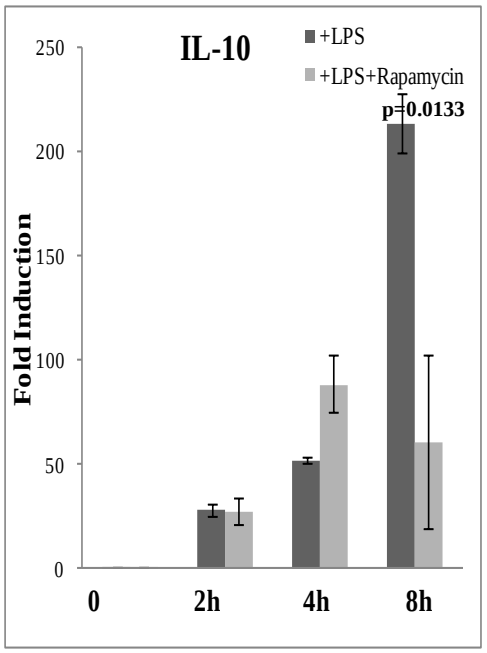
0
2h
4h
8h

D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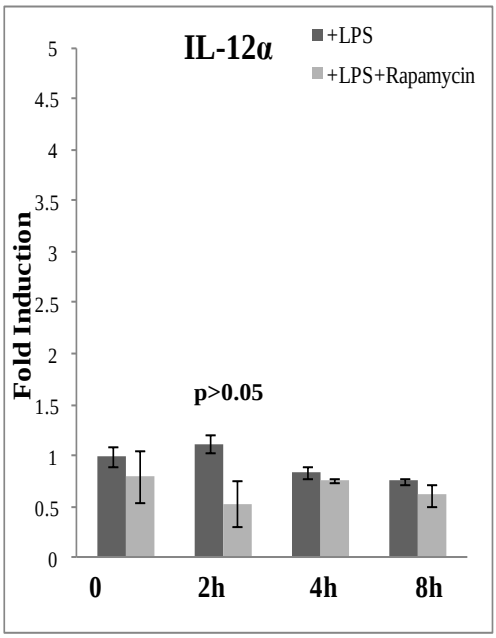
8h

E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+LPS+Rapamycin
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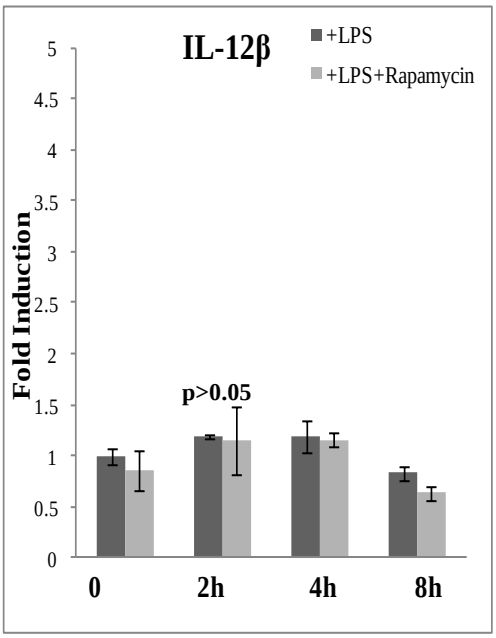
p>0.05
0
2h
4h
8h

F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+LPS+Rapamycin
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0
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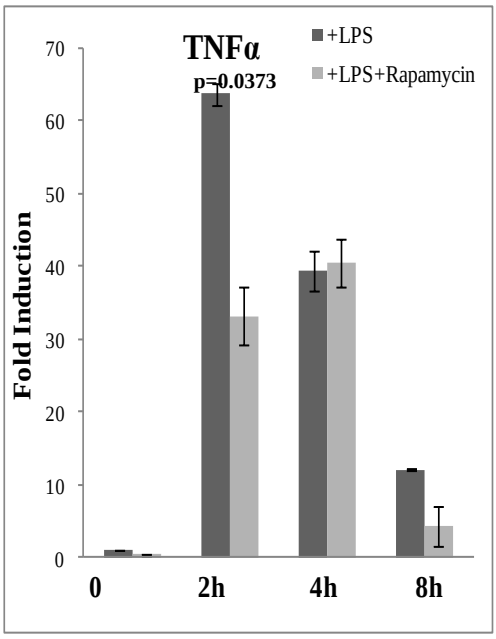
2h
4h
8h

G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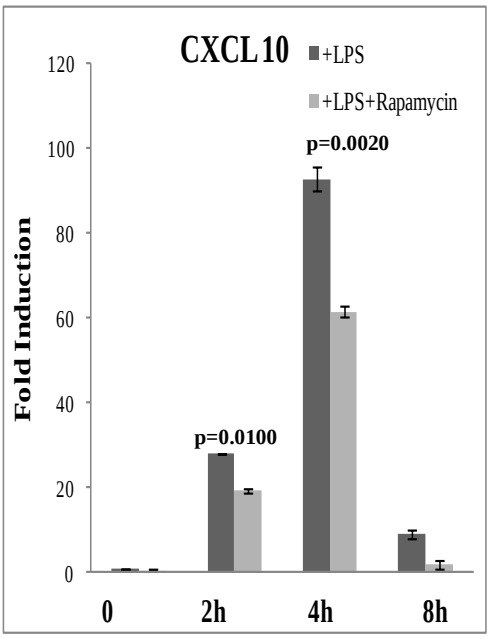
4h
8h

H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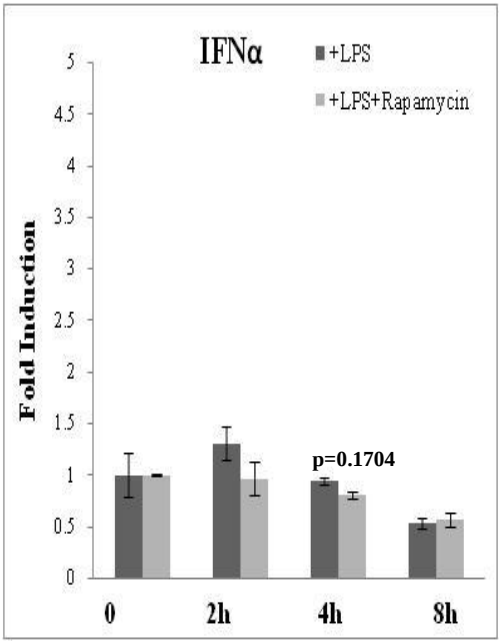
8h

I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+LPS+Rapamycin
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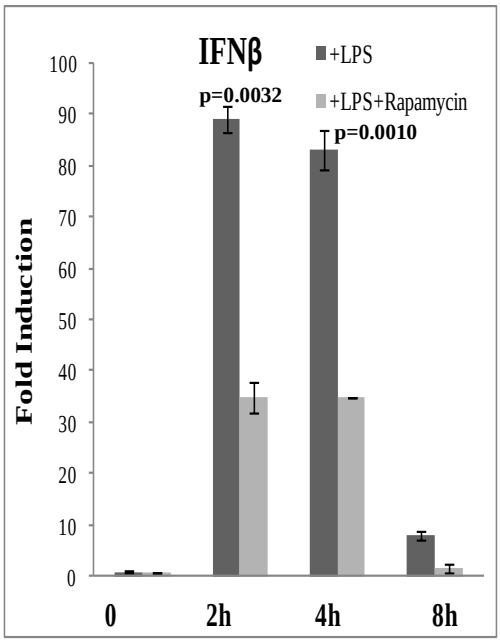
J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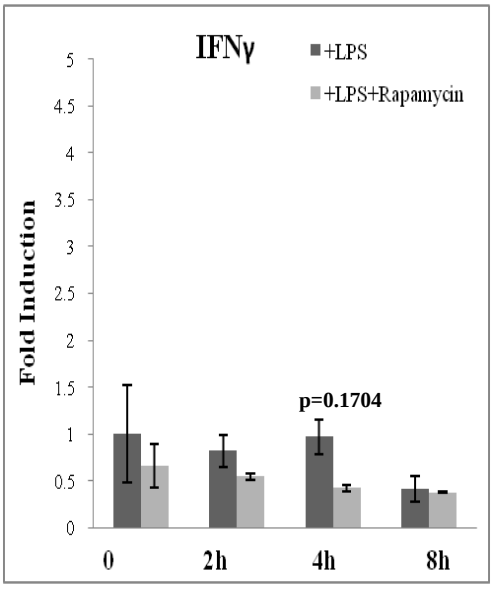


Figure 2.19 Impact of mTOR on mRNA synthesis of LPS induced cytokines

RAW264.7 cells were pre-incubated with or without rapamycin for 1 h, followed by stimulation with 100 ng/ml LPS for the indicated time. Cells were harvested with 1 ml of TRIzol® Reagent and RNA was purified. 2 µg of total RNA was reversed transcribed into cDNA for 1 h at 50°C using oligo dT primer for individual cytokine and reverse transcriptase in the presence of RNAse inhibitor. Transcribed cDNA template (50 ng) was incubated with 200 nM primers in a total volume of 20 µl using KAPA SYBR FAST qPCR kit. GAPDH was used as internal control to minimize the error of different input amount. For every cytokine, the readout of each time point was standardized with the readout from sample without LPS and rapamycin treatment. Every experiment was repeated for two times and standard deviation was labelled as the error bar. The significant differences between Rapamycin treated and untreated samples were labelled with statistic analysis p value.

