TRIM7, a Novel Binding Protein of the mTORC2 Component Sin1

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Contents

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List of Abbreviations

4E-BP1/2	4eIF4E-binding protein1/2
AKT/PKB	Protein kinase B
Ala	Alanine
AMPK	AMP-activated protein kinase
APL	Acute promyelocytic leukemia
APS	Ammonium persulfate
ARF	ADP ribosylation factor-like
ATM	Ataxia-telangiectasia mutated
ATR	Ataxia-telangiectasia and Rad3-related
BR	Bromodomain
BSA	Bovine serum albumin
BSTA	BSD domain-containing signal transducer and Akt interactor
C44A	Cystine to Alanine substitution at amino acid residue 44
CC	Coiled-coiled
cDNA	Complementary DNA
CKO	Conditional knockout
COS Box	C-terminal subgroup one signature
cPKC	Conventional protein kinase C
CQ	Chloroquine
Cys	Cystine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DR	Dietary restriction
DTT	Dithiothreitol
E1	Ubiquitin/SUMO-activating enzyme
E2	Ubiquitin/SUMO-conjugating enzyme
E3	Ubiquitin/SUMO ligase
EEA1	Early endosomal antigen-1
EMT	Epithelial-mesenchymal transition
FBS	Fetal bovine serum
FIL	Filamin-type immunoglobulin
FKBP12	FK506-binding protein 12
FN3	Fibronectin type 3
GAP	GTPase activating protein
GDP/GTP	Guanosine diphosphate/Guanosine triphosphate
GFP	Green fluorescent protein
GNIP	Glycogenin-interacting protein
GST	Glutathione S-transferase
H46A	Histidine to Alanine substitution at amino acid residue 46
HCC	Hepatocellular carcinoma
HDAC	Histone deacetylase

His	Histidine
HM	Hydrophobic motif
HRP	Horseradish peroxidase
Hsp90	Heat shock protein 90
IGF-1	Insulin-like growth factor-1
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRS-1	Insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
K48	Lysine residue 48
K48R	Lysine to Arginine substitution at amino acid residue 48
K63	Lysine residue 63
K7R	All seven Lysine residues substituted with Arginine
KD	Kinase-dead
LB	Liquid broth
LRS	Leucyl-tRNA synthetase
Lys	Lysine
MAPK	Mitogen-activated protein kinase
MATH	Meprin and tumor-necrosis factor receptor-associated factor homology
MBP	Mmaltose binding protein
MEFs	Mouse embryonic fibroblasts
MID	Midline
MK2	MAPK-activated protein kinase 2
MMP9	Matrix metalloprotease 9
MOTC	Microtubule-organizing center
mRNA/rRNA	Messenger ribonucleic acid/Ribosomal ribonucleic acid
mTOR	Mammalian target of rapamycin
mTORC1/2	Mammalian target of rapamycin complex1/2
MTT	Thiazolyl blue tetrazolium bromide
NBs	Nuclear bodies
NDRG1	N-myc downregulated gene 1
NIP7	Nuclear import 7
NP-40	Nondet P-40
PBS	Phosphate-buffered saline
PC-3 cells	Prostate cancer cells null for *PTEN*
PCBP2	Poly(rC) binding protein 2
PCR	Polymerase chain reaction
PD	Pull down
PDK1	Phosphoinositide-dependent kinase 1
PFA	Paraformaldehyde
PH	Pleckstrin homology
PHD	Plant homeodomain
PI3K	Phosphatidylinositol 3-kinase
PIKKs	PI3K-related protein kinases
PIP_2	Phosphatidylinositol-4,5-biphosphate

PIP_3	Phosphoinositol triphosphate
PKC	Protein kinase C
PML	Promyelocytic leukemia
PRAK	p38-regulated/activated kinase
PRAS40	Proline-rich Akt substrate 40 kilo Dalton protein
Protor	Proline-rich 5 observed with Rictor
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative real-time PCR
RARα	PML-retinoic acid receptor-α
RBCC	RING domain, B-box domain, and CC domain motif
REDD1	Regulated in development and DNA damage responses 1
RING	Really interesting gene
RNAi	Ribonucleic acid interference
ROS	Reactive oxygen species
Rpl17	60S ribosomal protein L17
Rps16	40S ribosomal protein S16
S20	Serine residue 20
S292	Serine residue 292
S422	Serine residue 422
S473	Serine residue 473
S6K1/2	p70 S6 kinase 1/2
SAPK	Stress-activated MAP kinases
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SGK1	Serum- and glucocorticoid-induced protein kinase 1
shRNA	Short hairpin RNA
Sin1	Stress-activated protein kinase-interacting protein 1
siRNA	Small interfering RNA
SREBP-1c	Sterol regulatory element-binding protein 1c
STAT3	Signal transducer and activator of transcription 3
SUMO	Small ubiquitin-like modifier
T1135	Threonine residue 1135
T18	Threonine residue 18
T256	Threonine residue 256
T256	Threonine residue 256
T308	Threonine residue 308
T449	Threonine residue 449
T450	Threonine residue 450
TBC1D7	Tre2-Bub2-Cdc16 (TBC) 1 domain family, member 7
TBS	Tris-buffered saline
Tel2	Telomere maintenance 2
TEMED	N,N,N',N'-Tetramethylethylenediamine
TGF-β	Transforming growth factor receptor-β
TGF-βR	Transforming growth factor receptor-β receptor
TIF1	Transcriptional intermediary factor 1
TLB	Tris lysis buffer
TM	Turn motif

TM	Transmembrane
TR	Transferrin receptor
Trc	Triconered
TRIM	Tripartite motif-containing
TRIM7.1ΔR	RING-deleted TRIM7.1
TSC1/2	Tuberous sclerosis complex1/2
Tti1	Tel2-interacting protein 1
UBCs	Ubiquitin-conjugating cores
WCL	Whole cell lysate
WT	Wild-type
YFP	Yellow fluorescent protein

Abstract

TRIM7 is a member of the TRIM (tripartite motif-containing) protein superfamily. This family has been implicated in many disorders such as genetic diseases, neurological diseases, and cancers. Little is known about the function of TRIM7 except that it interacts with glycogenin and may regulate glycogen biosynthesis. Recently, a yeast two-hybrid protein-protein interaction screen revealed the binding of TRIM7 to Sin1, a protein found in a complex with the mammalian target of rapamycin (mTOR) protein kinase. mTOR can form two complexes, mTORC1 and mTORC2, which are important for cell growth, differentiation, and survival. Sin1 is a core component of mTORC2 and is critical for mTORC2 stability and activity. It was confirmed by co-immunoprecipitation that TRIM7 associates with Sin1 and mTOR in cultured mammalian cells. Furthermore, it was demonstrated that TRIM7 is a phosphoprotein, although it was not directly targeted by mTOR *in vitro*. Similar to some other TRIM family proteins, it was demonstrated that TRIM7 has a ubiquitin E3 ligase function allowing it to autoubiquitinate both *in vitro* and in cells. The autoubiquitination of TRIM7 was dependent on its RING domain. Further characterization of TRIM7 indicated that it can both homo-oligomerise as well as hetero-oligomerise with other members of its sub-class of TRIM proteins and that it co-localises with them into discrete cytoplasmic loci. To determine the cellular function of TRIM7, a stable cell line expressing an shRNA directed against TRIM7 was generated. Successful knock down of TRIM7 was achieved and this led to an increase in the protein levels of components of the mTORC2 complex, including Sin1. This coincided with an increase in cell proliferation. In conclusion, this research identifies a novel role for TRIM7 as a ubiquitin ligase involved in regulating cell proliferation and provides a potential link between TRIM7 and the mTOR pathway, a major transducer of proliferative and cell survival signals.

Declaration

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Chapter One

Introduction

1. Introduction

1.1 The Discovery of mTOR

Cancers arise from a combination of gene mutations that impact on the activity of a variety of signalling pathways within the cells. Some pathways are involved in cell growth and proliferation whereas others are involved in other cellular processes such as apoptosis or survival. One of the main pathways exploited by many human cancers is the mammalian target of rapamycin (mTOR) pathway that plays an important role in cell proliferation, differentiation, and survival (Guertin and Sabatini 2005). Early studies in the 1990s on the drug rapamycin using yeast and mammalian models, revealed its 250 kDa binding target to be a serine/threonine protein kinase, that was subsequently named target of rapamycin (or mammalian target of rapamycin in mammals) (Kunz and Hall 1993). Since its discovery, many cellular processes were shown to be regulated by mTOR such as ribosome biogenesis, autophagy, and metabolism (Guertin and Sabatini 2005, Sarbassov et al. 2005a). mTOR is mainly regulated by growth factors and nutrients, and it was originally shown to form a rapamycin-sensitive complex that is now known as mTOR complex 1 (mTORC1) (Hara et al. 2002, Kim et al. 2002). A few years later it was revealed that another complex is formed which is less sensitive to rapamycin, the mTOR complex 2 (mTORC2) (Jacinto et al. 2004, Loewith et al. 2002).

1.2 Regulation of mTORC1 Signalling

1.2.1 The mTORC1 pathway

The two mTOR complexes share some common components, but they also consist of proteins specific to each complex and the mode of activation for each complex is slightly different. mTORC1 is composed of Raptor, mLST8, and proline-rich Akt substrate 40 kDa (PRAS40) (Hara et al. 2002) that is activated by responding to changes in energy levels, amino acids, growth factors, and oxygen levels (Figure 1.1). Less is known about mTORC2, but studies have shown that it

is activated by growth factors (Ma and Blenis 2009). Both the telomere maintenance 2 (Tel2) and Tel2-interacting protein 1 (Tti1) have been recently shown to be important novel components of mTORC1 by interacting with Raptor (Kaizuka et al. 2010). Tel2 was first characterized in budding yeast (Kota and Runge 1999) and has been shown to regulate the stability of PI3K-related protein kinases (PIKKs) including mTOR, ATM/ATR (ataxia-telangiectasia mutated/ataxia-telangiectasia and Rad3-related), and DNA-PKcs (DNA-dependent protein kinase catalytic subunit) (Lovejoy and Cortez 2009).

One of the main upstream regulators of mTOR signalling is the phosphatidylinositol 3 kinase (PI3K) (Manning and Cantley 2007). PI3K is a lipid kinase that targets the 3'-hydroxyl ring of the phosphoinositides for phosphorylation. PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP_2) converting it to phosphatidylinositol-3,4,5-triphosphate (PIP_3) creating a membrane docking site for pleckstrin homology (PH) domain-containing proteins including PDK1 and AKT. PI3K activity is countered by PTEN (phosphatase and tensin homolog) a lipid phosphatase that dephosphorylates PIP_3 to PIP_2 (Cantley and Neel 1999). Akt, also known as protein kinase B (PKB), is a serine/threonine kinase downstream of PI3K (Burgering and Coffer 1995). It is part of the AGC kinase family which also includes p70 S6-Kinase (S6K), serum- and glucocorticoid-induced protein kinase (SGK), and protein kinase C (PKC) (Peterson and Schreiber 1999). Akt has a role in cell metabolism, survival, growth, and proliferation which allows cancer cells to promote angiogenesis and metastases only under conditions when it is constitutively active (Manning and Cantley 2007). In mammals, Akt has three main isoforms encoded by separate genes; *Akt1*, *Akt2*, and *Akt3*. Akt1 and Akt2 are ubiquitous, while Akt3 is mainly found in the brain and testes (Walker et al. 1998). Upon PI3K activation, Akt is recruited to the plasma membrane by PIP_3 and binds to it via its PH domain. The binding to PIP_3 is the rate-limiting step in Akt activation (Yang et al. 2002). In order for Akt to be fully active, it requires to be phosphorylated both at its threonine (T308) and serine (S473) residues. T308 is localised in the activation loop (A-Loop) of the catalytic domain whereas S473 is in the hydrophobic motif

(HM) towards the carboxy-terminus. Phosphoinositide-dependent kinase 1 (PDK1) phosphorylates the activation loop for Akt and it was recently found that the HM kinase is mTORC2 which will be discussed in Section 1.3.1 (Sarbassov and Sabatini 2005).

Akt is negatively regulated by PTEN (Sulis and Parsons 2003) where *PTEN* mutations and deletion have been demonstrated in many human cancers leading to PI3K hyperactivity promoting carcinogenesis (Manning and Cantley 2007). Similarly, Wang and colleagues have shown that *PTEN* deletion in mice leads to Akt hyper-phosphorylation in the prostate epithelium, and eventually, prostate cancer (Wang et al. 2003). It has been reported that Akt activates mTORC1 by phosphorylating tuberous sclerosis complex 2 (TSC2) and liberating its inhibitory effect on Rheb (Hahn-Windgassen et al. 2005). TSC1-TSC2 forms a heterodimer that prevents continuous activation of mTORC1, a feature in many types of cancers. The discovery of the association between TSC1-TSC2 with mTORC1 was the first to link mTORC1 to cancer (Crino et al. 2006). TSC2 is a GTPase-activating protein (GAP) that prevents mTORC1 activation by inhibiting Rheb (converts it from its GTP-bound to GDP-bound form) (Figure 1.1). Studies on mice have shown that deleting either *Tsc1* or *Tsc2* genes leads to continuous activation of mTORC1 (Bhaskar and Hay 2007). A recent study has demonstrated a novel third component of the TSC1/TSC2 complex, Tre2-Bub2-Cdc16 (TBC) 1 domain family, member 7 (TBC1D7). TBC1D7 is required to maintain the TSC1-TSC2 complex and promote its GAP activity (Dibble et al. 2012). However, Haar et al. have shown that Akt can activate mTORC1 by mechanisms independent of TSC1-TSC2. One example is by phosphorylating PRAS40 liberating its inhibitory binding effect on Raptor and allowing enhanced growth factor-induced mTORC1 activation (Vander Haar et al. 2007).

Signals can be transmitted to mTORC1 in at least three ways; the Rag GTPase proteins that monitor the levels of amino acids, the Rheb GTPase which transmits signals emitted by growth factors and changes in energy levels, and finally the signals that directly activate mTORC1 by interacting with its

components (Figure 1.1) (Guertin and Sabatini 2007). mTORC1 is mainly diffused throughout the cytoplasm (Sancak et al. 2008), but in the presence of amino acids, it is recruited to the cell's endomembrane compartments (i.e. endoplasmic reticulum and Golgi apparatus) where Rag interacts with Raptor. This re-localisation of mTORC1 is not sufficient for its full activation as binding to Rheb is also required (Long et al. 2005). This activation through Rheb occurs independently from the regulation of Rheb activity by the Akt-TSC axis (explained above), but the exact mechanism is not fully understood.

There are four members of mammalian Rag proteins; A, B, C, and D which form a heterodimer complex (Schürmann et al. 1995). RagA/B is the GTP-charged complex whereas RagC/D is the GDP-bound complex (Nakashima et al. 1999). It has been shown that GTP-bound RagB interacts with the mTORC1 component Raptor upon increased amino acid availability and the complex then translocates to endomembrane compartments (Sancak et al. 2008). A complex known as Ragulator further interacts with the Rag proteins and recruits them to the lysosome triggering mTORC1 activation. It is not clear yet exactly how mTORC1 activation is mediated by this process (Sancak et al. 2010). Recently, it has been demonstrated that leucyl-tRNA synthetase (LRS) acts as a sensor of the cellular leucine concentration and when it is bound to leucine it binds to Rag and acts as a GAP leading to activation of mTORC1 signalling (Han et al. 2012). A member of a different family of GTPases, the Rho GTPases, has also been found to regulate mTORC1 activity. Rac GTPase directly interacts with Rheb and Raptor independently of GTP indicating that there is collaboration between GTPases in the regulation of mTORC1 (Saci et al. 2011). Once mTORC1 is active, it phosphorylates its downstream target molecules. The two best characterized targets of mTORC1 are the S6K (S6K1 and S6K2) and 4E (eIF4E)-binding proteins 1 and 2 (4E-BP1 and 4E-BP2). Their phosphorylation regulates translation initiation, protein synthesis, and leads to increased cell mass (Figure 1.1) (Ma and Blenis 2009).