Table 6 Impact of mTOR on mRNA synthesis of LPS induced cytokines

Cytokine	0h	2h	4h	8h
IL-1α	1/0.82	804.9/596.6	5595/4931	4828/2906
IL-1β	1/0.88	5615/5586	15483/32963	8957/6825
IL-2	1/0.9	5.8/2.5	10.3/6	1.0/0.4
IL-10	1/0.8	28,3/27.5	51.6/88.6	213.6/61
IL-12α(p35)	1/0.80	1.12/0.54	0.84/0.77	0.76/0.62
IL-12β(p40)	1/0.86	1.19/1.15	1.19/1.17	0.84/0.64
TNFα	1/0.4	63.8/33.3	39.5/40.5	12.1/4.4
Interferon α	1.0/1.0	1.3/0.96	0.94/0.8	0.53/0.56
Interferon β	1/0.73	89.3/35	83/34	8/1.7
Interferon γ	1/0.66	0.82/0.54	0.97/0.42	0.41/0.37
CXCL10	1/0.73	28.2/19.3	92.9/61.6	9.1/1.9

Note: The ratio equals to mRNA from LPS stimulated sample divided by mRNA from Rapamycin and LPS stimulated sample

LPS generally induced mRNA synthesis of cytokines in RAW264.7 cells, including pro-inflammatory cytokines IL-1α and β, IL-2, TNFα, CXCL10, as well as anti-inflammatory cytokine Interferon β, IL-10. LPS poorly affected the mRNA synthesis of cytokine IL-12α (p35) and β (p40) while LPS stimulation attenuated RNA synthesis of interferon α and γ.

However, mTORC1 inhibitor rapamycin specifically inhibited the mRNA synthesis of IL-1α, IL-2, TNFα, CXCL10, interferon β as well as the IL-10. Rapamycin treatment further suppressed the mRNA synthesis of interferon γ, compared with samples without rapamycin treatment. It is worth noticing that mRNA synthesis of IL-1β was greatly enhanced by rapamycin, in contrast to its impact on other pro-inflammatory cytokines induced by LPS. On the other hand, rapamycin barely affected RNA synthesis of interferon α, as well as IL-12A and B, indicating that the mRNA synthesis of interferon α、IL-12α and β was irresponsive to LPS stimulation and rapamycin treatment.

Collectively, LPS induced mTOR activation showed its impacts on the mRNA synthesis of various cytokines in a discrimination manner. mTOR activation specifically enhanced the synthesis of interferonβ while suppressed the synthesis of IL-1β, indicated that mTOR activation may attenuate cells' inflammatory response and play an anti-inflammatory role.

3.7 LPS induced mTOR activation affects cell viability

Since mTOR pathway regulates protein synthesis and cell growth, its effects on cell viability was examined after stimulations with IGF, LPS, TNFα and IL-1β.

Firstly, IGF, TNFα and IL-1β were examined for their impacts on cell viability. To address this question, MCF7 cell line was used since it was responsive to all these three agonists. And the cell viability was measured by MTT assay and shown in Figure 2.20A. Compared with unstimulated cells, the viability of cells treated with IGF was higher, up to 17.8%. However, the induction of IGF on cell viability was totally abolished by rapamycin treatment, which suppressed the growth of IGF treated cells and untreated cells to the same level, as shown in Figure 2. 20A. Treatment of TNFα resulted in reduced cell viability, which was even lower when cells were co-treated with rapamycin and TNFα. On the other hand, IL-1β did not show significant impact on cell growth and there was no difference on viability between the cells co-treated with rapamycin/IL-1β and cells treated with rapamycin alone.

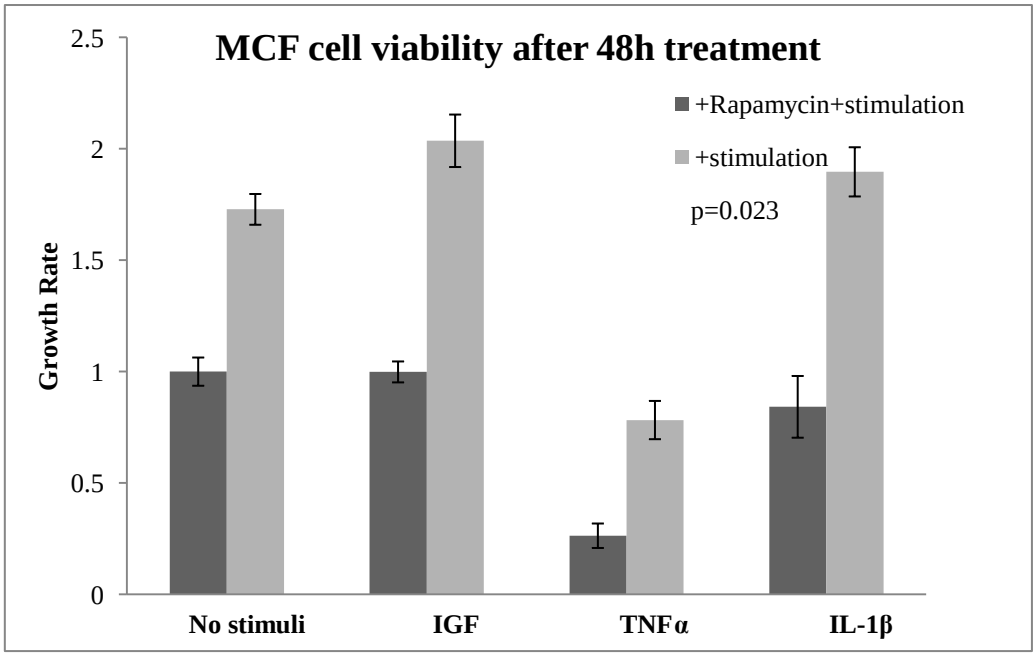
These data implied: long term IGF treatment on MCF7 cells increased cell viability in a manner depending on mTOR pathway; long term treatment of TNFα on MCF7 cells strongly suppressed cell viability in an mTOR pathway independent manner; while long term treatment of IL-1β on MCF7 cells did not affect cell viability in both mTOR active and inactive conditions.

Secondly, LPS was examined for its effect on cell viability. To address this point, RAW264.7 cell was studied as it was responsive to LPS stimulation. As shown in Figure 2.20B, cells naturally grew in forty-eight hours when they were given no treatment. Rapamycin blocked cell growth in forty-eight hours. When cells were treated with LPS at 100 ng/ml, 200 ng/ml or 400 ng/ml, the viability of cells decreased according to LPS dose. When cells were treated with LPS in the presence of rapamycin, the decrease of cell viability was attenuated. This part indicated that LPS induced cell death in a dose dependent

and mTOR pathway dependent manner; it also suggested that LPS treatment would increase cells' sensitivity to rapamycin.

This would be considered for the clinical study on tumorigenesis induced by chronic inflammation. Chronic inflammation is associated with long-term cytokine secretion and, endurable TAK1 activation. Rapamycin treatment could be a good therapy given that it effectively blocked cell growth and reduced cell viability. On the other hand, LPS could be considered as an adjuvant for rapamycin conducted cancer treatment to achieve better effects.

A



B

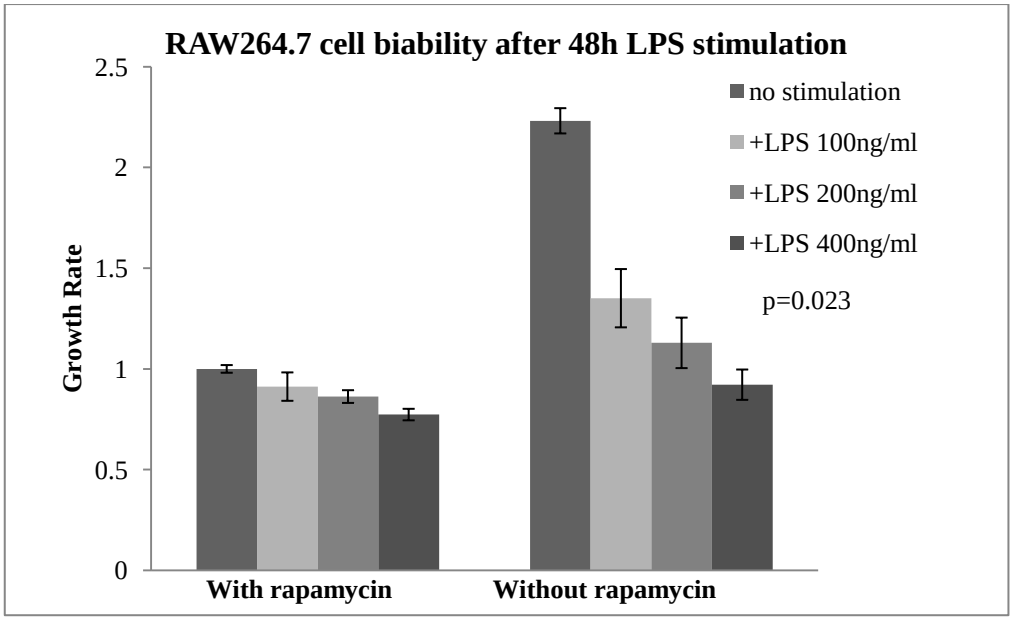


Figure 2.20 Cell viability changes upon different stimulations.

A The viability of MEF7 cells changes upon different stimulations. MEF7 cells were seeded in 96 well-plate at 2500 cells/well in 100μl media. 18h later, cells were cultured in serum free media and incubated with IGF at 25 ng/ml, TNFα at 100 ng/ml or IL-1β at 100 ng/ml alone or together with 100 nM rapamycin. 48h later, 20μl of 5 mg/ml MTT was added to each well and incubated with cells for 2h. The media was removed and 150μl of MTT solvent was added to each plate. 30 min later, the absorbance of the solution at 590 nm was measured. The reading from the sample without LPS and rapamycin treatment was used to standardize other readings. Results are shown as the average from three independent experiments and everyment was repeated for two times. The standard deviation of three sets experiments was labelled as error bar, the t-test was applied and $p=0.023<0.05$.

B The viability of RAW264.7 cells reduces upon LPS stimulation. RAW264.7 cells were seeded in 96 well-plate at 2500 cells/well in 100 μl media. 18h later, cells were incubated with LPS at 100 ng/ml, 200 ng/ml or 400ng/ml as indicated. 48 h later, cells were processed as A. Results are shown as the average from three independent experiments and everyment was repeated for two times. The standard deviation of three sets experiments was labelled as error bar, the t-test was applied and $p=0.023<0.05$.

3.8 Summary

This part of study focused on investigating the molecular connections between inflammatory signaling and mTOR pathway. Upon the identification of TSC2 from TAK1 pulldown complex by Mass Spectrometry, physical interaction between TSC2 and TAK1 was substantiated by GST-pulldown and immunoprecipitation, followed by mapping the kinase domain of TAK1 as the binding domain for TSC2. Phosphorylation of TSC2 was also observed upon

LPS, TNFα and IL-1β stimulations. LPS induced phosphorylation of TSC2 was inhibited by TAK1 inhibitor as well as λ phosphatase. Using phosphorylation antibodies, Ser939 and Thr1462 of TSC2 were found phosphorylated upon LPS, TNFα and IL-1β stimulations. The phosphorylation of TSC2 on Thr1462 upon LPS, TNFα and IL-1β were even attenuated in TAK1 deficient MEF cells. When immunoprecipitation-mass spectrometry approach was used to identify the novel phosphorylation sites on TSC2 mediated by TAK1, Ser1365 of TSC2 was identified as a potential candidate from both endogenous and over-expressed TSC2. Moreover, the downstream of TSC2 -mTOR pathway- was activated upon LPS, TNFα and IL-1β stimulations, as indicated by the phosphorylation of p70S6K1 on Thr389 and 4EBP1 on Thr70. Activation of mTOR was associated with TAK1 activity upon LPS, TNFα and IL-1β stimulations. Inhibition of TAK1activity by TAK1 inhibitor reduced mTOR activation and suppression of TAK1 expression by shRNA attenuated mTOR activation upon TNFα and LPS stimulations. Activation of mTOR pathway was even reduced in TAK1 deficient MEF cells. Subsequently, mTOR activation upon LPS stimulation regulated mRNA synthesis of various cytokines in a cytokine specific manner. LPS stimulation also reduced cell viability in a mTOR activity dependent manner.

With this information, we proposed that: together with Akt/PKB and IKKβ, protein kinase TAK1 phosphorylates TSC2 on Thr1462 and Ser1365 upon LPS, TNFα and IL-1β stimulations to activate mTOR pathway and phosphorylate p70S6K1 and 4EBP1, which in turn regulate cytokines' synthesis and cell viability upon inflammatory signals. This idea is simply summarized in Figure 2.21.

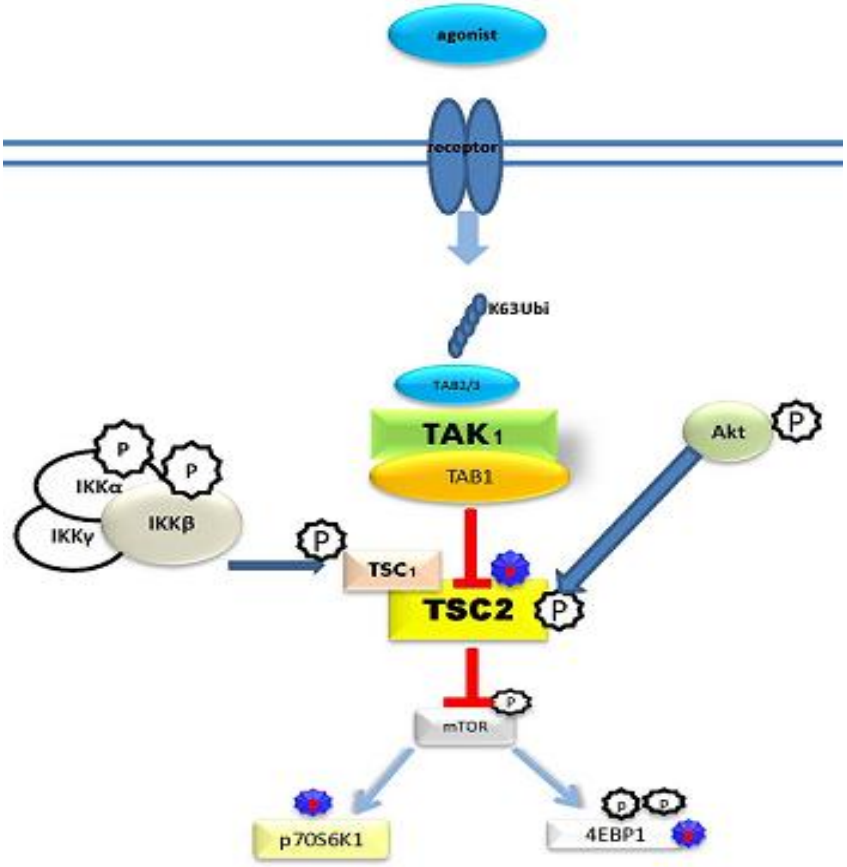


Figure 2.21 Inflammatory signals activate mTOR pathway via TAK1

With association of Akt/PKB and IKKβ, TAK1 phosphorylates TSC2 on Thr1462 and Ser1365 upon LPS, TNFα and IL-1β stimulations, which subsequently activates mTOR pathway and leads to phosphorylation of p70S6K1 and 4EBP1.

Part II HIPK2 and TAK1 pathway

3.9 HIPK2 suppresses activation of TAK1pathway

3.9.1 HIPK2 physically interacts with TAB2/TAB3

TAK1 was previously proposed as an upstream kinase of HIPK2, we firstly examined the direct interaction between HIPK2 and TAK1 complex. When Myc tagged TAB2/3 was over expressed together with GST tagged TAK1 or HIPK2 in HEK 293 cells, cell lysate was subjected to GST pulldown. By probing with Myc antibody, TAB2 and TAB3 were detected with association of HIPK2, as well as TAK1, as shown in Figure 3.1. This data confirmed the strong physical association between HIPK2 and TAB2/3. On the other hand, there is no report about physical association of TAK1 and HIPK2 to date. And we did not detect the interaction between TAK1 and HIPK2, either.

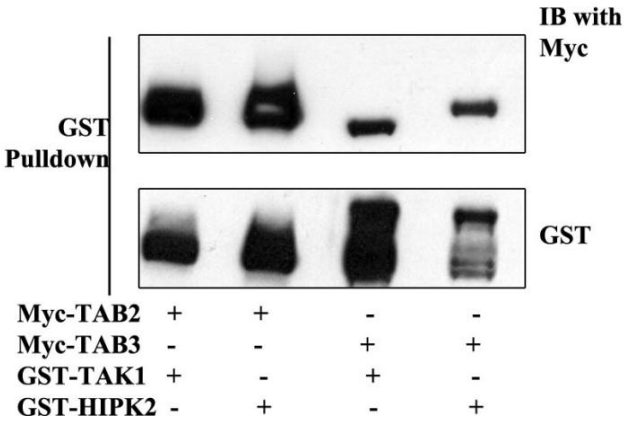


Figure 3.1 HIPK2 interacts with TAB2/TAB3

HEK293 cells were transfected with Myc-TAB2/Myc-TAB3 together with either GST-TAK1 or GST-HIPK. Cell lysate was subjected to GST pull down, followed by probing with Myc antibody and GST antibody.