The role of mTORC1 in protein synthesis has been shown to be crucial during muscle remodeling (Quy et al. 2013). It has been implicated that autophagy-related genes are activated during muscle denervation (Mammucari et al. 2007, Zhao et al. 2007). However, a recent study has shown the involvement of mTORC1 in suppressing autophagy during muscle denervation. Moreover, mTORC1 was required for muscle remodeling following denervation by promoting protein synthesis and ribosome biogenesis (Quy et al. 2013). Other downstream targets of mTORC1 include the hypoxia-inducible factor (HIF) that has been shown to play a role in angiogenesis by forming a HIF1α-HIF1β dimer (Hickey and Simon 2006, Land and Tee 2007). A study using zebrafish exhibiting mutated *tsc2* and *p53* has shown increased levels of HIF1α that promoted tumor growth and angiogenesis. These affects were reduced upon rapamycin treatment suggesting a role for mTORC1 (Kim et al. 2013) as it has been previously shown that HIF1α is rapamycin-sensitive (Land and Tee 2007). Studies on *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$ cells have shown a protective mechanism exerted by mTORC1 preventing its hyper-activity by phosphorylating the transcription factor STAT3 (signal transducer and activator of transcription 3). STAT3 induces PTEN expression, and thereby, prevents Akt activation inhibiting hyper-activity mTORC1-mediated tumorigenesis (Zha et al. 2011). The p38 mitogen-activated protein kinase (MAPK) is another upstream regulator of mTORC1 signalling under stress conditions. There are four p38 isoforms: α, β, γ, δ, each with specific functions (Jiang et al. 1997, Zarubin and Han 2005). For instance, p38α stimulates mTORC1 activation via its downstream target MK2 (MAPK-activated protein kinase 2) that phosphorylates TSC2 preventing its inhibitory effect on mTORC1 signalling (Li et al. 2003).

1.2.2 Negative regulators of mTORC1

While p38α may support mTORC1 activity, a second p38 isoform, p38β, has been reported to exert an opposite effect by inactivating mTORC1. This modulation is induced by the p38-regulated/activated kinase (PRAK), in energy-starved conditions. PRAK binds directly to Rheb and phosphorylates it causing

the release of the GTP bound to Rheb and inhibiting its nucleotide-binding capabilities (Zheng et al. 2011). The role of p38β in regulating mTORC1 may be dependent upon the stimulus as a separate study has shown that arsenite enhances mTORC1 activity via p38β phosphorylation of Raptor (X. N. Wu et al. 2011).

The AMP-activated protein kinase (AMPK) is another kinase that negatively regulates mTORC1 activity under low energy levels (i.e. low ATP levels) by phosphorylating and activating TSC2 (Inoki et al. 2003). It was also demonstrated that GSK3 increases the activity of TSC2 preventing mTORC1 activation. It was concluded that in order for TSC2 to achieve full activation, phosphorylation by both AMPK and GSK3 are required. In addition, AMPK has also been shown to directly phosphorylate Raptor suppressing mTORC1 activity (Gwinn et al. 2008). In order to prevent continuous activation of mTORC1, S6K1 triggers a series of negative feedback loops and the insulin receptor substrate 1 (IRS-1) is one of its targets (Figure 1.1) (Harrington et al. 2004). The inhibitory effect of S6K1 on IRS-1 controls the duration of activation of the PI3K thus dampening the cell proliferation signal (Bhaskar and Hay 2007). Other negative regulators of mTORC1 include REDD1 (regulated in development and DNA damage responses 1) (Inoki et al. 2006) and Promyelocytic leukemia (PML) tumor suppressor (Figure 1.1) (Bernardi et al. 2006). DEPTOR is another regulator of mTORC1. DEPTOR is an mTOR-binding protein that inhibits both mTORC1 and mTORC2 suppressing their kinase activity (Figures 1.1 and 1.2). DEPTOR reduction in cells had an effect on increasing cell size and protecting them from apoptosis (Peterson et al. 2009).

One of the most widely used negative regulators of mTORC1 signalling is rapamycin. Rapamycin is a Food and Drug Administration approved drug which is widely used to prevent organ transplant rejection (Chueh and Kahan 2005). Rapamycin has a role in reducing the growth rate of cancer cells, promoting apoptosis, and has anitangiogenic features making it a good antitumor agent (Guertin and Sabatini 2005). It initially binds to an intracellular protein, FKBP12

(FK506-binding protein 12), forming a complex which then binds to the FRB domain upstream the mTOR kinase domain (Bhaskar and Hay 2007). Only mTORC1 has a freely exposed FRB docking site for the FKBP12-rapamycin complex, whereas the FRB domain on mTORC2 might be masked by the other components of the complex or by post-translational modifications making it rapamycin-insensitive (Jacinto et al. 2004, Sarbassov et al. 2004). The inhibitory effects of rapamycin on mTORC1 causes a weakening and dissociation of the complex preventing its activity (Kim et al. 2002).

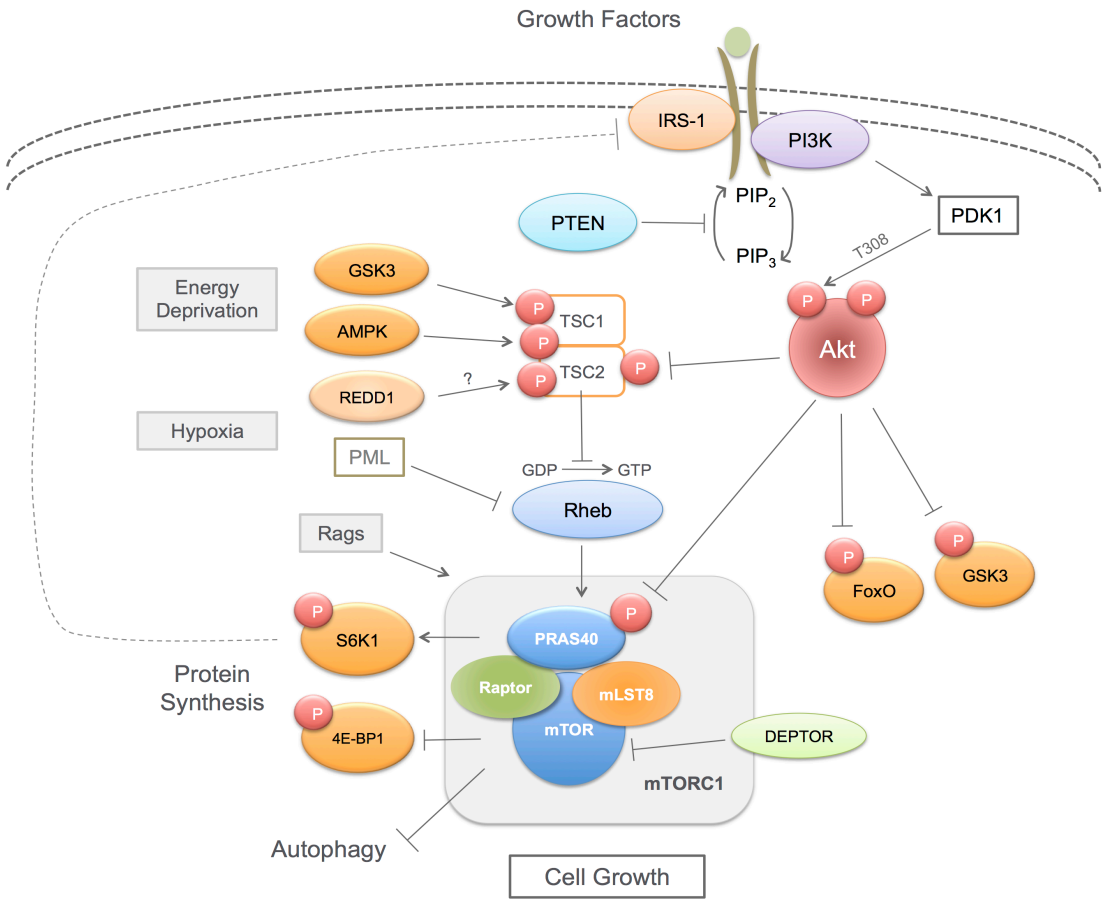


Figure 1.1 **Positive and negative regulators of mTORC1 and their role in cell growth.** Akt positively regulates mTORC1 by phosphorylating TSC2 and PRAS40, whereas mTORC1 negative regulators include GSK3, AMPK, REDD1, and PML which activate TSC2 under low energy and oxygen levels. Rags and DEPTOR are further regulators of mTORC1 signalling. Other targets of Akt include FoxO and GSK3.

1.3 Regulation of mTORC2 Signalling

1.3.1 The mTORC2 pathway

mTORC2 consists of mLST8 plus Rictor, mammalian stress-activated protein kinase-interacting protein 1 (Sin1), and proline-rich 5 observed with Rictor (Protor) (Frias et al. 2006). Like mTORC1, Tel2 and Tti1 have also been shown to be important components of mTORC2 by interacting with Rictor (Kaizuka et al. 2010). Knocking down either of Tel2 or Tti1 blocked the assembly of both mTORCs preventing them from phosphorylating their downstream targets. How Tel2/Tti1 regulate mTORC1/2 association is still not clear but provides new evidence of novel components for the two mTOR complexes (Kaizuka et al. 2010).

There is strong evidence that mTORC2 is the major kinase responsible for Akt phosphorylation within its HM domain at S473 and its turn motif (TM) domain at T450 (explained below) (Hresko and Mueckler 2005). mTORC2 activation allows Akt to phosphorylate its target substrates such as FoxO, GSK3, and BAD (Figure 1.2), and it has been shown that it plays a role in cell survival (Dada et al. 2008), metabolism (Sarbassov et al. 2005b), and the organization of the actin cytoskeleton (Wullschleger et al. 2005). Knockout experiments on mTORC2 components in mice have shown their effect on Akt S473 phosphorylation. For example, deleting any of *Rictor*, *mLST8*, or *SIN1* abolishes Akt's S473 phosphorylation preventing it from phosphorylating its downstream target FoxO3 at T32 (DA Guertin et al. 2006, Jacinto et al. 2006). Deleting the *mTOR* gene itself in mice is embryonic lethal during implantation (Gangloff et al. 2004, Murakami et al. 2004). In contrast, mTORC2-deficient mice survive for a longer period up to mid-gestation (DA Guertin et al. 2006, Jacinto et al. 2006). In addition to Rictor's role in Akt S473 phosphorylation, it also plays a role in the organization of the actin cytoskeleton. Knocking down Rictor increases the spreading of the actin fibers within the cell's cytoplasm rather than being restricted to the cell's cortex (Yang et al. 2006). Conventional *Rictor* knockout mice die around embryonic day 11 (D. A. Guertin et al. 2006). However, Carson

and colleagues generated conditional *Rictor* knockout (CKO) in dorsal neural progenitor cells that remained viable, fertile, but smaller in size with reduced mTORC2 activity (Carson et al. 2013). Similar to mTORC1, Rac1 has also been shown to interact with Rictor and Sin1, and co-localise to the plasma membrane indicating its potential role in regulating mTORC2. In addition, abolishing Rac1 decreases Akt's S473 phosphorylation. The co-localisation with mTORC2 to the plasma membrane could be an explanation for the Rac1-induced S473 phosphorylation of Akt (Saci et al. 2011).

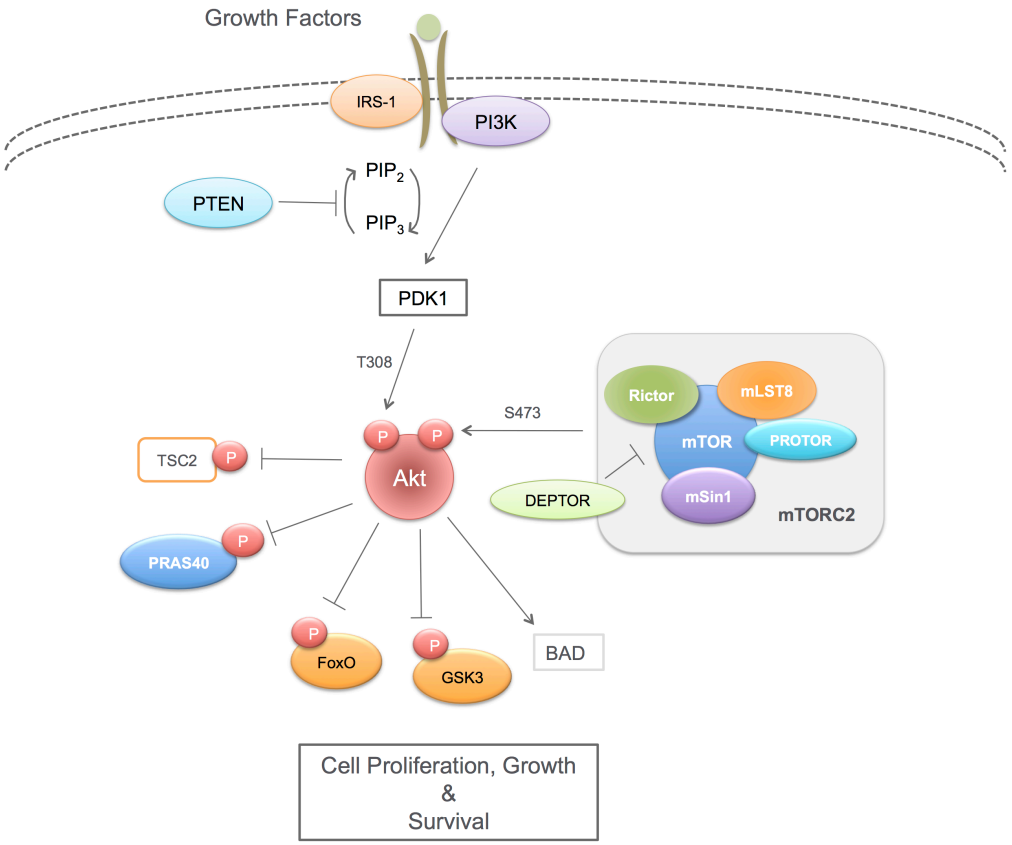


***Figure 1.2* mTORC2 activation and its role in Akt S473 phosphorylation.** Full activation of Akt allows it to phosphorylate its targets such as TSC2, PRAS40, FoxO, GSK3, and the apoptotic protein BAD leading to cell proliferation, growth, and survival. DEPTOR negatively regulates both mTORC2.

It has been implicated that TM phosphorylation at T450 on Akt plays an important role in stabilizing the kinase (Bornancin and Parker 1996, Yonemoto et al. 1997). In the absence of TM phosphorylation, the kinase depends on Hsp90 (heat shock protein 90) for stability. Therefore, mTORC2 regulates Akt phosphorylation both dependent (HM S473) and independent (TM T450) of growth factors (Figure 1.3). Moreover, studies where the HM and TM sites of Akt were mutated in both stimulated and unstimulated cells revealed that the two phosphorylation events occur independently of each other. This evidence provides a novel role for mTORC2 in protein stability (Facchinetti et al. 2008).

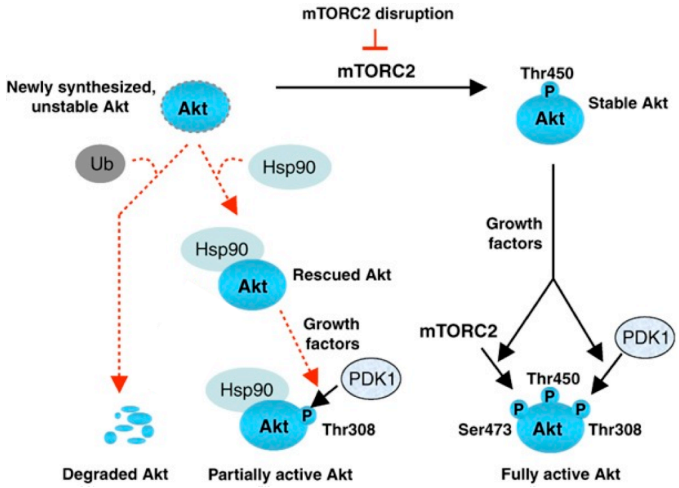


Figure 1.3 **mTORC2 phosphorylates Akt at both HM and TM sites.** Akt's TM is phosphorylated by mTORC2 at T450 independent of growth factor stimulation, whereas in the absence of mTORC2, Akt depends on Hsp90 for stabilization and remains partly activate by PDK1. Upon induction by growth factors, Akt becomes fully active (Facchinetti et al. 2008).

Surprisingly, it has been recently implicated that the phosphorylation of the HM site on Akt by mTORC2 destabilizes the protein. The destabilizing effect of Akt's HM phosphorylation was independent of the TM phosphorylation. This suggests a new model where Akt exerts a negative feedback once fully activated by PDK1 and mTORC2 in order to maintain a physiological balance within the cell (Figure 1.4) (Y. T. Wu et al. 2011).