3.9.2 HIPK2 stabilizes TAB2/TAB3

HEK293 cells were transiently transfected with TAB2 or TAB3 plasmid together with Myc tagged TAK1, HIPK2 or p38α respectively. TAB2 and TAB3 are initially identified as TAK1 binding proteins and they can barely express alone. As reported before: TAK1 stabilized TAB2 and TAB3 while p38α did not. HIPK2 greatly stabilized TAB2 and TAB3 when they were transiently expressed in HEK293 cells, even better than TAK1, as show in Figure 3.2. This confirmed a tight interaction between HIPK2 and TAB2/3.

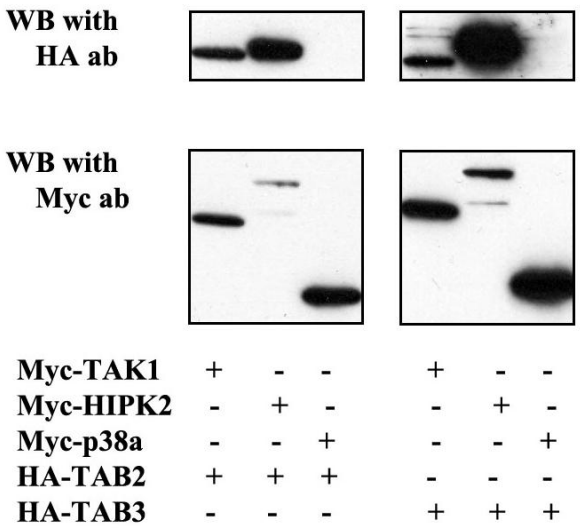


Figure 3.2 HIPK2 stabilizes TAB2 and TAB3 as TAK1 does.

HEK293 cells were transfected with HA tagged TAB2 or TAB3 together with Myc-TAK1, Myc-HIPK2 or Myc-p38α. Cell lysate was subjected to western blot with both HA antibody and Myc antibody.

3.9.3 HIPK2 suppresses ubiquitin binding to TAK1 complex

Although HIPK2 interacted physically with TAB2 and TAB3, the function of this interaction was still unknown. It was reported that TAB2 and TAB3 are recruited by E3 ligase TRAF6 to mediate the ubiquitin binding and activate

TAK1 with polyubiquitin binding. We examined whether HIPK2 plays roles in modulating polyubiquitin chain attachment to the TAK1 complex. HIPK2 was over expressed with TAK1 complex and cell lysate was subjected to GST-pulldown. The endogenous ubiquitin binding on TAK1 was dramatically reduced in the presence of HIPK2 when the blot was probed for ubiquitin. Here, protein kinase NLK (Nemo Like Kinase) was used as a control, and our unpublished data suggest that NLK could effectively reduce the polyubiquitin binding of TAK1 complex, as shown in Figure 3.3.

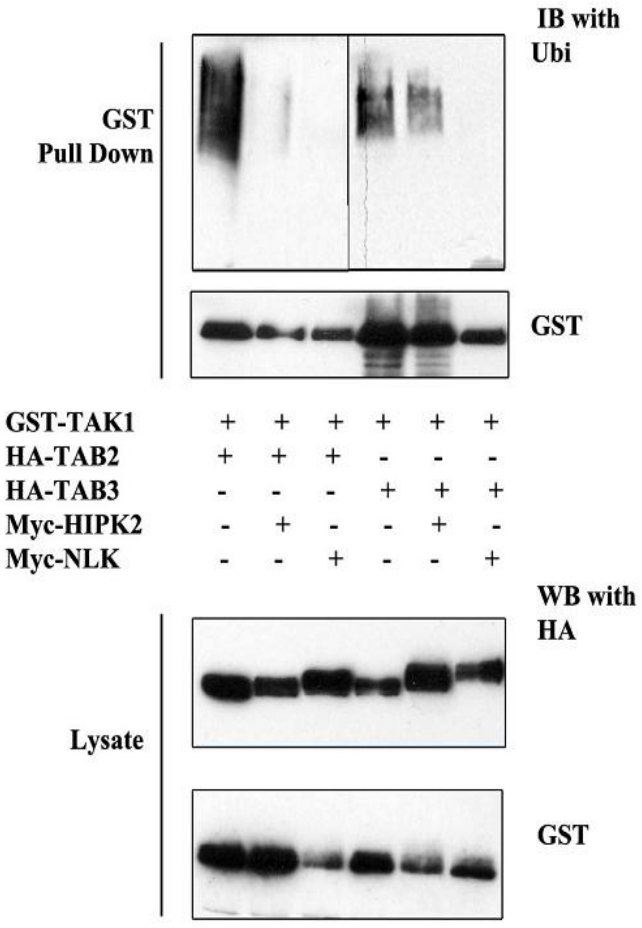


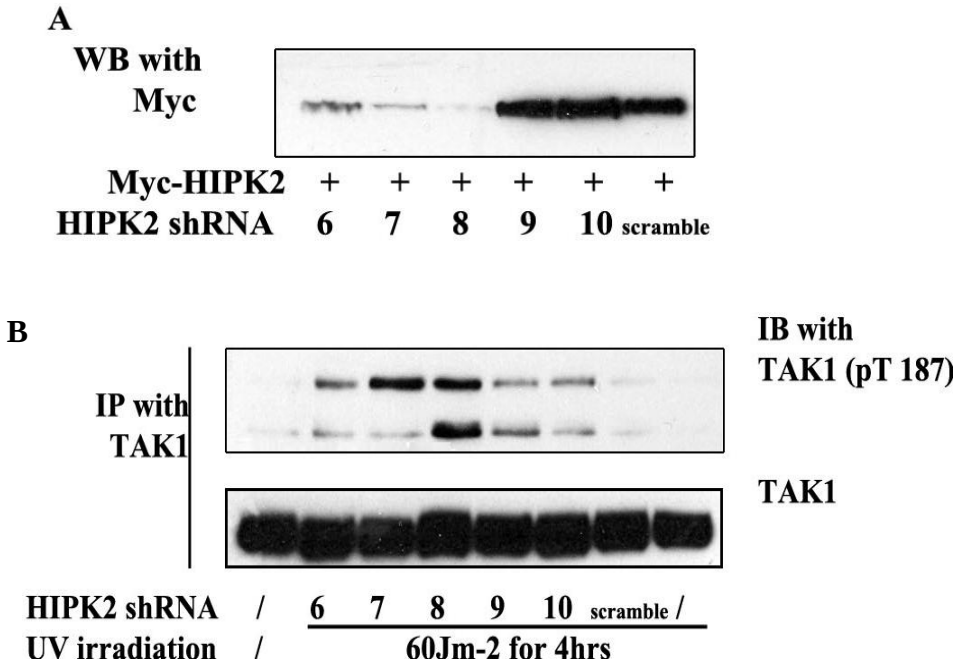
Figure 3.3 HIPK2 reduces the polyubiqitin binding of TAK1 complex.

HEK293 cells were transfected with GST-TAK1 and HA-TAB2 or HA-TAB3 together with either Myc-HIPK2 or Myc-NLK. Cell lysate was subjected to GST pull down, followed by probing with ubiquitin (Ubi) and GST antibody. Cell lysate was also directly probed with HA antibody and GST antibody to detect the expression of transfected protein.

3.9.4 Suppression of HIPK2 enhances TAK1 activity and downstream signal activation

It was reported that TAB2 and TAB3 functioned as adaptor proteins and mediated polyubiquitin chain binding to TAK1complex, which in turn triggered the activation of TAK1. Since HIPK2 interacted with TAB2 and TAB3 and interrupted the polyubiquitin binding of TAK1 complex, we looked for physiological effects of HIPK2 in TAK1 pathway.

To this point, HIPK2 shRNA was used to knockdown HIPK2 expression. Five shRNAs targeting different regions of the HIPK2 cDNA were tested. Firstly, HEK293 cells were transiently transfected with HIPK2 plasmid and each of shRNAs respectively. By monitoring the expression level of HIPK2, we hoped to determine the knock down efficiency of these shRNA and select the best one for further study. It was showed that shRNA 6, 7 and 8 had the best knock down efficiency in Figure 3.4A. We then transiently knocked down HIPK2 in HEK293 cells, 48 h later, the cells were exposed to UV irradiation and TAK1 activity was examined. UV irradiation has been shown to activate both HIPK2 and TAK1. If HIPK2 affected TAK1 activity physiologically, we expected to see an effect on TAK1 activity. As shown in Figure 3.4.B, TAK1 activity was greatly increased once expression of endogenous HIPK2 was suppressed, compared with that from control transfected and untransfected cells. Among five shRNAs, shRNA 8 lifted the TAK1 activity even more. Thus, shRNA 8 was chosen for further study.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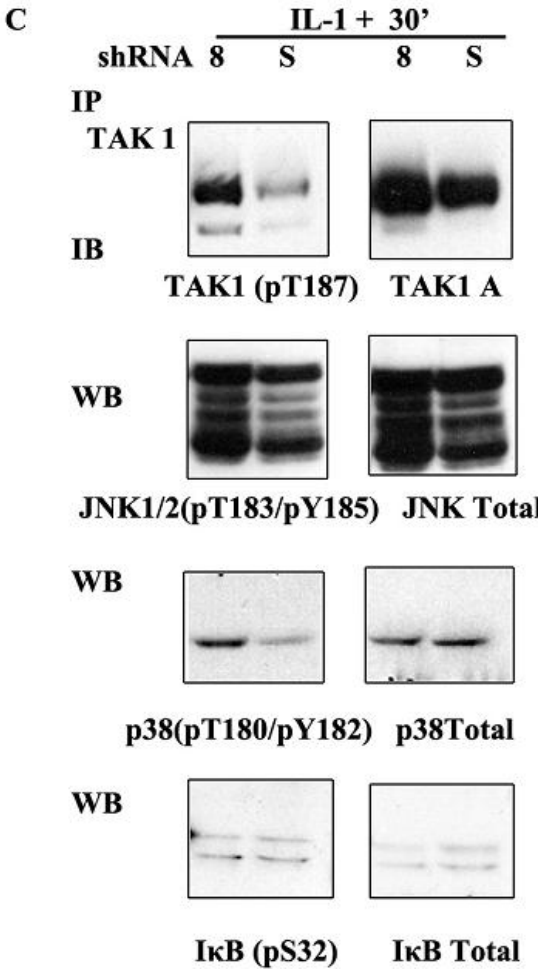
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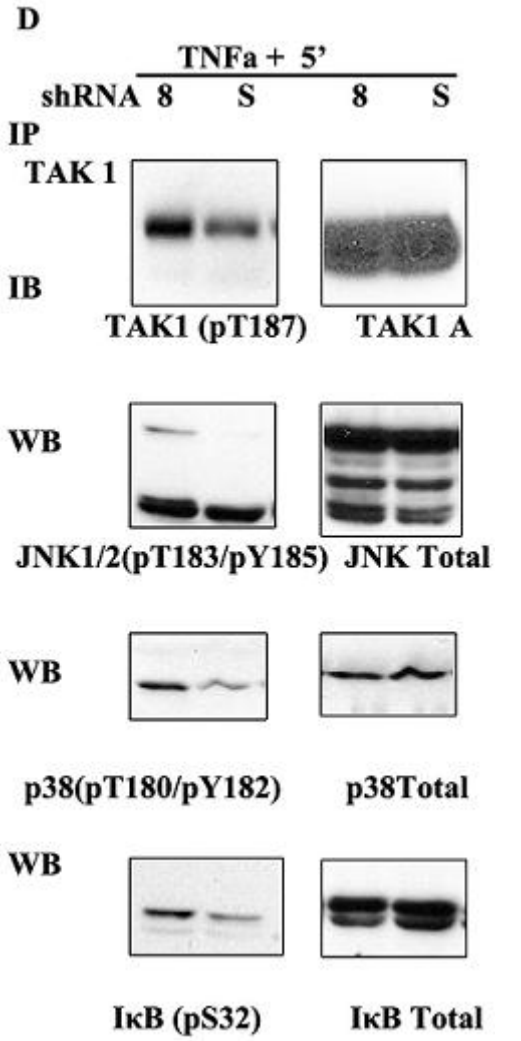
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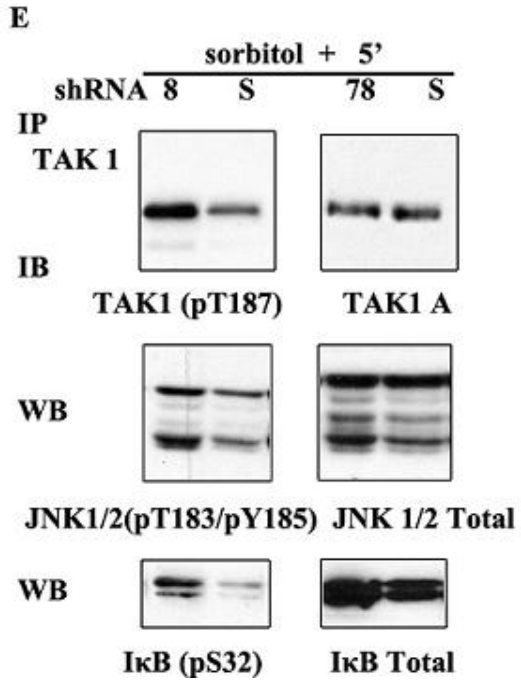
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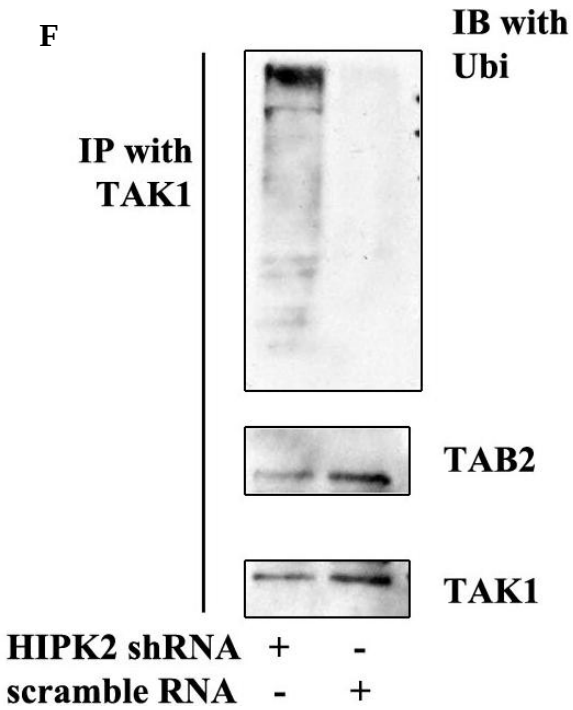

Fig. 3.4 Suppression of HIPK2 enhances TAK1 activity

A Selection of HIPK2 shRNA *in vitro*; HEK293 cells were transfected with Myc-HIPK2 and HIPK2 shRNA 6-10 or scramble shRNA. Cells were harvested 48 hours later, and cell lysate was probed with Myc antibody;

B Selection of HIPK2 shRNA *in vivo*; HEK293 cells were transfected with HIPK2 shRNA 6-10 or scramble shRNA. 48 hours after transfection, cells were stimulated with $60Jm^{-2}$ UV irradiation for 4 hours. Cell lysate was subjected to IP with TAK1 antibody, followed by the probing with TAK1 (pT187) antibody;

C Suppression of HIPK2 lifted TAK1 activity induced by IL-1β; HEK293 cells were transfected with HIPK2 shRNA 8 or scramble shRNA, 36 hours after transfection, cells were cultured in serum-free medium for 12 hours, followed by 50 ng/ml IL-1β stimulation for 30 minutes. Cell lysate was either subjected to IP with TAK1 antibody and IB with TAK1 (pT187) antibody, or probed with JNK1/2 (pT183/pY185), JNK total, p38 (pT180/pY182), p38α total, IκB (pS32), IκB total antibodies.

D Suppression of HIPK2 enhances TAK1 activity induced by TNFα; HEK293 cells were transfected with HIPK2 shRNA 8 or scramble shRNA, 36 hours after transfection, cells were cultured in serum-free medium for 12 hours, followed by 50 ng/ml TNFα stimulation for 5 minutes. Cell lysate was either subjected to IP with TAK1 antibody and IB with TAK1 (pT187) antibody, or probed with JNK1/2 (pT183/pY185), JNK total, p38(pT180/pY182), p38α total, IκB(pS32), IκB total antibodies;

E Suppression of HIPK2 enhances TAK1 activity induced by sorbitol; HEK293 cells were transfected with HIPK2 shRNA 8 or scramble shRNA, 48 hours after transfection, cells were incubated with 0.5M sorbitol for 5 minutes. Cell lysate was either subjected to IP with TAK1 antibody and IB with TAK1(pT187) antibody, or probed with JNK1/2 (pT183/pY185), JNK total, IκB(pS32), IκB total antibodies;

F Suppression of HIPK2 enhances the polyubiqintin binding of TAK1 complex; HEK293 cells were transfected with HIPK2 shRNA 8 or scramble shRNA, 36 hours after transfection, cells were cultured in serum-free medium for 12 hours, followed by 50 ng/ml TNFα stimulation for 5 minutes. Cell lysate was subjected to IP with TAK1 antibody and IB with ubiquitin (Ubi), TAB2 and TAK1 antibodies.