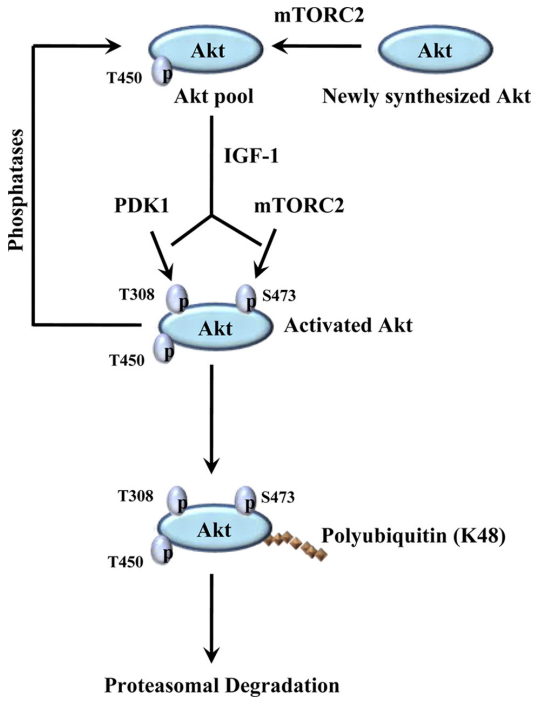


***Figure 1.4* A model of mTORC2-induced Akt degradation.** mTORC2 phosphorylates newly synthesized Akt at its TM site which is then followed by both A-loop and HM site phosphorylation by PDK1 and mTORC2, respectively, upon IGF-1 induction. Akt is then inactivated either by phosphatases or targeted by Lysine 48-linked polyubiquitin chains for proteasomal degradation (Wu et al. 2011).

A recent study has demonstrated a role for FoxO transcription factors in regulating mTORC2 activity. An increase in Rictor mRNA and protein levels were observed upon FoxO activation that consequently led to an increase in mTORC2 activity. However, this elevation in Rictor levels was independent of FoxO binding to DNA indicating that FoxO might associate with other transcription factors that can promote Rictor levels. Moreover, FoxO activation had a negative effect on mTORC1 activity. It was suggested that this might have been as a consequence of the increased Rictor levels that compete with Raptor for mTOR binding affecting the assembly and overall levels of mTORC1. This also suggests that FoxO itself can promote Akt activation by either affecting mTORC1 activity, and therefore, inhibiting its negative feedback loop on Akt, or by increasing Rictor levels promoting mTORC2 activity (Chen et al. 2010).

1.3.2 mTORC2 regulates other members of the AGC kinase family

In addition to Akt, mTORC2 regulates other members of the AGC kinase family including SGK1 and PKC (García-Martínez and Alessi 2008, Kamada et al. 2005). Similar to the other members of the AGC kinase family, SGK1 is mainly activated by insulin and growth factors (Kobayashi and Cohen 1999, Park et al. 1999). Upon its activation, it phosphorylates one of its main target substrates N-myc downregulated gene 1 (NDRG1) at three threonine sites; 346, 356, and 366 (Murray et al. 2004). PDK1 phosphorylates the activation loop of SGK1 at T256 but, unlike Akt, SGK1 does not bind to PIP_3 (Bayascas et al. 2008). The first indication that mTORC2 regulated SGK1 was demonstrated in mouse embryonic fibroblasts (MEFs) deficient in *Rictor*, *mLst8*, or *Sin1* which also displayed a reduction in the phosphorylation of NDRG1. Moreover, experiments revealed that mTORC2 phosphorylates the HM domain of SGK1 at S422 (García-Martínez and Alessi 2008). Like Akt, previous studies have indicated the ability of SGK isoforms to phosphorylate FoxO transcription factors upon their activation (Brunet et al. 2001, Tullet et al. 2008). Recently, it has been implicated that the mTORC2 component Protor plays an important role in the activation of SGK1. In the absence of Protor, both a reduction in the phosphorylation of SGK1 HM site and the phosphorylation of SGK1's substrate NDRG1 was observed. These findings indicate Protor's specific role in SGK1 activation and not other members of the AGC kinase family downstream mTORC2 signalling such as Akt and PKC. However, Protor is not important for mTORC2 assembly nor its kinase activity (Pearce et al. 2011).

Another member of the AGC kinase family that is regulated by mTORC2 is PKC. It has been implicated that mTORC2 phosphorylates the HM of conventional (c)PKCs independent of growth factors, unlike the case with Akt (Kamada et al. 2005, D. A. Guertin et al. 2006). In addition, the TM site which is a conserved domain in the AGC kinase family of both Akt and cPKC is also phosphorylated by mTORC2 (Hauge et al. 2007). Studies on MEFs null for either *Sin1* or *Rictor* have shown reduced phosphorylation of the TM in cPKC indicating the

importance of mTORC2. Degradation of PKC via the ubiquitin-proteasome pathway was observed in *Sin1*$^{-/-}$ MEFs due to the lack of TM phosphorylation (Facchinetti et al. 2008).

1.3.3 Regulators of mTORC2

A number of mechanisms have been proposed for regulating mTORC2 activity. A study by Glidden and colleagues demonstrated a link between acetylation and mTORC2. They have shown that Rictor can be acetylated by p300 which promoted mTORC2 activity. Moreover, overexpressing Sin1 further induced Rictor acetylation. Residues 975-1039 have been shown to be important for Rictor interaction with Sin1 and mLST8 which is required for mTORC2 stability, whereas residues within the region 1041-1137 are required for its acetylation (Figure 1.5). The acetylation domain is also required for the kinase activity of mTORC2 but the mechanism by which mTORC2 is stimulated by acetylation is not fully understood. However, the assembly of mTORC2 is thought to be crucial for Rictor acetylation (Glidden et al. 2012).

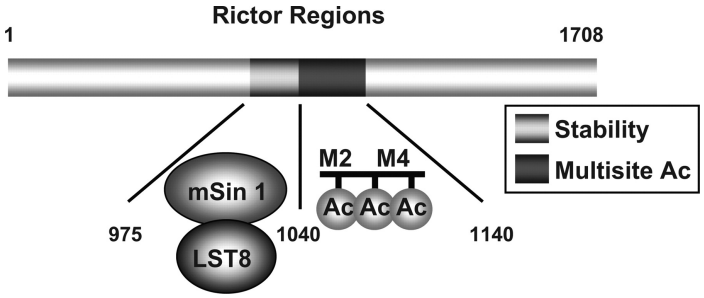


***Figure 1.5* The two functional domains of Rictor.** Residues 975-1039 are important for Sin1 and mLST8 binding to form mTORC2, whereas residues 1041-1140 are essential for Rictor acetylation by p300 inducing mTORC2 activity (Glidden et al. 2011).

New evidence shows that TSC1/2 may positively regulate mTORC2 signalling independent of its GAP activity, but the mechanism of how it does so is not fully known. This suggests that TSC1/2 can be placed both upstream and downstream of Akt (Huang et al. 2008). In addition, S6K1 has also been shown to directly phosphorylate Rictor at T1135 thereby regulating mTORC2

phosphorylation of Akt. Rictor's T1135 phosphorylation was further promoted in *Tsc2*-null MEFs in a mTORC1-dependent manner (Dibble et al. 2009). These findings propose a new mechanism where mTORC1 directly regulates mTORC2 affecting Akt's S473 phosphorylation and preventing the activation of both mTORCs simultaneously.

The BSD domain-containing signal transducer and Akt interactor (BSTA) is one of the most recently identified regulators of mTORC2 signalling that has been shown to be specifically important for Akt1 S473 phosphorylation. Upon growth factor induction, mTORC2 directly phosphorylates BSTA which facilitates the interaction between BSTA and Akt1 via its BSD domain thus promoting S473 phosphorylation. BSTA was shown to be a key player in Akt1-induced adipocyte differentiation as in its absence, cells do not differentiate and less Akt1 S473 phosphorylation was observed. The forkhead transcription factor FoxC2, which induces brown fat adipogenesis was shown to be a key regulatory target of the BSTA-Akt1 complex (Yao et al. 2013).

Although mTORC2 is rapamycin-insensitive, prolonged rapamycin treatment has been shown to affect the assembly of mTORC2 in certain cell types. The mechanism on how rapamycin acts on mTORC2 is different from mTORC1. Rather than directly binding to the complex, it binds to the free mTOR molecules preventing it from complexing with Rictor, and eventually, form forming mTORC2. However, mTORC2 can be formed despite rapamycin treatment in some cell types (Sarbassov et al. 2006). It is not clear why rapamycin inhibits mTORC2 in certain cell types, nevertheless, this provides an insight of how it acts differently in regulating both mTORCs.

1.3.4 mTORC2 is activated by ribosomes

A recent study has shown that mTORC2 associates with ribosomes and that this is required for mTORC2 activity. Specifically, mTORC2 was associated with NIP7 (nuclear import 7), a protein required for the maturation of the ribosomal RNA (rRNA) of the 60S subunit, as well as the ribosomal proteins 60S ribosomal

protein L17 (Rpl17) and 40S ribosomal protein S16 (Rps16). Drugs that prevent protein synthesis demonstrated that mTORC2 activity mediated by ribosomes occur independently of protein synthesis. In addition to the three ribosomal proteins mentioned above, mTORC2 was found to interact with Rpl26. This interaction is regulated in an insulin and PI3K-dependent manner. The elevated association between mTORC2 and ribosomal proteins has been shown in both melanoma and colon cancer cells that display increased mTORC2 activity which is important for them to survive. These findings have led to a new model being proposed where ribosomes regulate mTORC2 dependent upon the ribosome content in order to determine the growth capacity of cells. This may also clarify the fact that prolonged rapamycin treatment inhibits mTORC2 by affecting the levels of ribosome synthesis via the inactivation of mTORC1 signalling (Zinzalla et al. 2011).

1.3.5 Functional roles of mTORC2 signalling

Many functions for mTORC2 are emerging. It has been recently demonstrated that mTORC2 signalling is required for insulin-induced lipogenesis in the liver (Hagiwara et al. 2012). Downstream of mTORC2, Akt regulates gluconeogenesis by phosphorylating FoxO transcription factors (Puigserver et al. 2003) allowing the 14-3-3 chaperone protein to bind FoxO promoting its nuclear export and suppressing its DNA binding activity (Brunet et al. 1999). It has been shown that reducing mTORC2 activity in the liver impairs satiety sensing, promotes hyperglycemia, hyperinsulinemia, and hypolipidemia (Figure 1.6). Moreover, a reduction in the size of the liver was observed in addition to glucose intolerance indicating the importance of mTORC2 signalling in glucose homeostasis (Hagiwara et al. 2012). Another key player downstream mTORC2 signalling is the sterol regulatory element-binding protein 1c (SREBP-1c). SREBP-1c, along with glucokinase, is activated by Akt upon insulin induction thereby regulating metabolic processes such as glycolysis and lipogenesis (Foufelle and Ferré 2002, Horton et al. 2002). Supporting these findings, conditional *Rictor* knockout mice were generated that demonstrated the requirement of mTORC2 activity for

insulin-induced Akt activation in the liver. However, constitutively active Akt2 rescued the metabolic imbalance in the *Rictor* knockout mice indicating the importance of mTORC2-mediated phosphorylation of Akt to achieve metabolic homeostasis (Figure 1.6) (Hagiwara et al. 2012).

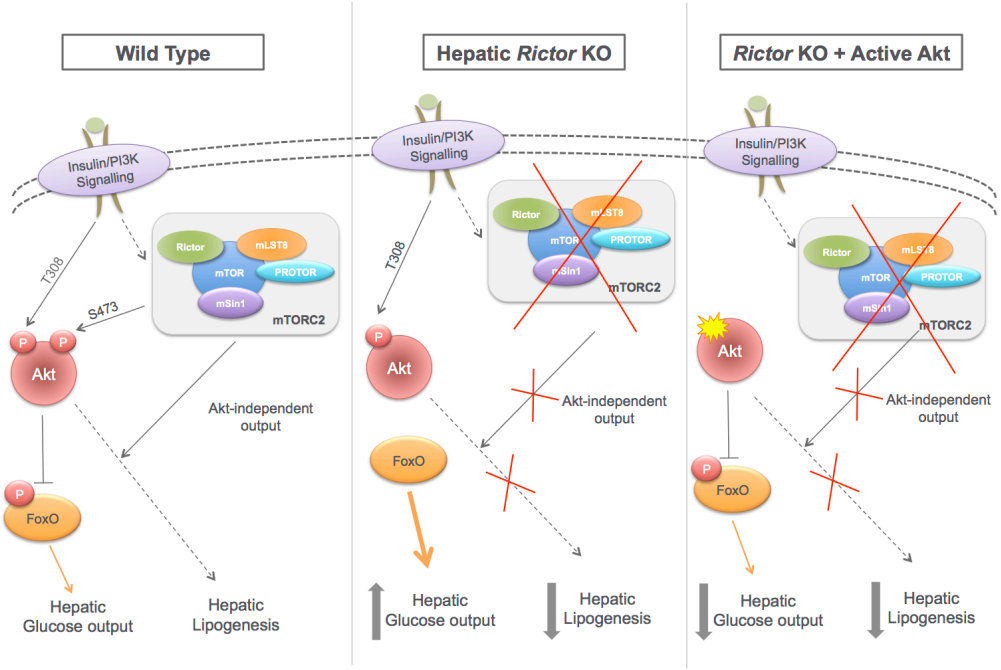


***Figure 1.6* The role of mTORC2 signalling in metabolism.** Under normal conditions, mTORC2 regulates hepatic glucose levels and lipogenesis via Akt where glucose homeostasis is achieved *(left).* Upon mTORC2 impairment, Akt partially loses its activity promoting glucose levels and reducing lipogenesis *(middle)*, whereas glucose levels are rescued upon constitutive active Akt in the absence of mTORC2 activity indicating the importance of the mTORC2-Akt axis in regulating glucose levels in the liver *(right)* (Adapted from Hagiwara et al. 2012).

Further functional roles of mTORC2 signalling have been recently reported where it has been shown to be involved in epithelial-mesenchymal transition (EMT), a main hallmark of cancer progression and metastasis. EMT is a reversible process where cells undergo changes in morphology and become more invasive (Thiery and Sleeman 2006). Transforming growth factor-β (TGF-β) is a key player in promoting EMT (Moustakas and Heldin 2007, Xu et al. 2009). It has been implicated to promote mTORC2-induced phosphorylation of Akt S473 via PI3K signalling. This activation is dependent on the TGF-β receptor (TGF-βR). Upon TGF-β activation, Smad controls the expression of the transcription factor Snail that is involved in EMT and represses the cell wall junction marker E-

cadherin. In order for EMT to complete, mTORC2 activity is required. Knocking down Rictor caused cells to arrest between epithelia and mesenchymal states. Moreover, cells appeared less motile indicated by the reduction of paxillin dynamics, and less invasive demonstrated by a reduction in matrix metalloprotease 9 (MMP9) levels that is involved in degrading surrounding matrix. TGF-β-induced S473 phosphorylation of Akt was reduced in the absence of Rictor affecting downstream targets of Akt such as GSK-3β (Lamouille et al. 2012). Rho GTPases play an important role during EMT as they reorganize actin into stress fibers that are important for cell migration (Yilmaz and Christofori 2009). RhoA activation by TGF-β is inhibited in the absence of mTORC2. These findings indicated the importance of mTORC2 activity for TGF-β signalling in order for cancer cells to progress, invade, and metastasize (Lamouille et al. 2012).

The mTOR pathway has been reported to be involved in nerve system processes such as dendrite protein synthesis (Raab-Graham et al. 2006), synaptic plasticity (Tang et al. 2002), and dendrite arborization (Jaworski et al. 2005). Recently, it has been implicated that there might be a cross-talk between both the Triconered (Trc) and the TOR pathway in *Drosophila*'s dendrite development via mTORC2 (Koike-Kumagai et al. 2009). Trc is a member of the NDR kinase family that is important for signalling pathways regulating dendrite arborization (Emoto et al. 2004, Gao 2007). Both *Sin1* and *Rictor* mutants demonstrated defects in dendrite tilling indicating that TORC2 signalling may be involved. Knocking down Trc itself demonstrated defects in the cytoskeleton organization similar to Sin1/Rictor knockdown indicating that Trc and TORC2 might both have a role in actin cytoskeleton organization (Koike-Kumagai et al. 2009). Trc is phosphorylated at two conserved sites, S292 and T449 (Mah et al. 2001, Tamaskovic et al. 2003). Upon TORC2 depletion, Trc T449 phosphorylation was abolished (Koike-Kumagai et al. 2009). While there are extensive studies on the role of mTORC1 in the nervous system (Makino et al. 2006), the evidence above indicates the importance of both TORCs in regulating nerve system processes. This was

further supported in mammalian systems where the *Rictor* CKO mice (explained earlier in Section 1.3.1) had decreased brain weight and reduced neuron myelination in the cerebral cortex (Carson et al. 2013).

1.4 mTORC1 and mTORC2 Orthologues in Yeast and Worms

Unlike mammals, yeasts have two *TOR* genes, *TOR1* and *TOR2* encoding two distinct serine/threonine kinases TOR1 and TOR2, respectively (Helliwell et al. 1994). Like mammalian cells, two TOR complexes are formed, TORC1 and TORC2, where either TOR1 or TOR2 can form TORC1 whereas TORC2 is only formed by TOR2. Both complexes were first discovered in the budding yeast *Saccharomyces cerevisiae* where TORC1 consists of KOG1/Raptor, LST8 and TCO89 and is rapamycin-sensitive, while TORC2 consists of AVO1/Sin1, AVO2, AVO3/Rictor, LST8, and BIT61 and is rapamycin-insensitive (Loewith et al. 2002). Both TORCs are involved in cell-cycle regulation but have distinct functions, many of which are conserved from yeasts to mammals. For example, both mTORC1 and yeast TORC1 function in regulating autophagy whereas mTORC2 and yeast TORC2 are involved the actin cytoskeleton organization (Bhaskar and Hay 2007).