Following suppression of HIPK2 expression by HIPK2 shRNA 8, HEK293 cells were stimulated with IL-1β, TNFα and sorbitol to activate TAK1. The activity of TAK1 and downstream proteins were monitored under these stimulations. As shown in Figure 3.4C, D and E, knocking down of HIPK2 significantly increased TAK1 activity and its downstream phosphorylation of p38α, IκB as well as JNK upon different stimulations. This indicated that HIPK2 suppressed the activity of TAK1 complex and its downstream signalling.

Also in Figure 3.4 B, where HIPK2 was knocked down with different shRNA and cells were stimulated with UV irradiation, the increased TAK1 phosphorylation on Thr187 in HIPK2 knockdown samples implied that presence of HIPK2 suppressed TAK1 ability in response to UV irradiation.

These data suggested that HIPK2 generally suppressed TAK1 downstream signal cascade upon cytokine stimulation, hyperosmotic stress and UV irradiation.

In paragraph 3.9.3, we mentioned that overexpressed HIPK2 reduced polyubiquitin binding of TAK1 complex. The polyubiquitin binding of TAK1 complex was considered as the important clue for TAK1 activation. To look for the mechanism of how HIPK2 regulated TAK1 complex *in vivo*, HEK293 cells were transfected with HIPK2 shRNA, followed by stimulation with TNFα. The cell lysate was then subjected to immunoprecipitation with TAK1 antibody and immunoblot for ubiquitin. As shown in Figure 3.4F, with the equal input of TAK1 complex, the poly ubiquitin binding of TAK1 was much stronger in the

HIPK2 knockdown cells than that of control cells, implying that presence of HIPK2 may inhibit binding of polyubiquitin chains to TAK1 complex.

Collectively, these results suggested that HIPK1 suppressed activity of TAK1 and its downstream by inhibiting complex binding with polyubiquitin chains.

3.9.5 IL-1β induces phosphorylation of p53 on Ser46

It was well known that HIPK2 was activated upon UV irradiation. However, the activation status of HIPK2 upon other stimulations which induces TAK1 activity was not clear. Since we proposed that HIPK2 suppressed TAK1 activity upon different stimulations, it is critical to find out HIPK2 activity status in these conditions.

Using phosphorylation of p53 at Ser46 as an indicator of HIPK2, we looked for the HIPK2 activation upon IL-1β stimulation. As shown in Figure 3.5, IL-1β induced phosphorylation of p53 at Ser46 strongly after 30minute or 1h. This implied that IL-1β stimulation activated HIPK2. But so far, after stimulation of LPS or TNFα, the phosphorylation of p53 on Ser46 had not been detected in our system or reported by any groups.

This part of study suggested that HIPK2 was activated in IL-1β stimulation but not in LPS or TNFα stimulations.

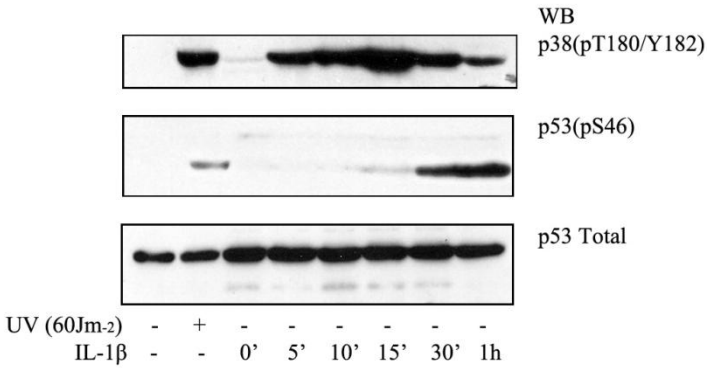

Figure 3.5 IL-1β induces phosphorylation of p53 at Ser 46

One part of HEK293 cells were stimulated with 60Jm^{-2} UV irradiation. The other part of HEK293 cells were starved overnight, followed by IL-1β stimulation for the indicated time. Cell lysate was separated by SDS-PAGE and probed with p38(pT180/pY182), p53 (pS46) and p53 antibodies.

3.9.6 Summary

This part of study focused on the characterization of interaction between HIPK2 and TAK2/3. HIPK2 stabilized TAB2 and TAB3, which mediated polyubiquitin binding to TAK1 complex. When expression of HIPK2 was suppressed by shRNA, the polyubiquitin binding of TAK1 complex was enhanced, as well as the activation of TAK1 and downstream MAPK and IκB. Moreover, activity of HIPK2 was observed upon IL-1β stimulation, indicated by phosphorylation of p53 on Ser46. Taken together, these data suggested an proposal that HIPK2 generally suppresses activation of TAK1 and its downstream by phosphorylating TAB2/TAB3 and interrupting the polyubiqutin binding of TAK1 complex. This signalling is summarized in Figure 3.6.

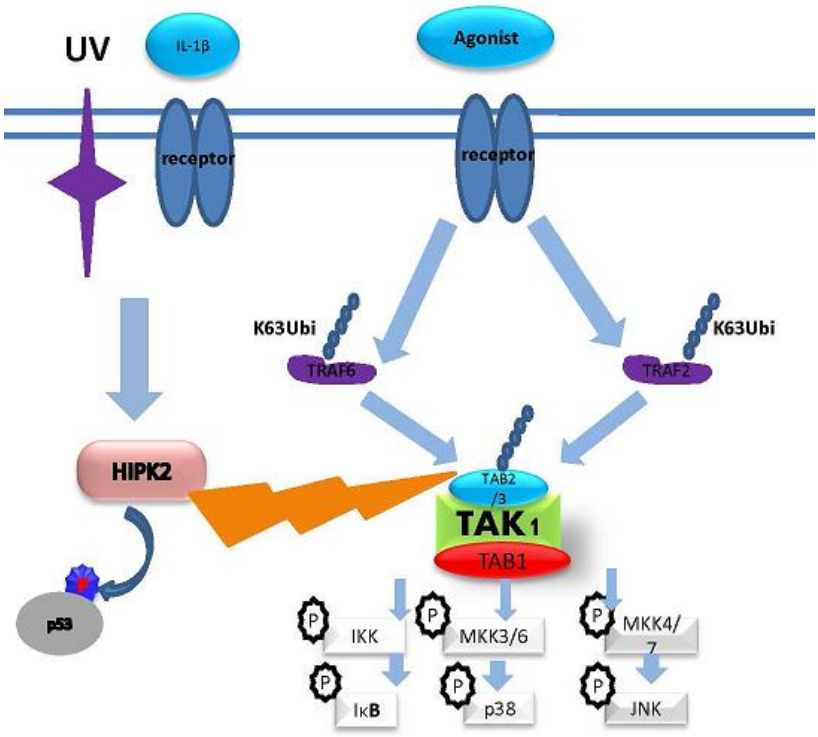

Figure 3.6 HIPK2 negatively regulates TAK1 signalling pathway.

Activation of HIPK2 generally disrupts the polyubiqutin binding of TAK1 complex and suppresses the activation of TAK1 and its downstream signalling.

Chapter VI Perspectives and Discussions

With the improvement of understanding on inflammation and cell growth control, research sheds lights on tumorigenesis resulted from chronic inflammation. Development of tumorigenesis from chronic inflammation contains two key points: the long term activation of inflammation signaling and uncontrolled cell growth. Given the important role of TAK1 and mTOR pathway in these two fields respectively, we studied the regulation of TAK1-mTOR signalin and proposed the connection between TAK1 activation and upregulation of mTOR pathway.

We firstly investigated TAK1 mediated activation of mTOR pathway in this study. We discovered the direct interaction between TSC2 and TAK1, especially the endogenous TSC2 and kinase domain of TAK1, which mediates the phosphorylation of TSC2. Our study on phosphorylation sites of TSC2 mediated by TAK1 identifies Thr1462 and Ser1365 on TSC2 as potential sites. We found that LPS, TNFα and IL-1β stimulations activates mTOR pathway as indicated by phosphorylation of p70S6K1 and 4EBP1, which is associated with TAK1 kinase activity. We then studied effects of mTOR on mRNA synthesis of LPS induced cytokines. We were also concerned with the fact that LPS affected cell growth in a manner dependent on mTOR activity. As conclusion, we reported here that TAK1 interacts with TSC2 and phosphorylates TSC2 to activate mTOR pathway, which subsequently regulates cell growth and cytokines synthesis in response to LPS, TNFα and IL-β.

Compared with other studies on mTOR activation and inflammation signaling pathway, our study provides new insights for the whole picture of mTOR activation on following points:

4.1 Identification of novel phosphorylation sites on TSC2

TSC2 is phosphorylated in response to LPS, TNFα and IL-1β stimulations. Especially upon LPS stimulation, TSC2 shows mobility shift in SDS-PAGE. And TAK1 is proposed as the kinase to mediate TSC2 phosphorylation under these conditions. However, using commercial phospho-antibody against TSC2, only phosphorylation on Thr1462 is confirmed to be responsive to stimulations and sensitive to TAK1 inhibitor and TAK1 deficiency.