Studies on *Caenorhabditis elegans* have shown that TORC1 shares similar functions as in yeasts (Hara et al. 2002, Long et al. 2002). Other studies on *C. elegans* have shown the involvement of TORC2 signalling in lipid storage. *CeRictor* mutants demonstrated an increase in lipid storage and developmental delay. This affect was also observed with *sgk1* mutants were gain-of-function mutations rescued the effects indicating the importance of SGK1 as a *CeRictor* mediator. However, unlike mammalian studies, this affect on lipid storage is independent of the Akt-Daf16/FoxO axis (Jones et al. 2009). Similar results were demonstrated with *S. cerevisiae* Ypk2/SGK1 in regulating ceramide synthesis indicating that the role of TORC2 signalling in lipogenesis is conserved from yeast, worms, to mammals (Aronova et al. 2008).

1.5 Anti-Cancer Drugs that Target mTORC1 and mTORC2

Many studies have been dedicated towards developing drugs against cancer. The PI3K pathway is shown to be hyper-activated in many human cancers mainly due to loss of the tumor suppressor *PTEN* or an activating mutation in the *PI3K* itself (Manning and Cantley 2007). Studies using human prostate cancer cells null for *PTEN* (PC-3 cells) have demonstrated that mTORC2 is required for tumor growth *in vivo*. PC-3 cell line experiments where Rictor was knocked out also showed that injecting nude mice with the Rictor-deficient cells reduced tumor size formation (Guertin et al. 2009). In addition, growing evidence indicates that the link between Akt and TSC2-Rheb-mTORC1 is a crucial step towards PI3K-mediated tumorigenesis (Guertin and Sabatini 2005).

According to the information mentioned above, developing inhibitors against mTORC2 could be a good approach to target cancer cells without having an effect on normal ones, since mTORC2 activity has been demonstrated to be important for the transformation of prostate epithelial cells when *PTEN* is deleted and not for normal cell growth. Discriminating normal cells from cancerous ones has always been a challenge in drug development, and constructing mTORC2 inhibitors might help to open new windows in understanding how cancer cells can be controlled. Although rapamycin has shown to have anti-proliferative properties *in vitro,* its use has been limited to very few cancers in clinical trials. It would be useful to find drugs that target both mTORC1 and mTORC2 preventing the hyper-activation of PI3K as seen in many human cancers. Rapalogs, drugs that act similar to rapamycin, have been derived and when combined with certain chemotherapeutic drugs have shown to increases the apoptotic rate in the tumors reducing their size (Mondesire et al. 2004). Other drugs, such as PI-103, have been demonstrated to have a dual inhibitory effect on both mTOR and PI3K reducing the proliferation rate in cancers (Fan et al. 2007). Torin 1, an ATP-competitive inhibitor, has been shown to inhibit the activity of both mTORC1 and mTORC2. (Thoreen et al. 2009). Another example of an mTOR selective inhibitor is INK128. INK128 was used in breast cancer xenograft studies and tends to

inhibit tumor growth and showed enhanced efficacy when combined with chemotherapy drugs (Jessen et al. 2009).

1.6 TOR and Aging

There has been much interest is studies that link various signalling pathways to lifespan extension. Aging is a consequence of an increase in the metabolic rate that leads to increase reactive oxygen species (ROS), which in turn increases cellular toxicity, and eventually reduces lifespan (Gems and Doonan 2009). Dietary restriction (DR) has been shown to reduce the levels of oxidatively damaged proteins, lipids, and DNA thereby, prolonging life (Masoro 2000). However, ROS is not the only driving force towards aging (Blagosklonny 2006). The TOR pathway has been shown to play a role in increasing lifespan upon dietary restriction in two ways. First, it can affect mRNA translation by regulating its target substrates S6K and 4E-BP1, hence, less protein synthesis and translation leads to a slower aging process (Silver et al. 2010). Secondly, it can trigger autophagy in nutrient-deprived conditions preventing the accumulation of damaged proteins thus reducing cellular toxicity (Ling et al. 2011). Another regulator of aging upon DR is the *Sirt-1* gene. Sirt-1 acts as a sensor for measuring levels of NAD^+ resulting from NADH oxidation, and acts as a histone deacetylase (HDAC) as well involved in glucose homeostasis (Medvedik et al. 2007). It has been implicated that DR inhibits the TOR pathway by activating Sirt-1 (Tucci 2012).

Studies on *C. elegans* have demonstrated a role of Rictor/TORC2 in lifespan extension. Ce*Rictor* mutants demonstrated defective feeding behavior and a reduced lifespan. However, Ce*Rictor* mutants affected *C. elegans* lifespan in a diet-dependent manner, and not through *akt*. When fed with a nutrient-poor diet, *rictor*-mutants showed an acceleration in their rate of aging reducing their lifespan, whereas when on a nutrient-rich diet, they demonstrated the opposite phenotype. It is not clear what signal in the diet that allows Ce*Rictor*-mutants to alter lifespan this way but suggests that Rictor/TORC2 plays a role in the aging of

C. elegans. *sgk1* mutants phenocopied Ce*Rictor* mutants suggesting that the two genes fall in the same genetic pathway regulating lifespan (Soukas et al. 2009).

1.7 Sin1 - An mTORC2 Component Involved in the SAPK Pathway

1.7.1 Sin1 is part of mTORC2

Sin1 has been shown to be important for mTORC2 assembly and activity (Frias et al. 2006). It is phosphorylated by mTOR and this stabilizes it by preventing its rapid turnover and degradation by the lysosome (Chen and Sarbassov 2011). *SIN1* alternative splicing generates five isoforms (Schroder et al. 2004) and at least three of them form distinct mTORC2s (Figure 1.7). Frias and colleagues were the first to identify mammalian Sin1 as part of mTORC2 (Frias et al. 2006) and it has been speculated that it might act as the main regulator of mTORC2's kinase activity responsible for the S473 phosphorylation of Akt (Jacinto et al. 2006). Sin1 is conserved from yeast to mammals and has been shown to have homologues in *Schizosaccharomyces pombe* (Sin1), *S. cerevisae* (AVO1), and *Drosophila discoideum* (RIP3) (Schroder et al. 2004). In addition, Sin1 RNA interference (RNAi) in cells reduced Rictor protein levels preventing the assembly of mTORC2, and vice versa, indicating the importance of both components for mTORC2 formation (Frias et al. 2006). It has been reported that Sin1 might have a role in regulating Akt-induced apoptosis where its knockdown increased sensitivity towards the apoptotic inducer etoposide. Moreover, BAD phosphorylation by Akt (which prevents apoptosis) was also decreased upon knocking down Sin1 (Yang et al. 2006). Although Sin1 is important for mTORC2 activity (Frias et al. 2006), we cannot rule out the fact that Sin1 might potentially have other functions independent of mTORC2. Rictor's ability to form an E3 complex is a good example where it demonstrates its independent roles of mTORC2 signalling despite being a key player in the pathway (Gao et al. 2010).

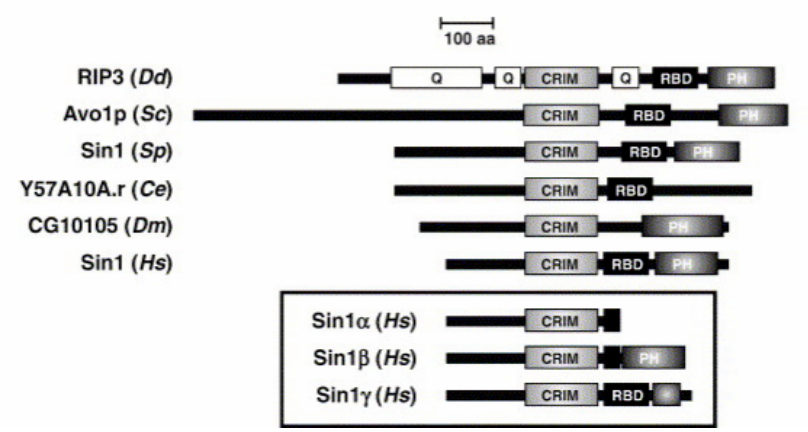


***Figure 1.7* A schematic representation of the Sin1 orthologues and their domains.** The Sin1 domains appear to be conserved throughout different species (*Dd, Dictyostelium discoideum; Sc, S. cerevisiae; Sp, S. pombe; Ce, C. elegans; Dm, Drosophila melanogaster; Hs, Homo sapiens*). The three human Sin1 splice variants are shown (boxed) containing a CRIM (conserved region in the middle) domain in all three isoforms, while isoforms β and γ contain PH (pleckstrin homology) and a RBD (Ras-binding domain) domains, respectively (Q, polyglutamine stretches) (Schroder et al. 2007).

1.7.2 Sin1 and the SAPK pathway

It is clear that Sin1 is an important regulator of mTORC2 assembly and activity. However, Sin1 has been found to have roles independent of TORC2 signalling. Sin1 associates with members of the MAPK family. MAPK pathways are essential for the transduction of environmental signals from the cytoplasm to the nucleus causing changes in gene expression (Marshall 1994). Under stress condition, a subgroup of the MAP kinase family is activated, the stress-activated MAP kinases (SAPKs). They have been found to play a role in the adaptive immune response, T-cell activation, inflammation, and stress-induced apoptosis. The SAPKs are classified into two main sub-classes based on their sequence; the c-Jun N-terminal kinase (JNK) and p38 kinase (Waskiewicz and Cooper 1995, Davis 1994, Verheij et al. 1998). In *S. cerevisiae*, Sty1/Spc1, which is a member of the SAPK family, has been implicated to bind to Sin1. Sty1 is involved in sexual conjugation and differentiation in yeast, and also has a similar structure to mammalian SAPKs that are activated by similar environmental stress (Millar et al. 1995, Shiozaki and Russell 1995). It has been demonstrated that *Sin1* deletion leads to phenotypes similar to *Sty1* deletion in yeast. Phenotypes

include sensitivity to environmental stress, a delay in mitotic initiation, and a decreased efficiency in both sexual conjugation and differentiation. Sin1 is not required for the activation of Sty1, but may be required to facilitate the translocation of Sty1 from the cytoplasm to the nucleus under environmental stress conditions (Wilkinson et al. 1999). However, Ikeda and colleagues have conflicting data and have demonstrated that Sin1 is not required for the stress-regulated Sty1-Atf1 pathway in *S. pombe* so its precise role in Sty1 signalling is unclear (Ikeda et al. 2008).

Other studies have reported that Sin1 binds to mammalian JNK and p38. Sin1 regulates JNK by inhibiting its basal activity *in vitro* (Schroder et al. 2005) while it has been demonstrated to bind p38 and link it to one of its transcription factor targets ATF2. This was shown to be important for its transcriptional activity in regulating apoptosis under stress conditions (Makino et al. 2006). Another binding partner of Sin1 which has been identified in a yeast two-hybrid screen is the poly(rC) binding protein 2 (PCBP2) which specifically binds to its N-terminus. Similar to Sin1 RNAi, knocking down PCBP2 promoted apoptosis indicating that both Sin1 and PCBP2 protect cells from apoptosis (Ghosh et al. 2008). These different studies demonstrate that Sin1 might have different roles and might be involved in more than one stress-induced pathway both in yeasts and mammals.

1.8 TRIM7 - A Novel Sin1-Binding Protein

The tripartite motif-containing (TRIM) superfamily of proteins have been reported to be involved in the innate immune response in addition to their involvement in many disorders such as genetic diseases, neurological diseases, and cancers (Meroni and Diez-Roux 2005). More than 60 TRIM proteins have been identified to date in humans and mice and their numbers vary in different species but increase through evolution. They have been classified into nine family groups (CI-CXI) based on the composition of their C-terminal region (Figure 1.8) (Short and Cox 2006). Their structure mainly consists of a RING domain, one or two B-box domains, and a coiled-coiled (CC) domain at their amino-terminal region (also known as RBCC motif) (Reymond et al. 2001). The RBCC motif is highly

conserved in different species making it a defining characteristic of the TRIM proteins. The RING domain contains a zinc-binding motif that allows protein-ubiquitin association and many of the TRIM proteins exhibit E3 ubiquitin ligase activity which promotes ubiquitination (Sabile et al. 2006). The B-boxes as well have a zinc-binding motif and play a role in the identification and binding of viral proteins (e.g. TRIM5) (Li et al. 2007b), whereas the CC region allows protein-protein interactions to form larger protein complexes (i.e. homo- and hetero-oligomers) (Minucci et al. 2000).

The C-terminal domains of the TRIM proteins have been implicated in various cellular functions such as those involved in subcellular localisation, cell-specific expression, and transcriptional regulation (Ozato et al. 2008). For example, the COS (C-terminal subgroup one signature) box has been shown to be involved in microtubule binding (Bernardi and Pandolfi 2007), while other domains such as the FN3 (fibronectin type 3) domain acts as a DNA binding site. Members of class VI nuclear TRIM proteins exhibit plant homeodomains (PHDs) that are involved in chromatin-mediated transcription regulation. Moreover, PHD-containing TRIMs can also be paired with bromodomains (BR) that recognize acetylated Lysines such as those found on histones. The pairing of PHDs with bromodomains has been shown to promote transcription repression (Le Douarin et al. 1997, Klugbauer and Rabes 1999, Ivanov et al. 2007). Other C-terminal domains within TRIM proteins have been implicated in intracellular trafficking and inducing self-association such as ARF (ADP ribosylation factor-like) and MATH (meprin and tumor-necrosis factor receptor-associated factor homology) domains, respectively (Ozato el a. 2008).

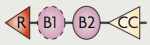
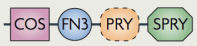
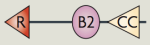
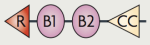
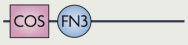
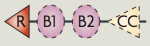
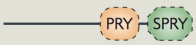
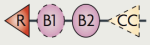
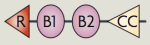
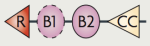
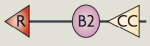
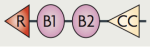
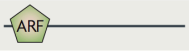
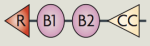
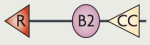
Family‡	N-terminal region (RBCC motif)‖	C-terminal region	Family members
C-I			MID1, MID2, TRIM9, TRIM36, TRIM46, TRIM67
C-II			TRIM54, TRIM55, TRIM63
C-III			TRIM42
C-IV			TRIML1, TRIM4, TRIM5α, TRIM6, TRIM7, TRIM10, TRIM11, TRIM15, TRIM17, TRIM21, TRIM22, TRIM25, TRIM26, TRIM27, TRIM34, TRIM35, TRIM38, TRIM39, TRIM41, TRIM43, TRIM47, TRIM48, TRIM49, TRIM50, TRIM53, TRIM58, TRIM60, TRIM62, TRIM64, TRIM65, TRIM68, TRIM69, TRIM72, TRIM75
C-V			PML, TRIM8, TRIM31, TRIM40, TRIM52, TRIM56, TRIM61, TRIM73, TRIM74
C-VI			TRIM24, TRIM28, TRIM33
C-VII			TRIM2, TRIM3, TRIM32, TRIM71
C-VIII			TRIM37
C-IX			TRIM23
C-X§			TRIM45
C-XI§			TRIM13, TRIM59

***Figure 1.8* TRIM proteins are classified based on their C-terminal region.** Depending on the composition of the C-terminal region, TRIM proteins have been divided into nine classes (CI-CXI). The N-terminal region of the TRIM proteins contain the RBCC motif, whereas they differ towards their carboxy-terminus where each class contains a different set of domains (ARF, ADP ribosylation factor-like; BR, bromodomain; COS, C-terminal subgroup one signature; FN3, fibronectin type 3; FIL, filamin-type immunoglobulin; MATH, meprin and tumour-necrosis factor receptor-associated factor homology; MID, midline; PHD, plant homeodomain; PML, promyelocytic leukaemia; TM, transmembrane). (‡, classification of TRIMs defined by Short and Cox; §, new families identified to reclassify TRIMs; ‖, the dotted outlines indicate that domains not present in all family members) (Ozato et al. 2008).

Recently, the Whitmarsh group identified a novel protein, TRIM7, that interacts with Sin1 in a yeast two-hybrid screen. Very little is currently known about the function of TRIM7.