We use two methods to search for the phospho-sites on TSC2 mediated by TAK1. Initially, we expressed the kinase and substrate together in mammalian cells, where both kinase and substrate can be fold properly with full function and kinase could also phosphorylate substrate in the proper manner. The second method for phospho-sites search was to express the truncate of TSC2 in *E.coli* while express TAK1 in mammalian cells. The kinase and substrate were reconstituted *in vitro* for kinase assay; this is a more common approach to identify phosphorylation sites [66, 93]. Compared with the mammalian cell system, the disadvantage of expression in *E.coli* system is that proteins may not be present in their physiological form due to improper folding or missing the post translational modifications. For the substrate and kinase over expressed in mammalian cells, because many other kinases are present in the cell, the over expressed TSC2 may upset the balance in the cell and lead to artefacts such as

non-specific phosphorylation in TSC2. Thus, the results from the two different approaches should be considered together and validate each other.

The phosphorylation sites identified from mammalian expression system are all located at the middle region of TSC2, from 960 to 1448. This agrees with the theory that the middle region of TSC2 is responsible for the interaction with other proteins and modified by regulatory proteins to control the GAP activity of TSC2 [152]. A total of seven potential sites were identified in this approach, which supports the huge molecular shift of TSC2 upon LPS stimulation. However, there are no antibodies available that recognize any one of these sites.

Facing the problem to express the full length 200 kDa TSC2 in *E.coli*, we chose to express the TSC2 fragment where the potential phosphorylation sites located. However, whether the fragment of TSC2 could form proper three dimensional structure is not sure. This affects the efficiency of *in vitro* kinase assay and may explain why only one of three truncates is phosphorylated *in vitro*. It is worth noting that the phosphorylation sites confirmed by phosphorylation antibodies were not found in the *in vitro* phosphorylation results.

For site Thr1462, it could be because that GST-TSC-B16 truncate (residues 1397 to 1807) has the same molecular weight as TAK1 does and the expected position for GST-TSC2-B16 on the auto exposure was obscured by strong TAK1 auto-phosphorylation signals. Besides, this truncate could be folded improperly in *E.coli* so that the potential sites are not exposed to the kinase. Thr 1462 is not captured from mammalian expressed TSC2, either. This is probably due to the low sequence coverage of sample.

For site Ser939, the phosphorylation signal is too weak to detect. After long time exposure for *in vitro* kinase assay, the GST-TSC2 truncate (residues 910 to 1112) was visible on the film, as shown in Figure 2.10B. However, after the substrate was processed for digestion and HPLC, the radioactive signal is no longer detectable in the sample any more. We thus lost the opportunity of finding out any potential sites among residue 910 to 1112. This indicates that Ser939 is not a favourite site for TAK1 kinase activity.

TSC2 phosphorylation on Thr1462 and Ser939 may not be mediated by TAK1. We report the activation of Akt/PKB in response to LPS. Thr1462 and S939 are the favourite sites for Akt/PKB kinase activity. Thus, the phosphorylation of Thr1462 and Ser939 could be mediated by LPS induced Akt/PKB activation. This also agrees with the observation that phosphorylation of TSC2 on Ser939 does not change upon LPS, TNFα and IL-1β in TAK1 deficient cells.

To elucidate the phosphorylation of TSC2 mediated by TAK1, the more sensitive as well as effective approaches are required in future. One of the ideas is using peptides. The peptides containing potential phosphorylation sites could be considered as replacement of TSC2 truncates in kinase assay since the peptide is presented with simple three-dimensional structure and potential sites are fully exposed in peptides.

4.2 The Kinases that are involved in LPS, TNFα and IL-1β induced mTOR activation

p70S6K1 is an important kinase in cell signaling cascade. p70S6K1 phosphorylation on Thr267 is due to activation of Akt/PKB while p70S6K1

phosphorylation on Thr389 is due to activation of mTORC1. In this study, we find that LPS stimulation results in the phosphorylation of p70S6K1 on Thr389.

Previously, there is a report that LPS stimulation induces mTOR activation in an IKKβ dependent manner [93]. In this paper, researchers reported that TNFα stimulation leads to IKKβ mediated phosphorylation of TSC1 and relocalization of TSC2, resulting activation of the mTOR pathway. They found that both TNFα and LPS can activate p70S6K1 and IKKβ, and proposed that LPS may activate the mTOR in the same way as TNFα does.

However, there are two points in their report which differ from our study. Firstly, using *in vitro* kinase assay, they reported that Akt/PKB is not activated in response to TNFα. However, using phospho-antibody, we find that Akt/PKB in MCF7 cells is phosphorylated at both Ser308 and Thr473 upon TNFα stimulation, which are abolished by Akt/PKB inhibitor VIII. Secondly, we did not notice the significant phosphorylation status of TSC1 on western blot; instead, we noticed the electrophoretic mobility change of TSC2. It is also important to point out that in our study, the IKKα and IKKβ double knockout MEFs express TSC2 at low levels (the unpublished data), which results in high activation of p70S6K1 even without stimulation. Since the paper has already shown that only IKKβ but not IKKα is involved in p70S6K1 activation, it is critical to clarify whether IKKβ affects mTOR activation by regulating TSC2 in addition to TSC1. Our results in paragraph 3.4.3 suggest that IKKβ regulates activation of mTOR and p70S6K1 without affecting phosphorylation of TSC2.

Recently, another paper reports that LPS stimulation activates mTOR pathway and their study attributes mTOR activation to Cot/Tpl2 kinase (also known as MAPKKK8 that is responsible for the activation of the MKK1-Erk1/2) by regulating phosphorylation of Akt on Ser473 and p70S6K1 on Thr389 in LPS-stimulated macrophages [144]. In this paper, Alemany, S. *et al* proposed that LPS stimulation on macrophage leads to phosphorylation of Cot/Tpl2, which in turn phosphorylates MKK1 and Erk1/2 to activate the PI3K-Akt-p70S6K1 signaling pathway; subsequently, the phosphorylation of JNK and p38α and recovery of IκB are repressed. This paper also reports that Cot/Tpl2 is widely involved in TLR4, TLR3 and TLR2/6 mediated PI3K-Akt-p70S6K1 activation. However, in this paper, no clear evidence is presented about the direct interaction between Cot/Tpl2 and any component of the PI3K-mTOR-p70S6K1 axis. All evidences Alemany, S. *et al* reported are from Cot/Tpl2 knockout macrophage cells and Cot/Tpl2 kinase deficient cells. It is hard to connect Cot/Tpl2, which is at the end of NFκB signaling, with PI3K, which is on the very top of mTOR pathway, given that a number of kinase might be affected in the knockout cells and deficient cells.

And so far, our data firmly suggested that TAK1 is involved in mTOR activation upon LPS, TNFα and IL-1β stimulations.

4.3 mTOR responds differently among various cell lines to different stimulations.

In our study, we tried to compare the effect of LPS, TNFα/IL-1β as well as IGF on activating mTOR pathway. However, it is hard to either compare the intensity of phosphorylation of p70S6K1 to certain stimulation among different cell lines or compare the intensity of phosphorylation signals in the same cell

line by different stimulations. LPS fails to do activate mTOR pathway in MCF7 cells and HEK293 cells. TNFα and IL-1β fail to induce the phosphorylation of p70S6K1 in RAW264.7 cells, either. The failure of mTOR pathway activation on certain cell lines in response to LPS, TNFα or IL-1β may be due to the poor expression of specific receptors and the inefficient transmission of stimulation signals

4.4 The effect of mTORC1 in LPS induced the cytokine synthesis

The detection of LPS by immune cells activates cytokine synthesis. Since the mTOR and TAK1 activity are related, we are keen to reveal the impact of mTOR on cytokine synthesis.

The ultimate reflection of the impact of mTOR on cytokine synthesis should be the measurement of secreted cytokines by ELISA or western blot. However, mTOR controls the phosphorylation of p70S6K1 and 4EBP1 to regulate the assembling of translation machinery. The translation of mRNA during protein synthesis will be disrupted by inhibition of mTOR and this generally affect global protein synthesis, without discrimination on specific cytokines. Therefore, we measured the mRNA synthesis of cytokines when mTOR activity was inhibited by rapamycin.

In 2008, Weichhart, T. *et al* [153] reported that the TSC2-mTOR signalling pathway can regulate the innate inflammatory response. They proposed that TSC2-mTOR pathway would have an anti-inflammatory effect by promoting the anti-inflammatory cytokine (e.g. IL-10) synthesis and inhibiting the pro-inflammatory cytokine (e.g. IL-12) synthesis. The impact of TSC2-mTOR pathway on cytokine synthesis was assessed by measuring the amount of

secreted cytokines in the media, 24 h post treatment with LPS and rapamycin. However, there are a few points in the paper which require attention.