1.8.1 TRIM proteins exhibit ubiquitin and SUMO E3 ligase activities

One of the most important physiological regulating signals in eukaryotes is ubiquitination. It is a post-translational modification that targets to eliminate short-lived proteins involved in different parts of the cell such as cell signalling, DNA repair, cell cycle regulation, and transcriptional regulation, as well as having roles in protein trafficking. Since many oncogenes and tumor suppressor genes are post-translationally modified by ubiquitin-conjugation, it is important to determine

the role of these ubiquitin E3 ligases in order to understand the mechanism of action of these modifiers (Weissman 1997). TRIM proteins are the largest family of proteins that possess RING domains, and since proteins containing a RING domain tend to be E3 ligases, it would be crucial to understand how these TRIM proteins contribute to cellular functions and diseases (Reymond et al. 2001). The three main players that catalyze the ubiquitination process are the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin ligase (E3). Upon ubiquitin activation by the E1 enzyme, the ubiquitin is transferred to the E2 enzyme. In turn, the E3 enzyme attaches the ubiquitin to its target protein on a Lysine residue by forming an isopeptide bond (Figure 1.9A) (Pickart 2001, Pickart and Eddins 2004). A polyubiquitin chain can be formed where more isopeptide bonds are formed between the internal Lysine residue of the ubiquitin and the Glycine residue of the C-terminal region of another ubiquitin molecule (Pickart 2001). The ubiquitin chain formed can either target the protein for degradation by the proteasome or target it for other cellular functions involved in trafficking (Weissman et al. 2011). There are two main classes of ubiquitin chains formed by TRIM proteins. The first is the classical Lysine 48-linked polyubiquitination which targets the protein for proteasomal degradation. The second type is Lysine 63-linked chain that targets the protein to the lysosomes (Figure 1.9B) (Ye and Rape 2009). There are many types of E2 enzymes that feature ubiquitin-conjugating cores (UBCs) which are classified into four groups (van Wijk and Timmers 2010). TRIM proteins have been shown to favor two main classes of UBCs; classes D and E. Immunofluorescent microscopy studies have demonstrated the co-localisation of TRIM proteins with UBCs and that different E3 ligases can share the same classes of E2 enzymes (Napolitano et al. 2011). TRIM5 is an example of an ubiquitin E3 ligase. It has been found that TRIM5 autoubiquitinates itself by forming polyubiquitin chains and is degraded by the proteasome (Diaz-Griffero et al. 2006).

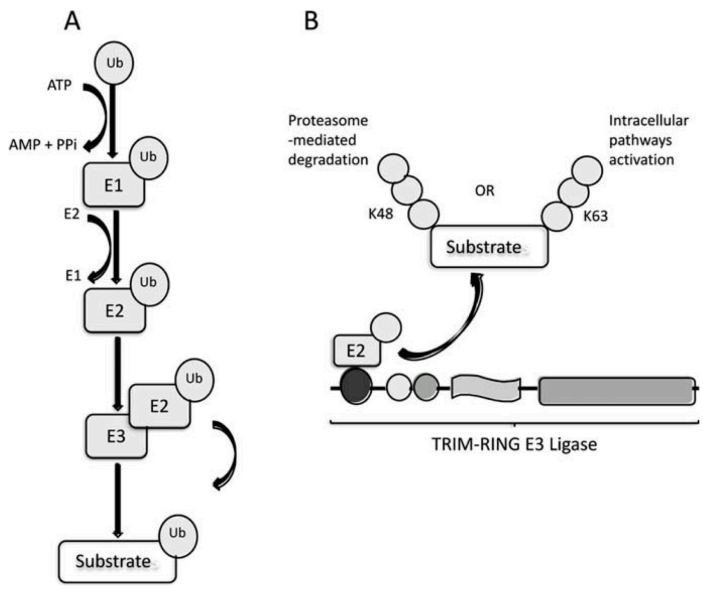


Figure 1.9 **TRIM proteins as ubiquitin E3 ligases. (A)** A schematic representation of the ubiquitination pathway where the E1-activating enzyme activates and transfers the ubiquitin molecule to the E2-conjugating enzyme, which in turn, is transferred to the E3 ligase. The E3 ligase then targets its substrate for ubiquitination. **(B)** Depending on the type of ubiquitin chain, TRIM proteins can target their substrates for degradation (K48-linked chain) or for other intracellular roles (K63-linked chain) (Napolitano and Meroni 2012).

Another main feature of TRIM proteins is their tendency to form complexes between family members via their CC domain. Immunofluorescent microscopy has shown that TRIM5 co-localises with other TRIMs such as TRIM4, 6, 22, 27, and 34 forming cytoplasmic bodies also known as aggresomes. Moreover, studies where the crosslinker glutaraldehyde was used showed the formation of a 150-180 kDa complex by Western blotting which might indicate the formation of trimers (Li et al. 2007a). It has been suggested that dimer or trimer formation might allow one TRIM protein to regulate the other. One example is the degradation effect exerted by TRIM21 on TRIM5 when it complexes with it and targets it for ubiquitination (Yamauchi et al. 2008).

Recently, a novel function of TRIM proteins has been identified. Some TRIM proteins can possess SUMO E3 ligase activity. Like ubiquitin, these small ubiquitin-like modifier (SUMO) proteins are post-translational modifiers. Unlike the ubiquitination pathway that mainly targets proteins for degradation, SUMOylation is required for protein stability, activity, localisation, and to facilitate

protein-protein interactions (Johnson 2004, Hay 2005, Geiss-Friedlander and Melchior 2007). Similar to the ubiquitin pathway, SUMOylation is catalyzed by three enzymatic steps that involve a SUMO-activating enzyme E1, a SUMO-conjugating enzyme E2 (Ubc9), and a SUMO E3 ligase (Deshaies and Joazeiro 2009). PML (TRIM19) and TRIM27 are two examples of SUMO E3 ligases where their ligase activities are RING-dependent. Unlike their ubiquitin E3 activity, an intact B-box domain was also required for the SUMO E3 activity. PML SUMOylates p53, Mdm2, and c-Jun, while TRIM27 binds directly to p53 and SUMOylates Mdm2 as well indicating that some TRIM proteins can interact with both Ubc9 and the target substrate. PML and TRIM27 are also able to interact within nuclear bodies, and when present together, Mdm2 SUMOylation and stability increase. This shows that some TRIM proteins can posses dual E3 functions but whether a target substrate is simultaneously ubiquitinated and SUMOylated is still unclear (Chu and Yang 2011).

1.8.2 TRIM proteins and cancer

Despite its role as a SUMO E3 ligase, PML is involved in many other cellular processes such as oncogenesis, DNA-damage and stress response pathways, apoptosis, senescence, and defense against viral infections (Regad et al. 2001). Many human cancers such as breast, colon, and prostate cancers exhibit partial or complete loss of *PML* indicating its important role a tumor suppressor. Moreover, PML induces p53 phosphorylation at its amino-terminal region (i.e. S20 and T18) (Louria-Hayon et al. 2003, Alsheich-Bartok et al. 2008). However, conflicting studies have implicated the involvement of PML in promoting carcinogenesis. PML can enhance the transcriptional activity of p53 gain-of-function mutants that are found in many human cancers promoting the formation of cancer colonies. The increase in cancer colonies was dependent on PML, where in its absence, a reduced growth rate of the cancer cells bearing p53 mutants was observed. This suggests that in certain cancers, PML's role as a tumor suppressor depends on the status of p53 (Haupt et al. 2009). The TRIM family genes tend to translocate between chromosomes playing an important role

in carcinogenesis. *PML* translocation occurs in acute promyelocytic leukemia (APL) forming a PML-retinoic acid receptor-α (RARα) fusion protein (de Thé et al. 1991, Kakizuka et al. 1991). This fusion protein prevents the formation of PML-nuclear bodies (PML-NBs). In addition, PML-RARα has other detrimental affects by acetylating enzymes involved in chromatin modification such as DNA methyltransferases and histone demethylases, and affects the DNA repair capabilities that are performed by wild-type PML (Martens et al. 2010).

Another group of TRIM protein that have been linked to cancer, specifically hepatocellular carcinoma (HCC) in mice, are the transcriptional intermediary factor 1 (TIF1) protein family. These include TRIM24 (TIF1α), TRIM28 (TIF1β), and TRIM33 (TIF1γ) each interacting with distinct transcription factors (Khetchoumian et al. 2007, He et al. 2006, Allton et al. 2009). TRIM24 acts as a tumor suppressor by preventing cells from progressing to the S phase of the cell cycle thereby reducing growth and preventing the formation of anchorage-independent colonies (Parada et al. 1998, Wong et al. 2000). As mentioned earlier in this report, TRIM proteins form homo- or hetero-complexes between each other via their CC region. This feature applies to the TIF1 family of proteins as well. TRIM24 has been show to form either a dimer with TRIM28 or a trimer with both TRIM28 and TRIM33. However, it is not clear whether TRIM24 acts as a tumor suppressor against HCC on its own or as part of a complex with its other family members (Herquel et al. 2011). Surprisingly, some studies have shown the ability of TRIM24 to act as an oncogene as well by promoting the transcriptional activity of the oestrogen-dependent genes associated with proliferation and tumorigenesis. These findings were consistent with the overexpressed TRIM24 observed in breast cancer patients (Tsai et al. 2010). TRIM29 is another example of a TRIM protein that can have a dual role in carcinogenesis. On one hand, it acts as an oncogene by increasing cell proliferation via the Wnt signalling pathway thorough β-catenin stabilization (Wang et al. 2009). On the other hand, it acts as a tumor suppressor by directly interacting with p53 and relocating it to the cytoplasm thus reducing p21 transcriptional activation (Yuan et al. 2010).

1.8.3 TRIM7 is a member of the TRIM proteins superfamily

TRIM7 lies in class IV of the TRIM family of proteins (Short and Cox 2006). Members of this group contain PRY and SPRY motifs forming a PRYSPRY domain (also known as B30.2 domain). It has been reported that the B30.2 domain only exist in vertebrates (Rhodes et al. 2005). *TRIM7* (also known as glycogenin-interacting protein "*GNIP*") alternative splicing generates four isoforms; *TRIM7.1*, *TRIM7.2*, *TRIM7.3, and TRIM7.4*. TRIM7 has been shown to be involved in glycogenin regulation (Zhai et al. 2004), a protein involved in the biosynthesis of glycogen (Alonso et al. 1995). All isoforms of TRIM7 contain a B30.2-like domain, except TRIM7.4, as well as a CC domain (Skurat et al. 2002). Despite the B30.2 domain being present in other TRIM proteins exhibiting RING domains, it's functional role mainly remains unknown and does not take part in the E3 ligase activity of TRIMs (Henry et al. 1998). However, efforts have been made to further characterise and understand the function and structure of the B30.2 domain where it's crystal structure has shown that it consists of a 13-stranded intact β-sandwich structure. This was the first study to determine the structure of the B30.2 domain indicating that it acts as a dimer allowing the formation of binding pockets for other proteins (Grütter et al. 2006). Other studies have implicated that the B30.2 domain acts as a binding site for glycogenin promoting its interaction with TRIM7 (Zhai et al. 2004). TRIM7.1 is the longest isoform whereas TRIM7.4 is the shortest. However, their amino-terminal regions are identical and only differ towards their coiled-coiled domain. On the other hand, isoforms 2 and 3 lack both the RING and B-box domains but have identical C-terminals as in TRIM7.1 (Figure 1.10). Although little is known about TRIM7, it has been shown that isoform 4 is found in skeletal muscles of adult mice and in embryonic mouse tissues, and is localised diffusely throughout the cytoplasm and nucleus (Reymond et al. 2001).

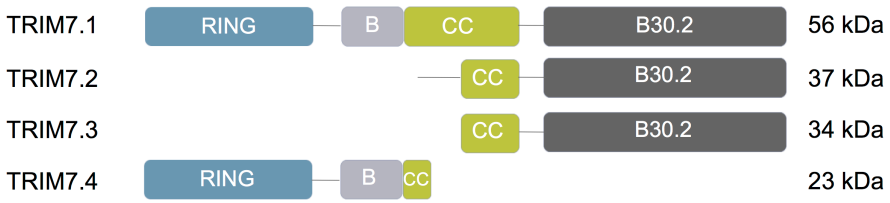


Figure 1.10 **TRIM7 alternative splicing generates four different isoforms.** Isoforms 1-3 all share a common B30.2 domain towards their carboxy-terminus which is not present in TRIM7.4 (the shortest of all isoforms). The amino terminus of both TRIM7.1 and TRIM7.4 are identical but differ in their coiled-coiled region. The protein size of each isoform is indicated in kilodaltons (kDa) (Adapted from Zhai et al. 2004).

1.9 Aims and Objectives of the Project

Preliminary data indicates that TRIM7 interacts with the mTORC2 component Sin1. The main aim of this project was to characterize the interaction between TRIM7 and Sin1 and determine the functional role of TRIM7 in regulating mTORC2 signalling. The objectives were to:

a) Confirm the binding of TRIM7 to Sin1 in mammalian cells by co-immunoprecipitation.
b) Determine the cellular localisation of TRIM7 by fluorescence microscopy.
c) Determine whether TRIM7 binds to and co-localises with related TRIM proteins.
d) Elucidate if TRIM7 has ubiquitin and/or SUMO E3 activity.
e) Generate cells with a knock-down of TRIM7 and determine the effect on mTORC2 signalling and cell behavior.

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Generating TRIM Constructs

The TRIM7.1 complementary DNA (cDNA) was amplified by PCR using pcDNA3-Flag-TRIM7.1 plasmid as a template for all constructs (Whitmarsh lab), whereas the MycGFP.pcDNA3-TRIM11/27 plasmids were kindly provided by Germana Meroni (Cluster in Biomedicine, Trieste, Italy) and were used as templates to generate the TRIM11/27 constructs. The different primers and cloning sites for each construct are shown in Table 2.1. The PCR reaction was carried out using the Pfu Ultra II Fusion HS DNA Polymerase Protocol (Agilent Technologies) following the manufacturer's instructions. The samples were then mixed with Crystal 5x DNA Loading Buffer Blue (Bioline, Cat. No. BIO-37045) and gel electrophoresis was performed at 100V where the samples were loaded on a 1% (w/v) agarose gel (Sigma) using the Hyperladder 1 (Bioline) as a marker. The target DNA was then extracted from the gel using the DNA Gel Extraction Kit (QIAGEN). Both the insert and its appropriate vector were digested with their corresponding restriction enzyme (New England Biolabs) for 3 hours at 37°C. Samples were then analyzed on a 1% (w/v) agarose gel and DNA was extracted as mentioned above. The digested insert and vector were ligated at a ratio of 1:5 (vector:insert) using T4 DNA Ligase enzyme and 10x T4 Ligation Buffer (both from New England Biolabs) overnight at room temperature. The ligation mixture was transformed into *E. coli* DH5α (F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–) competent cells via the calcium chloride approach (Dagert and Ehrlich 1979). The competent cells containing the ligation mix were incubated on ice for 30 minutes followed by a heat-shock process of 2 minutes at 42°C and 2 minutes on ice. Cells were then plated on agar plates containing the appropriate antibiotic.

The pcDNA3-Flag-TRIM7.1 plasmid was used as a template for mutagenesis (Table 2.1) with the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to manufacturer's instructions.

Table 2.1 A summary of the primers used to generate the TRIM constructs.

Construct	Forward Primer (5'→3')	Reverse Primer (5'→3')	Restriction Sites	Antibiotic
pEGFP-C2-TRIM7.1	GAGAAGATCTCGATGGCGGCTGTGGGACCG	GAGAAAGCTTTCAAGGCCAGATTCGCAAGT	*Bgl*II/*Hind*III	Kanamycin
pcDNA3.Flag-TRIM7.1ΔR	GAGAAAGCTTCGAGCCCGCGCGCCCCAGTC	GAGAGATCTGTCAAGGCCAGATTCGCAAGT	*Hind*III/*Xho*I	Ampicillin
p3xFlagCMV24-TRIM7.1ΔR	GAGAAAGCTTCGAGCCCGCGCGCCCCAGTC	GAGAGATCTGTCAAGGCCAGATTCGCAAGT	*Hind*III/*Xho*I	
p3xFlagCMV24-TRIM7.1	GAGAAAGCTTATGGCGGCTGTGGGACCGCG	GAGAGATCTGTCAAGGCCAGATTCGCAAGT	*Hind*III/*Xho*I	
p3xFlagCMV24-TRIM11	GAGAAAGCTTATGGCTGCCCCAGACTTGTC	GAGAGAATTCTCACTGCGGGCCAAGGGTGT	*Hind*III/*EcoR*I	
p3xFlagCMV24-TRIM27	GAGAAAGCTTATGGCCTCCGGGAGCGTGGC	GAGAGAATTCTCACGGAGAGGTCTCCATGG	*Hind*III/*EcoR*I	
pMAL-C2-TRIM7.1	GAGATCTAGAATGGCGGCTGTGGGACCGC	GAGAAAGCTTTCAAGGCCAGATTCGCAAGT	*Xba*I/*Hind*III	
pMAL-C2-TRIM11	GAGAGAATTCATGGCTGCCCCAGACTTGTC	GAGATCTAGATTACTGCGGGCCAAGGGTGT	*EcoR*I/*Xba*I	
Mutagenesis Constructs (Forward & Reverse Primers 5'→3')				
pcDNA3.Flag-TRIM7.1 C44A	CGTGAGCCGGTGTCCGTCGAGGCCGGCCACAGCTTC TGCCGCG	GCGCGGCAGAAGCTGTGGCCGGCCTCGACGGACACC GGCTCAC		
pcDNA3.Flag-TRIM7.1 C44A/H46A	GAGCCGGTGTCCGTCGAGGCCGGCGCCAGCTTCTGC CGCGCC	CAGGCGCGGCAGAAGCTGGCGCCGGCCTCGACGGA CACCGGC		

2.2 Cell Culture and Transfections

COS7, HeLa, and HEK293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplied with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin and streptomycin antibiotics, and 1% (v/v) glutamax (all from Life Technologies). All plasmids and constructs were transfected into cells using Polyplus JetPEI™ DNA Transfection Reagent (PeqLab) following the manufacturer's instructions. For cell treatments, 80 ng/ml of insulin-like growth factor-1 (IGF-1) (Calbiochem) and 250 μM H_2O_2 were used at different time points, 120 μM Chloroquine (Sigma) and 250 nM Torin 1 (provided by Nathanael S. Grey, Harvard Medical School, Boston, USA) were used both for 2 hours, and 20 μM MG132 (Calbiochem) for 4 hours.

2.3 Generating TRIM7 Knockdown Stable Cell Lines

Both TRIM7 short hairpin RNA (shRNA) (clone no. HSH019974-1-HIV1 [OS279065]) and scrambled shRNA plasmids from GeneCopoeia were used to generate stable knockdowns in HEK293T cells. Both shRNAs were transfected into cells as explained in Section 2.2 and puromycin was added 24 hours post-transfection at a concentration of 3 μg/ml. The media was replaced on a daily basis with fresh antibiotic until separate colonies were obtained. Different clones from each shRNA-transfected plate were recovered by trypsinization, transferred into new plates, and left to grow. The efficiency of the knockdown was tested by quantitative real-time polymerase chain reaction (qPCR).

2.4 qPCR Analysis for TRIM7 Knockdown Stable Cell Lines

qPCR was performed on RNAs extracted from the HEK293T stable cell lines using RNeasy® Mini Kit (QIAGEN) where TRIM7.1 primers (Forward: 5'-TCTTTTAAGAGACTGGGTCTTGC-3'; Reverse: 5'-CCAGCTCTTAGGGAGATGGA-3') were used, whereas RPL19 primers were used for normalization (Forward: 5'-GATGCCGGAAAAACACCTTG-3'; Reverse: 5'-TGGCTGTACCCTTCCGCTT-3'). qPCR was performed following

QuantiTect SYBR Green qPCR Handbook (QIAGEN) according to the manufacturer's instructions. RNA was used at a concentration of 100 ng/reaction and the qPCR was carried out in a C1000 Thermal Cycler with CFX96™ Real-Time System (Bio-Rad) using Bio-Rad CFX Manager software version 1.5.5.

2.5 GST (glutathione S-transferase) Pull Down and Immunoprecipitation

COS7 or HEK293T cells co-transfected with the appropriate plasmids shown in Table 2.2 were washed with 1x phosphate-buffered saline (PBS) composed of 150 mM NaCl and 20 mM Na_2HPO_4 pH 7.4, and then lysed in Tris lysis buffer (TLB) containing 20 mM Tris/HCl (pH 7.4), 137 mM NaCl, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 2 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM PMSF, 1 mM sodium orthovanadate, 5 μg/ml leupeptin, and 5 μg/ml aprotonin. A portion of the cell lysate was mixed with 6x SDS loading buffer [300 mM Tris-HCl pH 6.8, 300 mM SDS, 600 mM dithiothreitol, 30% (v/v) glycerol, and 0.1% (w/v) Bromophenol Blue]. For the GST pull downs, the remaining lysate was mixed with GSH-Agarose slurry solution (Generon) at 4°C for 3 hours with rotation to bind the GST proteins. The GST-bound proteins were washed four times with lysis buffer and eluted in 6x SDS loading buffer. For the immunoprecipitation experiments, the M2 antibody (Sigma) that recognizes the Flag epitope and Protein G-Sepharose (Generon) or Protein G Dynabeads (Invitrogen) were used. The protein-bound beads were washed as in the previous experiment.

Table 2.2 A complete list of constructs used during the experiments

Construct	Description
pcDNA3	Mammalian empty plasmid
pcDNA3-Flag-TRIM7.1	Mammalian plasmid expressing TRIM7.1 N-terminally tagged with 1xFlag
pcDNA3-Flag-TRIM7.1 C44A	Mammalian plasmid expressing TRIM7.1 with a Cys→Ala point mutation at amino acid position 44 N-terminally tagged with 1xFlag
pcDNA3-Flag-TRIM7.1 C44A/H46A	Mammalian plasmid expressing TRIM7.1 with a Cys→Ala and His→Ala point mutations at amino acid positions 44 and 46, respectively, N-terminally tagged with 1xFlag
pcDNA3-Flag-TRIM7.1ΔR	Mammalian plasmid expressing RING-deleted TRIM7.1 (amino acids 1-81 deleted) N-terminally tagged with 1xFlag
p3xFlagCMV24-TRIM7.1	Mammalian plasmid expressing TRIM7.1 N-terminally tagged with 3xFlag
p3xFlagCMV24-TRIM7.1ΔR	Mammalian plasmid expressing RING-deleted TRIM7.1 (amino acids 1-81 deleted) N-terminally tagged with 3xFlag
pEBG	Mammalian plasmid expressing GST-tag alone
pEBG-TRIM7.1	Mammalian plasmid expressing TRIM7.1 N-terminally tagged with GST
pEBG-TRIM7.3/7.4	Mammalian plasmid expressing TRIM7.3/7.4 N-terminally tagged with GST
pEBG-CLK-1	Mammalian plasmid expressing Clk-1 N-terminally tagged with GST
pEBG-hSin1	Mammalian plasmid expressing Sin1 N-terminally tagged with GST
pEBG-hSin1 (1-135,136-271, 272-372, and 372-511)	Mammalian plasmid expressing deletion-mutants of Sin1 N-terminally tagged with GST
pEGFP-C2	Mammalian plasmid expressing GFP-tag alone
pEGFP-C2-TRIM7.1	Mammalian plasmid expressing TRIM7.1 N-terminally tagged with GFP
pYFP-PML	Mammalian plasmid expressing PML.1 N-terminally tagged with YFP

pMAL-C2	Bacterial plasmid expressing MBP-tag alone
pMAL-C2-TRIM7.1	Bacterial plasmid expressing TRIM7.1 N-terminally tagged with MBP
pMAL-C2-TRIM11	Bacterial plasmid expressing TRIM11 N-terminally tagged with MBP
pGEX-6P1-hSinb	Bacterial plasmid expressing Sin1 N-terminally tagged with GST
pGEX-6P1-FOXO3A (1-80)	Bacterial plasmid expressing amino acids 1-80 of FoxO3a N-terminally tagged with GST
pGEX-6P1-CLK-1	Bacterial plasmid expressing Clk-1 N-terminally tagged with GST
pcDNA3-myc6-hSin1	Mammalian plasmid expressing Sin1 N-terminally tagged with Myc
pcDNA3-myc-Mdm2	Mammalian plasmid expressing Mdm2 N-terminally tagged with Myc
MycGFP.pcDNA3-TRIM11/27	Mammalian plasmid expressing TRIM11/27 N-terminally tagged with Myc and GFP
p3xFlagCMV24-TRIM11/27	Mammalian plasmid expressing TRIM11/27 N-terminally tagged with 3xFlag
pRh5-myc-mTOR	Mammalian plasmid expressing mTOR N-terminally tagged with Myc
pCMV-HA-mTOR/mTOR KD	Mammalian plasmid expressing wild-type or kinase dead mutant mTOR N-terminally tagged with Myc
pCMV5-HA-Aktα	Mammalian plasmid expressing Akt1 N-terminally tagged with HA
pM107-6xHis-Ub	Mammalian plasmid expressing ubiquitin N-terminally tagged with 6xHis
pcDNA3-6xHis-UBK48R	Mammalian plasmid expressing ubiquitin with a Lys→Arg point mutation at amino acid position 48 N-terminally tagged with 6xHis
pcDNA3-6xHis-UBK7R	Mammalian plasmid expressing ubiquitin with all seven Lysines mutated to Arginines N-terminally tagged with 6xHis
pcDNA3-His-SUMO1/2	Mammalian plasmid expressing SUMO1/2 N-terminally tagged with 1xHis

2.6 Antibodies

Table 2.3 A list of antibodies used for immunoprecipitation, Western blotting, and immunofluorescence

Antibody	Species	Used for	Dilution	Company	Catalog/Clone No.
GST-tag	Goat	WB	1:3000	GE Health Care	27457701V
GFP-tag	Rabbit	WB	1:1000	Invitrogen	G10362
Myc-tag	Mouse	WB	1:5000	Millipore	CA92590
M2 (Flag-tag)	Mouse	WB IF	1:2000 1:500	Sigma	F1804
HA-tag	Mouse	WB	1:1000	Sigma	H9658
His-tag	Mouse	WB	1:1000	GE Health Care	2747001
MBP-tag	Mouse	WB	1:1000	Cell Signaling Technology	2396S
mTOR	Goat	WB	1:1000	Santa Cruz Biotechnology	sc-1549
Rictor	Rabbit	WB	1:1000	Novus Biologicals	NB100-612
Sin1	Rabbit	WB	1:1000	Covlab	Clone 113
Akt	Rabbit	WB	1:4000	Cell Signaling Technology	9272S
pAkt S473	Rabbit	WB	1:2000	Cell Signaling Technology	40605
β-actin	Rabbit	WB	1:2000	Abcam	ab8227
ERK1/2	Rabbit	WB	1:1000	Santa Cruz Biotechnology	sc-93/sc-154
LC3	Mouse	WB	1:1000	MBL	M115-3
Ub	Mouse	WB	1:1000	Santa Cruz Biotechnology	sc-271289

EEA1	Mouse	IF	1:250	Prof. Philip Woodman (University of Manchester, Manchester, UK)	
TR	Mouse	IF	1:50		
Lamp1	Mouse	IF	1:250		
IR Dye® 680/800	Mouse/Rabbit Goat	WB	1:40000 1:10000	Li-Cor	926-68020/965-68021 926-32214
HRP-tag	Mouse/Rabbit Goat	WB	1:10000	GE Health Care Abcam	NA931V/NA934V ab6877-1
Alexa® Fluor^{594}	Mouse	IF	1:500	Invitrogen	A31624

2.7 SDS-PAGE and Western Blotting

All samples obtained from the binding/co-expression experiments were loaded on 8-15% (v/v) SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels depending of the protein size, using Precision Plus Protein as a marker (Bio-Rad) and electrophorased at 150V. The stacking gels were composed of 124 mM Tris pH 6.8, 3.72% Acrylamide (Life Science Products), 0.1% SDS, 0.1% ammonium persulfate (APS) (Sigma), and 0.2% N,N,N',N'-Tetramethylethylenediamine (TEMED) (Sigma), while the resolving gel consisted of 375 mM Tris pH 8.8, 8-15% Acrylamide, 0.1% SDS, 0.1% APS, and 0.1% TEMED. The proteins on the gels were then transferred to Immobilon-FL transfer membrane (Millipore) using Western blotting either by the semi-dry or the wet transfer method for 3 hours at 15V or 3 hours at 50V in a 4°C cold room, respectively. The buffers used for SDS-PAGE and Western blotting are shown in Table 2.4. The membranes were then blocked in 5% (w/v) non-fat milk containing 1x Tris-buffered saline (TBS) [15mM Tris-HCl (pH 7.4), 150mM NaCl] for 60 minutes with shaking followed by overnight incubations at 4°C with the appropriate primary antibody while shaking. Antibodies directed against epitopes on over-expressed proteins were made in 5% (w/v) milk/TBS, whereas 2.5% (w/v) milk/TBS was used for antibodies directed against endogenous proteins. 1x TBS containing 0.5% (v/v) Tween-20 was used to wash the membranes four times for 10 minutes with shaking. Anti-Mouse/Rabbit/Goat IR Dye® 680/800 or HRP-labeled secondary antibodies were used made up in 5% (w/v) milk/TBS containing 0.5% (v/v) Tween-20 and 0.01% (v/v) SDS. The IR-sensitive secondary antibodies were incubated for 30 minute at room temperature in the dark while shaking, whereas the HRP-secondary antibodies were incubated for 45 minutes. Membranes were washed four times as mentioned above. A Li-Cor Odyssey Infrared Imaging System was used to analyze the membranes with the IR-sensitive secondary antibodies using Odyssey software version 2.1. For the HRP-labeled membranes, Amersham ECL Prime Western Blotting Detection Reagent (GE Health Care) was used following the manufacturer's instructions and developed using X-ray film and a Mini Medical 90 Film Processor.

Table 2.4 Buffers used for SDS-PAGE and Western blotting

Buffer	Composition
1x SDS Running Buffer	24 mM Tris, 191 mM Glycine, 0.1% (v/v) SDS
1x Semi-dry Transfer Buffer	24 mM Tris, 191 mM Glycine, 20% (v/v) MeOH
1x Wet Transfer Buffer	24 mM Tris, 191 mM Glycine, 20% (v/v) MeOH, 0.05% (v/v) SDS

2.8 Immunofluorescence Microscopy

COS7 and HeLa cells were grown on glass coverslips and either the pEGFP-C2-TRIM7.1 construct or the pEGFP-C2 vector (Whitmarsh lab) were transfected into cells. Cells were fixed with 4% (w/v) paraformaldehyde (PFA) (Sigma) for 15 minutes at room temperature, permeabilized with 0.1% (v/v) Triton-X in 1x PBS, and washed in 1x PBS. The coverslips were then mounted on twin frosted glass slides using ProLong® Gold Antifade Reagent with DAPI (4',6-diamidino-2-phenylindole) (Invitrogen). HeLa cells transfected with pcDNA3-Flag-TRIM7.1, pcDNA3-Flag-TRIM7.1 C44A or C44A/H46A, and pcDNA3-Flag-TRIM7.1ΔR were then blocked and permeabilized with 1% (w/v) BSA (bovine serum albumin) 0.1% (v/v) Tween-20 in 1x PBS for 40 minutes at room temperature. The coverslip was then incubated with the M2 primary antibody made in 1% (w/v) BSA 0.1% (v/v) Tween-20 in 1x PBS for 1 hour at room temperature. Cells were washed four times with 1x PBS and then incubated with Alexa® Fluor^{594}-conjugated secondary antibody (Invitrogen) for 1 hour at room temperature.

For co-expressing TRIM constructs, p3xFlagCMV24-TRIM7.1 was co-transfected with either MycGFP.pcDNA3-TRIM11/27 or pYFP-PML (Whitmarsh lab) and processed as above.

Staining for endosomal markers was performed in both COS7 and HeLa cells transfected with the pEGFP-C2-TRIM7.1 construct. For the EEA1 (Early endosomal antigen-1) and transferrin receptor (TR) antibodies, cells were fixed in

4% (w/v) PFA but permeabilized with 0.1% (v/v) Triton-X in 1x PBS. For Lamp1 staining, methanol fixation was used instead where cells were incubated in absolute methanol (-21°C) for 5-10 minutes at -21°C. The remaining steps follow as for PFA fixation. The samples were all analyzed using an Olympus BX51 microscope equipped with a Photometrics Cool SNAP ES camera using MetaVue software version 6.3r6.

2.9 Expression and Purification of TRIM7.1 and TRIM11 From *E. coli*

The maltose binding protein (MBP) system was used for both TRIM7.1 and TRIM11 expression in *E. coli* and subsequent purification (constructs generated as described in Section 2.1). *E. coli* BL21 DE3 (F^- ompT gal dcm lon $hsdS_B(r_B^- m_B^-)$ λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) competent cells were transformed with pMAL-C2-TRIM7.1, pMAL-C2-TRIM7.11, and pMAL-C2 as explained in Section 2.1. After single colonies were picked, cells were left to grow in the presence of 2% (v/v) glucose in liquid broth (LB) until they reached optical density (OD_{600}) of 0.4-0.5 and then induced by 0.1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) overnight at room temperature while shaking. Cells were harvested, lysed with lysis buffer containing 20 mM Tris-HCl, 0.5 M NaCl pH 8.0, and sonicated four times with 10 second bursts allowing a one-minute interval between each sonication while on ice using Qsonica XL-2000 followed by 0.4% (v/v) N-Lauroylsarcosine sodium salt solution (Sigma) and 1 mM $ZnCl_2$ treatment. The cleared lysate was then transferred into a polypropylene column (QIAGEN) containing Amylose resin (New England Biolabs) that has been pre-washed with 12x its volume with the lysis buffer allowing it to flow by gravity. The column was eluted with 10 mM maltose in lysis buffer. A total concentration of 20% (v/v) glycerol was added to the elutions for long-term storage at -80^{o}C.