Firstly; IL-12 p40 was measured as the indicator of proinflammatory cytokines. IL-12 is a proinflammatory cytokine for its function to induce differentiation of naïve T cell, production of IFN-γ and TNFα and activate JAK-STAT pathway. However, IL-12 is a heterodimeric cytokine encoded by two separated genes, IL-12A or IL-12p35 and IL-12B or IL-12p40. The homodimer of IL-12 p40 is formed, but it does not function like active heterodimer IL-12. Thus, simple measurement of IL-12p40 may not be a proper assessment of proinflammatory cytokines;

Secondly; in this paper, the cell free media was collected 24 h post stimulation and used for cytokine secretion measured by ELISA. This is the reflection at only one time point.

Compared with this report, the RT-PCR results we provided are more comprehensive and dynamic.

For the pro-inflammatory cytokines, measurement was done on the mRNA level of TNFα, IL-1β and IL-1α, IL-12 p35 and p40, IL-2 as well as CXCL10; and for anti-inflammatory cytokines, it was the IL-10 that was taken for count. The synthesis of mRNA of interferon α, β and γ were also measured. For every cytokine, the mRNA levels at four time points post stimulation were measured. In our study, LPS treatment did induce the mRNA synthesis of IL-1β, IL-1α, IL-2, IL-10, TNFα, interferon β and CXCL10. While LPS treatment had little impact on IL-12A (p35), IL-12B (p40) and interferon α and interferon γ. When rapamycin was added in the stimulation to inhibit the effect of mTOR, most of

cytokines were downregulated at mRNA synthesis level, including IL-1α, IL-2, IL-10, TNFα, interferon β and CXCL10, while the upregulated cytokine was rare, which emphasizes the enhanced IL-1β expression be rapamycin. The mRNA of certain cytokines, like IL-12A (p35) and IL-12B (p40) and interferon α and γ, were not significantly affected by rapamycin treatment in addition to LPS stimulation. This result suggested that mTOR promoted the synthesis of mRNA of both pro-inflammatory and anti-inflammatory. In our study, the only cytokine which was suppressed by activation of mTORC1 is IL-1β, the pro-inflammatory cytokine. Thus, it is improper to simply sort the effect of mTORC1 on cytokines as promoting inflammatory or suppressing inflammatory. Due to the complexity of cytokine functions on different cell lines, the effect of mTOR on cytokine synthesis would rather be considered for its cell type specificity and cytokine preference.

mTOR inhibition suppresses mRNA synthesis of most cytokines; while the most significantly enhanced mRNA synthesis draws our attention: the proinflammatory cytokine IL-1β. In 2008, Wagner, H. [154] reported that mTOR suppresses the production of bioactive IL-1β. Turnquist, H.R. *et al* also reported that mTOR inhibition elicits *de novo* production of IL-1β by phenotypically immature, mouse bone marrow-derived dendritic cells both *in vitro* and *in vivo* [155]. Our study supports these observations and pushes the clue further to the mRNA level. Given enhanced mRNA synthesis of IL-1β and suppressed mRNA synthesis of interferon β upon mTOR inhibition, we suspect that activation of mTOR may attenuate the immune response of RAW264.7 cells upon LPS stimulation.

4.5 mTOR regulates cell growth

Cell growth usually refers to two aspects: increase of cell number and enlargement of cell size. mTOR regulates cell growth, reflected on both cell number and cell size. One of the common methods to measure cell size is FACS. It determines cell size and cell growth stage at the same time. Imaging is also a method to capture the cell growth in number and size. Another reflection of cell growth is the cell viability, especially upon cytotoxic stimulations. If mTOR activity is changed during stimulations or study, the growth of cells could be captured by one of these methods. Previously, for Akt/PKB mediated mTOR activation, Potter, C.J. *et al* [68] measured the size and cell number of Drosophila ommatidia by scanning electron micrographs; while, for IKKβ mediated mTOR activation, Lee, D.F. *et al*[93] measured the tumorigenesis and growth of blood vessels from 4T1 tumours by imaging; for AMPK mediated mTOR regulation, Inoki, K. *et al* [75] measured the size of HEK293 cells at G1 and G2 stage respectively by FACS, they also took pictures for cell viability. All these methods are considered for our study. Given the limitation to access the animal and the uncertainty about phospho-site on TSC2 mediated by TAK1, we did not prepare the animal assay to validate the physiological significance of LPS induced mTOR activation. In our study, we also noticed that LPS stimulation results in the increase of cell size, so does IGF stimulation (unpublished data). We measured cell viability upon different stimulation for a quantitive assessment of mTOR activity.

Cell viability was measured by MTT assay. Firstly, the long term treatment of IGF resulted in increase of cell viability, which is suppressed by rapamycin. This is in agreement with results from Potter, C.J. [68] and others.

Secondly, long term treatment of TNFα led to decrease of cell viability, which is different from what Lee, D.F. found [93]. Cell viability was even lower when they were treated with TNFα and rapamycin together. This seems different with Lee's proposal that TNFα plays a positive role in tumorigenesis, but it agrees with the established theory that prolonged treatment of TNFα leads to apoptosis by activating JNK and certain apoptotic events [156, 157]. Thus, the decreased cell viability upon TNFα treatment should be an integrated reflection of both cell growth regulation and cell death regulation; this also explains why cell viability is even lower when additional rapamycin abolished mTORC1 mediated cell growth.

Thirdly, long term treatment of IL-1β does not affect cell viability, which is a new discovery. Given the activated mTOR pathway upon IL-1β treatment, the unaffected cell viability is probably a balanced effect of activation of both mTOR pathway and cell death signals.

Fourthly, long term of LPS treatment results in decrease of viability of RAW264.7cell. Although LPS treatment activates mTOR signalling in RAW264.7 cell, but the dose dependent decrease of cell viability suggests that effect of LPS treatment is a comprehensive reflection of upregulated cell growth pathway (mTOR pathway) and activated cell death signals (JNK activation and NFκB activation). Cell viability still decreased at a small range when cells were treated with rapamycin in addition to LPS treatment, indicating that LPS treatment also promotes cell death in an mTOR independent manner. Thus, it is imprecise to simply consider cell viability as the reflection of mTOR activation.

Furthermore, when we tried to separate the role of TAK1 in control panel for cell viability, wild type cell line showed decreased viability upon LPS while TAK1 deficient cell line showed increased viability upon LPS. This suggests that activation of TAK1 has more impact on cell death than cell growth and that LPS may activate cell growth by other pathways independent of TAK1. Cell growth in response to LPS is sensitive to rapamycin, as others indicated; while it is worth pointing out that cell growth is more sensitive to rapamycin in TAK1 deficient cells.

4.6 HIPK2 generally regulates activity of TAK1 pathway

In this study, we also characterized regulation of TAK1 signaling pathway mediated by HIPK2. HIPK2 interacts with and stabilized TAB2/TAB3, instead of TAK1. Via interrupting the ubiquitin binding of TAB2/TAB3 in TAK1 complex, HIPK2 suppresses the activity of TAK1 signaling pathway upon UV irradiation, hyperosmotic strees, TNFα and IL-1β stimulations. Moreover, p53 is phosphorylated on Ser46 in response to IL-1β stimulation. Therefore, we proposed that HIPK2 generally suppresses TAK1 mediated signaling pathway by disturbing the polyubiquitin binding with TAB2/TAB3.

Since the proposal of TAK1-HIPK2 interaction, the details of this interaction had never been characterized. The major issue of this study is the existence of this interaction in nature situation. So far, we only observed the interaction between TAB2/TAB3 and HIPK2 in the over-expression study. There is no clue for the existence of physical interaction of endogenous proteins between any components from TAK1 complex and HIPK2. Thus, the uncertainty about this interaction severely questions the physiological significance of follow up study.

The other disadvantage in this study is lacking of effective HIPK2 antibody to recognize endogenous HIPK2, which leaves the doubt on efficiency of HIPK2 knockdown.

Given these missing points, the study on HIPK2 mediated regulation of TAK1 signaling still requires a lot of efforts to emphasize the specificity and efficiency of HIPK2 knockdown as well as the existence of interaction among endogenous proteins. However, thanks to shRNA approach and the significant changes of TAK1 signaling pathway in HIPK2 knockdown cells, the best shRNA for HIPK2 is selected and the role of HIPK2 in regulation of TAK1 signaling is revealed as a negative suppressor. Since the activity of HIPK2 as indicated by phosphorylation of p53 is induced by UV and IL-1β stimulation but not by TNFα or other stimulations, the elevated activation of TAK1 signaling upon UV, TNFα, IL-1β and sorbitol stimulations in HIPK2 knockdown cells suggests that HIPK2 generally suppresses TAK1 signaling. HIPK2 activation promotes apoptosis upon lethal DNA damage while TAK1 signaling mediates cell proper immune responses. The suppression of TAK1 signaling by HIPK2 is a possible mechanism by which the lethal DNA damage signal or other apoptotic signals shut down cell immune response and block the transmission of dangerous signals among cells.

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