2.10 TRIM7.1 Phosphorylation and *in vitro* Kinase Assays

pEBG-TRIM7.1 was expressed in HEK293T cells and a pull down was performed as in Section 2.5. The GSH-Agarose beads bound to GST-TRIM7.1 was washed three times with TLB and two times with the kinase buffer (30 mM Tris pH 7.4, 20

mM $MgCl_2$, 1mM DTT [Dithiothreitol]). A kinase reaction was carried out by adding a mixture of 0.4 mM ATP (Promega) and 1μl ATP [γ-^{32}P]-3000 Ci/mmol 10 mCi/ml (Perkin Elmer) to the kinase buffer and incubated for 30 minutes at 30°C with mixing every 10 minutes. Two additional washes with TLB were performed followed by mixing the samples with 6x SDS loading and running them on SDS-PAGE gels. Gels were stained, dried, and exposed to KODAK BioMax MR Films.

The MBP-TRIM7.1, purified as described in Section 2.9, was used for the *in vitro* kinase assay. The positive controls used for the mTOR, Akt1, and PKCα kinases were GST-Sin1, GST-FoxO3a (aa 1-80), and GST-Clk-1 (all from Whitmarsh lab), respectively. For the Akt1 kinase assay, pCMV5-HA-Aktα (kindly provided by D. Alessi, University of Dundee, UK) was transfected into HEK293T cells, induced with 80 ng/ml IGF, harvested, and immunoprecipitated as explained in Section 2.5. The total kinase reaction was made up to 30 μl composed of the purified proteins, 377 Units/mg of the mTOR or 3597 Units/mg of the PKCα kinases (both from Millipore) (the immunoprecipitate in the case of Akt1), 0.4 mM ATP and ATP [γ-^{32}P], and the kinase buffer. $MnCl_2$ was used in the kinase buffer instead of $MgCl_2$ for the mTOR kinase assay. The reaction was carried out at 30°C and terminated by mixing the samples with 6x SDS loading buffer. Samples were run on SDS-PAGE gels and the gels were stained, dried, and exposed to KODAK BioMax MR Films.

2.11 *in vivo* and *in vitro* Ubiquitination Assays

The constructs used for the *in vivo* ubiquitination assay were the following: Flag-TRIM7.1, 3xFlag-TRIM7.1, Flag-TRIM7.1ΔR, MycGFP.TRIM11/27, and 3xFlag-TRIM11/27 described in Table 2.2. The point mutants generated in Section 2.1 were used as well in addition to the pcDNA3-myc6-hSin1 construct. In addition, pM107-6xHis-Ub (provided by Clare Davies, University of Birmingham, Birmingham, UK) and pcDNA3-6xHis-UBK48R/K7R (provided by Miranda van Trienst, University Medical Center, Utrecht, Netherlands) were used. Cells were

treated with MG132 (Calbiochem) at a concentration of 20 μM for 4 hours post-transfection. A third of the harvested cells were lysed in TLB as in Section 2.5 for inputs, whereas the remaining two thirds of the cells were lysed under denaturing conditions. The denaturing buffer (Buffer A) consisted of 6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM imidazole pH 8.0. The lysates were then sonicated two times with 10-15 second bursts as in Section 2.9. A pull down was carried out using Ni-NTA Superflow (QIAGEN) mixed with the lysate for 3 hours at room temperature. Then, the beads were washed three times with Buffer A, two times with Buffer B (1:4 dilution of Buffer A in 25 mM Tris-HCl, 20 mM imidazole pH 6.8), and two final washes with Buffer C (25 mM Tris-HCl, 20 mM imidazole pH 6.8). Imidazole was added freshly to the buffers. Samples were then mixed with 6x SDS loading buffer containing 200 mM imidazole and analyzed by Western blotting.

For the *in vitro* ubiquitination assay, the purified proteins from Section 2.9 were used where MBP alone was used as a negative control while MBP-TRIM11 was used as a positive control. The *in vitro* reaction consisted of the 0.5-1 μM purified proteins (E3), 15 nM E1 enzyme, 1 μM E2 enzyme (His_6-UbcH-5a), 5 μM His_6.Ub (all from Biomol), 50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 4 mM ATP, 150 mM NaCl, and 2 mM DTT to a final volume of 60 μl. The reaction was incubated for 90 minutes at 37°C and terminated by adding 6x SDS loading buffer followed by SDS-PAGE.

2.12 *in vivo* SUMOylation Assays

p3xFlag.CMV24-TRIM7.1, MycGFPpcDNA3-TRIM27, pcDNA3-myc-Mdm2 (Whitmarsh lab), and pcDNA3-His-SUMO1/2 (provided by Prof. Andrew Sharrocks, University of Manchester, Manchester, UK) were used for the SUMOylation assays. The constructs were transfected into HEK293T cells and treated with 20 μM MG132 for 4 hours. Cells were harvested under denaturing conditions as in the previous section and a pull down was performed using Ni-NTA Superflow. The buffers used were slightly different from the ones of the

ubiquitination assays; Buffer S1 consisted of 6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl pH 8.0, 5 mM imidazole, and 10 mM β-mercaptoethanol, whereas Buffer S2 consisted of 8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl pH 8.0, and 10 mM β-mercaptoethanol. Buffer S3 was the same as Buffer S2 but with pH 6.3. The samples were then analyzed by Western blotting.

2.13 MTT Cell Proliferation Assay

Both the scrambled and TRIM7 stable cell lines from Section 2.3 were equally split (7,000 cells) onto 96-well plates in triplicates at three time points; Days 1, 2, and 3. A MTT (Thiazolyl Blue Tetrazolium Bromide) cell proliferation assay was performed following the protocol from Wallert and Provost Lab (van de Loosdrecht et al. 1994). MTT (Sigma) was made in 1x PBS to a final concentration of 5 mg/ml and the MTT solvent consisted of 4 mM HCl and 0.1% (v/v) Nondet P-40 (NP-40) made in isopropanol. Each day the absorbance of each individual plate was measured at 570 nm using a FLUOstar OPTIMA multimode plate reader running on FLUOstar OPTIMA software v. 1.32 (BMG LABTECH).

Chapter Three

Results

3.1 Characterisation of TRIM7 Binding Partners

3. Results

3.1 Characterisation of TRIM7 Binding Partners

3.1.1 Introduction

Little is known about TRIM7 apart from it featuring structural domains that are similar to those found in other TRIM family members and that there are a number of splice variants giving rise to distinct protein isoforms. The Whitmarsh group demonstrated an interaction between TRIM7 and the TORC2 component Sin1 in a yeast two-hybrid screen (unpublished data). The aim of this chapter is to demonstrate whether TRIM7 associates with Sin1 in mammalian cells and if it associates with other components of the TORC2 complex. Furthermore, it was investigated whether TRIM7 could form homo-oligomeric complexes or form hetero-oligomers with other TRIM family members. Understanding the binding capabilities of TRIM7 is crucial to clarify what pathways it may be involved in and eventually will allow us to build up a picture of its functional role in cell signalling. Different binding experiments were carried out that included GST pull downs and immunoprecipitations in order to achieve these goals. In addition, immunofluorescent microscopy was used to determine the localisation of TRIM7 in cells.

3.1.2 Characterising the binding of TRIM7 to Sin1

To follow up on the yeast two-hybrid screen and to confirm the binding of TRIM7 to Sin1, a GST pull down experiment was performed. The longest isoform of TRIM7, TRIM7.1 (see Figure 1.10), was used for these experiments. Constructs expressing myc-tagged Sin1 and GST-tagged TRIM7.1 were co-transfected into COS7 cells. As a positive control, GST-tagged Clk-1 was co-transfected with myc-tagged Sin1, as an interaction between these proteins has been demonstrated previously in the Whitmarsh lab (unpublished data). GST fusion proteins were isolated on GST-agarose beads and it was confirmed that Clk-1

binds to Sin1 (Figure 3.1, lane 2). GST-TRIM7.1 pulled down Sin1 confirming the results obtained from the yeast two-hybrid screen (Figure 3.1, lane 3).

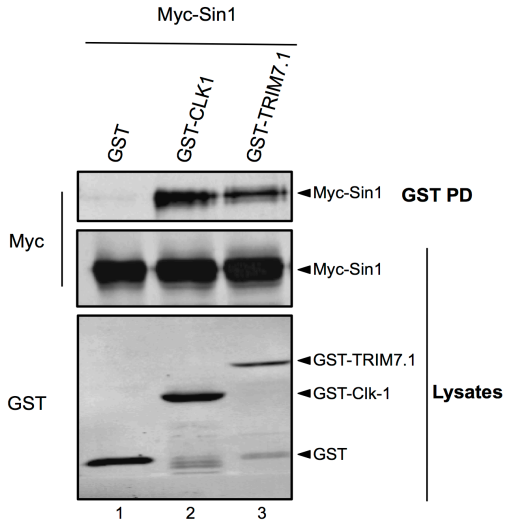


Figure 3.1 **TRIM7.1 binds to Sin1.** Immunoblot showing the binding of TRIM7.1 to Sin1. COS7 cells were co-transfected with pEBG (negative control), pEBG-CLK-1, or pEBG-TRIM7.1, and pcDNA3-myc6-hSin1. Clk-1 was used as a positive control that binds to Sin1. The top panel shows the pull down results confirming the binding of TRIM7.1 to Sin1. The protein levels of the myc-tagged Sin1 is shown in the middle panel using an anti-myc antibody while the bottom one illustrates the migration of the GST-tagged proteins as well as GST itself using an anti-GST antibody.

Following confirmation of the binding of TRIM7.1 to Sin1, the next step was to determine the effect of treating cells with different stimuli that are known to regulate the mTOR pathway: IGF-1 and H_2O_2. For this experiment, cells were transfected with constructs expressing Flag-tagged TRIM7.1 and myc-tagged Sin1 and cells were treated at different time points (5, 15, 30, and 60 minutes) with IGF-1 or H_2O_2 to determine whether the binding of TRIM7.1 to Sin1 changes following a stimulus. Flag-TRIM7.1 was immunoprecipitated from cell lysates with the M2 antibody that recognizes the Flag-tag. Figure 3.2A shows that the binding of TRIM7.1 to Sin1 appears to fluctuate slightly at different time points. However, while there was a trend towards this fluctuation it did not reach statistical significance (Figure 3.2C). The experiment was repeated under the same conditions and time points but stimulated with H_2O_2 instead, and similar to the IGF-1 treatment, there appeared to be some fluctuations in binding between TRIM7.1 and Sin1 binding but this was not statistically significant (Figure 3.2B).

These results suggest that stimuli that activate mTORC2 activity do not significantly affect the binding of Sin1 to TRIM7.

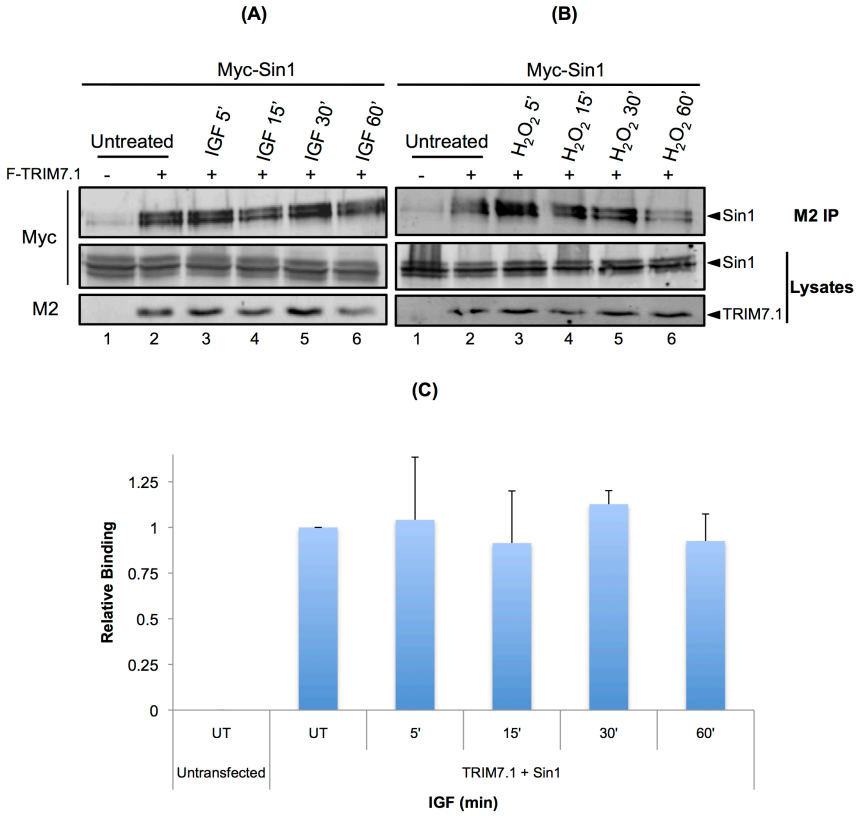


Figure 3.2 **IGF-1 and H_2O_2 do not modulate the binding of TRIM7.1 to Sin-1. (A)** Immunoblot showing the binding of Sin1 to TRIM7.1 following the co-transfection of pcDNA3-myc6-hSin1 and pcDNA3-Flag-TRIM7.1 in COS7 cells treated with 80 ng/ml IGF at the indicated time points. An M2 antibody was used to pull down the Flag-tagged TRIM7.1 *(top panel)* and a slight fluctuation between TRIM7.1/Sin1 binding over the time course is shown. The M2 antibody shows the protein input levels of TRIM7.1 *(bottom)*. **(B)** The same experiment was repeated using 250 μM H_2O_2 instead of IGF-1. **(C)** A bar chart representing the quantification (using Image J) of three repeats of the co-immunoprecipitation between TRIM7.1 and Sin1 at the different time points of IGF stimulation (UT = untreated). There was no significant change in TRIM7.1/Sin1 binding. The error bars represent mean ± standard deviation (SD).

To further characterise the TRIM7-Sin1 interaction, the region of Sin1 that bound to TRIM7 was investigated. To do this a GST pull down experiment was performed in HEK293T cells using GST-tagged Sin1-deletion mutants and a 3xFlag-tagged TRIM7.1 construct. As shown in Figure 3.3, TRIM7.1 interacts with Sin1 towards its C-terminal region between amino acids 372 and 522 (lane 5), which is consistent with findings from the initial yeast two-hybrid screen. The

full-length GST-Sin1 was used as a positive control where the binding between the two proteins had been established (Figure 3.3, lane 1).

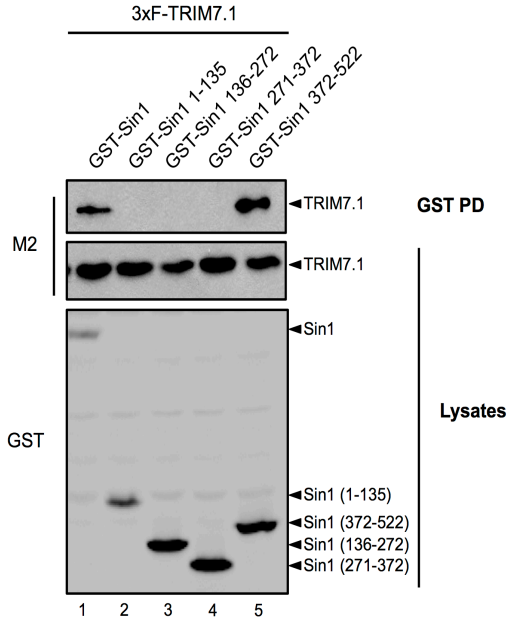


***Figure 3.3* TRIM7.1 binds to the C-terminal region of Sin1.** Immunoblot showing a GST pull down of the co-expressed GST-Sin1 (full length or deletion-mutants) and 3xFlag-TRIM7.1 in HEK293T cells. The top panel shows the pull down with the GSH-agarose beads, whereas both the middle and bottom panels show the expression levels of both F-TRIM7.1 and GST-Sin1 using antibodies against the Flag-tag and the GST-tag, respectively. The pull down panel demonstrates that TRIM7.1 binds to both full length and GST-Sin1 (amino acids 372-522).

The experiments performed so far have used the longest TRIM7 isoform, TRIM7.1. It was therefore of interest to determine if Sin1 could bind to other TRIM7 isoforms (see Figure 1.10). To do this, a GST pull down experiment was performed in HEK293T cells using GST-tagged TRIM7.1, 7.3, and 7.4 isoforms and the myc-tagged Sin1. Like TRIM7.1, TRIM7.3 was also able to interact with Sin1, but the shortest isoforms TRIM7.4 did not show significant binding to Sin1, being similar to GST alone. The binding between TRIM7.3 and Sin1 appeared to be stronger compared to TRIM7.1-Sin1 binding which can be explained by the GST-TRIM7.3 expressing to a higher level than GST-TRIM7.1 (Figure 3.4, compare lanes 2 and 3). These results indicate that there is differential binding amongst TRIM7 isoforms to Sin1 and that Sin1 does not bind to the RING domain or B-box of TRIM7.

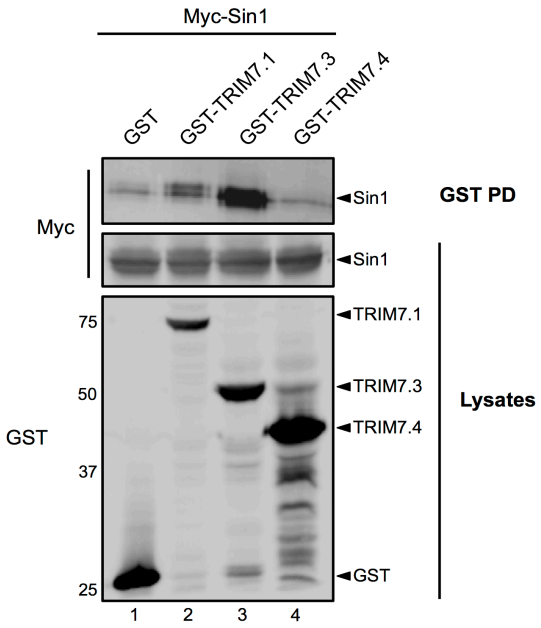


Figure 3.4 **Differential binding of TRIM7 isoforms to Sin1.** Immunoblot showing a GST-pull down of the co-expressed GST-TRIM7 isoforms 1, 3, or 4, and myc6-hSin1 in HEK293T cells. GST alone was used as a negative control. The top panel shows the GST pull down, whereas both the middle and bottom panels show the expression levels of both myc-Sin1 and GST-TRIM7 using antibodies against the myc-tag and the GST-tag, respectively. The pull down shows that in addition to TRIM7 isoform 1, TRIM7.3 binds to Sin1. TRIM7.4 shows no binding.

3.1.3 TRIM7 binds to mTOR

As Sin1 is a core component of the TORC2 complex, it was important to determine if TRIM7 might also be part of this complex. To achieve this, an immunoprecipitation was carried out using a 3x-Flag tagged TRIM7.1 and a HA-tagged mTOR in HEK293T cells. Figure 3.5A shows a weak interaction between TRIM7.1 and mTOR (lane 2) compared to the TRIM7.1-Sin1 interaction that was used as a positive control (lane 3). However, although weak, the TRIM7.1-mTOR interaction was reproducible (data not shown). Moreover, there was a reduction in TRIM7.1 protein levels observed in the presence of mTOR (bottom panel of Figure 3.5A, lane 2). To determine if TOR activity might be causing this reduction in TRIM7.1 protein level, the mTOR inhibitor Torin 1 was included in the experiment to see if it rescued TRIM7 levels. As seen in the left panel of Figure 3.5B, TRIM7.1 levels were significantly reduced in the presence of mTOR but this was not rescued by Torin 1 treatment (lanes 2 and 4). In agreement with this,

a kinase-dead (KD) mutant of mTOR also did not rescue the decrease in TRIM7.1 protein (Figure 3.5B right). The reduced phosphorylation of Akt at S473 was used as a positive control for demonstrating Torin 1 inhibition of mTORC2 activity (Thoreen et al. 2009). The reduced protein level of TRIM7 does not appear to be dependent on mTOR activity and the mechanisms involved remain unclear.

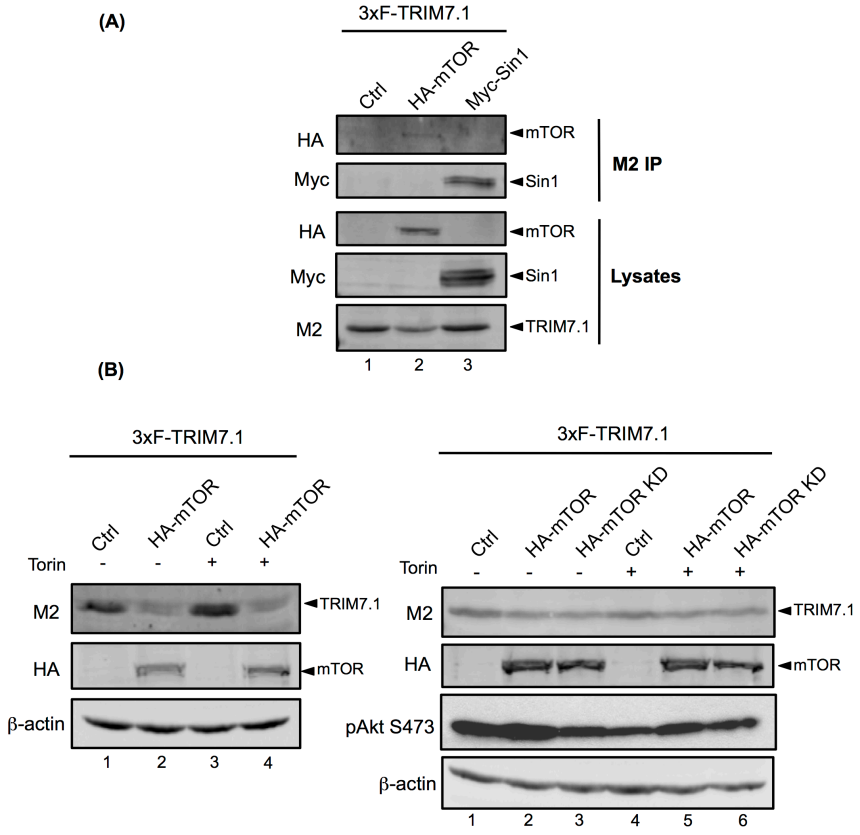


Figure 3.5 **mTOR binds to TRIM7.1. (A)** Immunoblot showing a co-immunoprecipitation between 3xFlag-TRIM7.1 and HA-mTOR in HEK293T cells. myc6-hSin1 was used as a positive control. The M2 antibody was used for the immunoprecipitation and the precipitates blotted with antibodies against both the HA-tag and the myc-tag. The top panel shows that TRIM7.1 binds mTOR. In the control experiment, TRIM7.1 binds to Sin1 *(second panel)*. The bottom three panels show the expression levels of proteins using antibodies against the HA-tag, the myc-tag, and the Flag-tag. TRIM7.1 expression appears to be reduced in the presence of mTOR *(bottom)* compared to the controls. **(B)** mTOR activity does not cause reduced TRIM7.1 protein levels. Blocking mTOR activity through either the mTOR inhibitor Torin 1 or a kinase-dead version of mTOR (KD) (right panel) did not recover the decreased TRIM7.1 levels caused by expression of HA-mTOR . pAkt S473 was used as a positive control for Torin 1 (*right panel*), while β-actin was used as a loading control.

3.1.4 TRIM7 is a phosphoprotein

Having established that TRIM7.1 interacts with two of the major mTORC2 components, we wanted to address whether TRIM7.1 is a phosphoprotein or not, and more importantly, if it is a potential substrate for mTOR or other kinases involved in mTORC2 signalling. To do this, an *in vitro* phosphorylation assay was performed on GST-TRIM7.1 that had been expressed in HEK293T and isolated by GST pull down. The GST-TRIM7.1 was incubated in a reaction mix containing both cold and hot ATP in addition to a kinase buffer. Figure 3.6 demonstrates that a band that correlates with the size of GST-TRIM7.1 is phosphorylated (lane 2). This suggests that TRIM7 associates with a protein kinase from the HEK293T lysate that is capable of phosphorylating it.

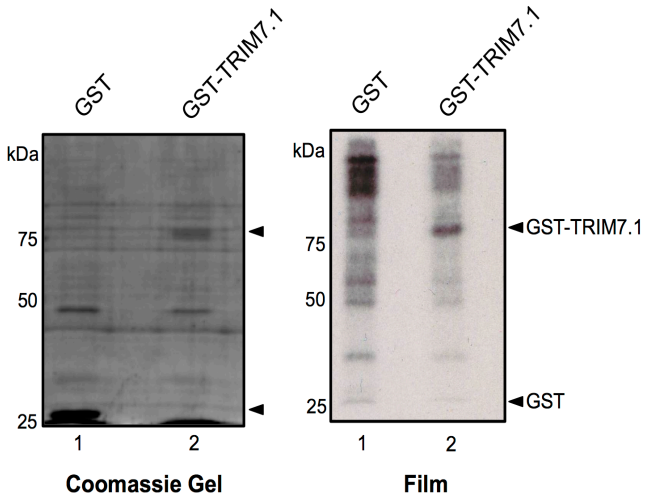


***Figure 3.6* TRIM7.1 is a phosphoprotein.** A 10% SDS-PAGE gel showing a GST pull down of GST or GST-TRIM7.1 in HEK293T cells. The pull-downs were incubated with [γ32P]ATP in a protein kinase reaction mix. GST alone was used as a negative control. The Coomassie-stained gel *(left)* shows the protein levels, whereas the overnight-exposed X-ray film *(right)* shows the phosphorylated proteins (indicated by arrowheads).

Knowing that TRIM7.1 is a phosphoprotein challenged us to determine the kinases responsible. Three potential kinases would be mTOR itself and the mTOR regulated kinases AKT and PKC. *In vitro* kinase assays were carried out to determine if these kinases phosphorylated TRIM7.1. Initial attempts to produce recombinant GST-TRIM7.1 expressed in *E. coli* were unsuccessful due to its lack of solubility. To overcome this, TRIM7.1 was fused to maltose-binding protein

(MBP), that has been shown to enhance the solubility of proteins expressed in *E. coli* (Fox et al. 2003). This allowed for the successful purification of TRIM7.1 as shown in Figure 3.7 (lanes 4-6).

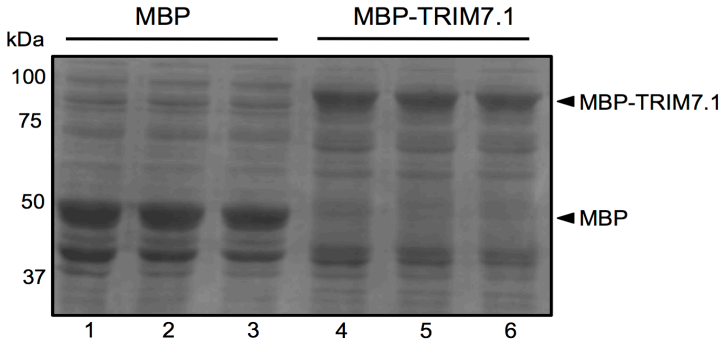


Figure 3.7 **Purification of MBP-TRIM7.1.** A Coomassie-stained 10% SDS-PAGE gel showing the eluted MBP and MBP-TRIM7.1 proteins. MBP was used as a control. *E. coli* strain BL21 DE3 competent cells transformed with plasmids expressing MBP or MBP-TRIM7.1were induced with 0.1 mM IPTG overnight at room temperature. Three fractions of the eluted MBP and MBP-TRIM7.1 proteins were collected by column purification as shown. Proteins were eluted with 10 mM maltose.

The MBP-TRIM7.1 was used as a substrate for the *in vitro* kinase assays (Figure 3.8). Sin1 was used as a positive control for the mTOR kinase assay, while FoxO3a was used as a control for Akt1, and Clk-1 for PKCα (Chen and Sarbassov 2011, Maira et al. 2001, Martin-Montalvo et al. 2011, Whitmarsh unpublished results). mTOR did not phosphorylate MBP-TRIM7.1 above the background level observed for the MBP tag alone, indicating that TRIM7.1 was unlikely to be a target (Figure 3.8, lanes 1 and 2). No phosphorylation of TRIM7.1 was seen with Akt1 but it did phosphorylate its well-characterised substrate FOXO3a. Similarly PKCα did not significantly phosphorylate TRIM7.1 but did phosphorylate the positive control (Figure 3.8, lanes 8 and 9). These findings indicate that TRIM7.1 is unlikely to be a direct substrate for mTOR, Akt1, or PKCα. The kinase(s) that phosphorylated TRIM7 therefore remained elusive so it was decided to focus on other aspects of TRM7 function and regulation.

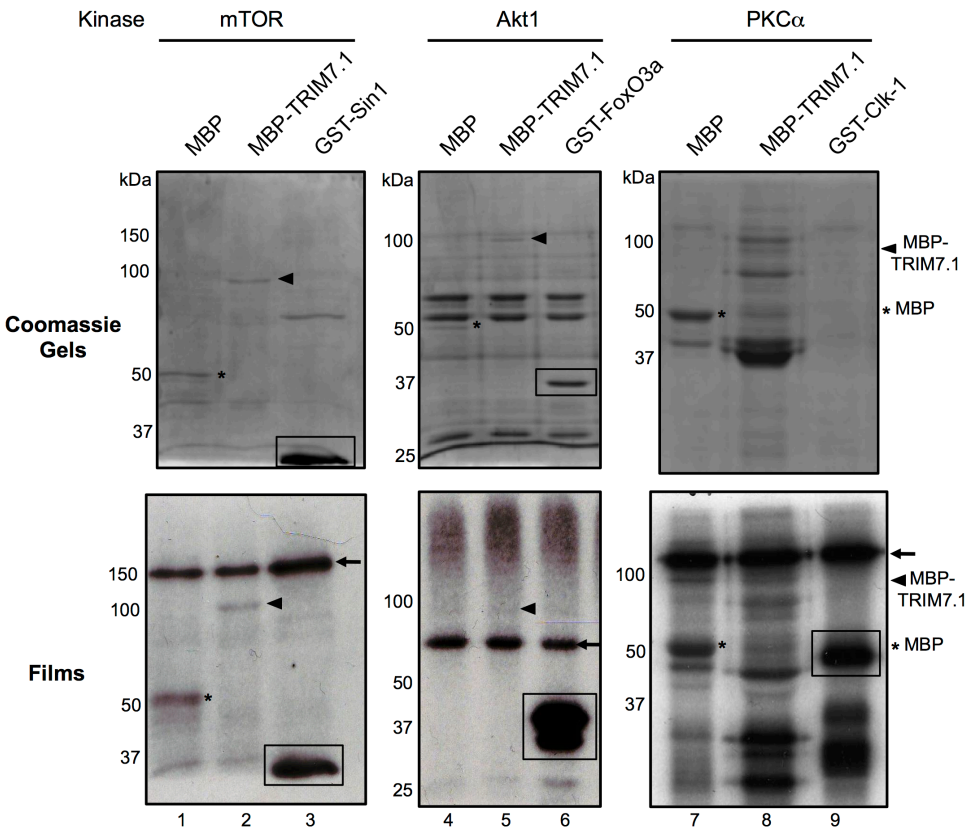


***Figure 3.8* TRIM7 is not a substrate for mTOR, Akt1, or PKCα in vitro.** The purified MBP proteins were used for the *in vitro* kinase assays and run on 10% SDS-PAGE gels following the kinase reactions. MBP was used as a negative control (asterisks), whereas GST-Sin1, GST-FoxO3a, and GST-Clk-1 were used as positive controls for mTOR, Akt1, and PKCα, respectively. The top panels show the protein levels on the Coomassie-stained gels, while the lower panels show the overnight-exposed X-ray films. TRIM7.1 is indicated by the arrowheads, the autophosphorylated kinases are indicated by the arrows, and the positive controls are "boxed". No significant phosphorylation of TRIM7.1 was detected in any of the kinase reactions. However, some degree of TRIM7.1 phosphorylation was observed for the mTOR and PKCα kinase reactions, but the phosphorylation was also observed in the MBP negative controls (lanes 1 and 7, respectively).

3.1.5 TRIM7 binds to other TRIM family members

One of the main features of the TRIM family of proteins is their ability to form homo- and hetero-complexes via their CC domain with members of different classes (Lee et al. 2011). Since TRIM7 possesses a CC domain, we were interested in determining its ability to form complexes with other family members. First, we tested the ability of TRIM7 to form homo-oligomers. HEK293T cells co-expressing GST-tagged TRIM7.1, 7.3, or 7.4 with 3xFlag-TRIM7.1 were subjected for GST pull down. Figure 3.9 demonstrates the ability of TRIM7

isoforms 1 and 3 to form a complex (lane 3). No interaction was observed between TRIM7.1 and TRIM7.4 or with TRIM7.1 itself. However, TRIM7.1 levels appeared to be significantly reduced when both the GST- and 3xFlag-tagged versions were present together making it difficult to conclude whether TRIM7.1 can homo-oligomerise or not.

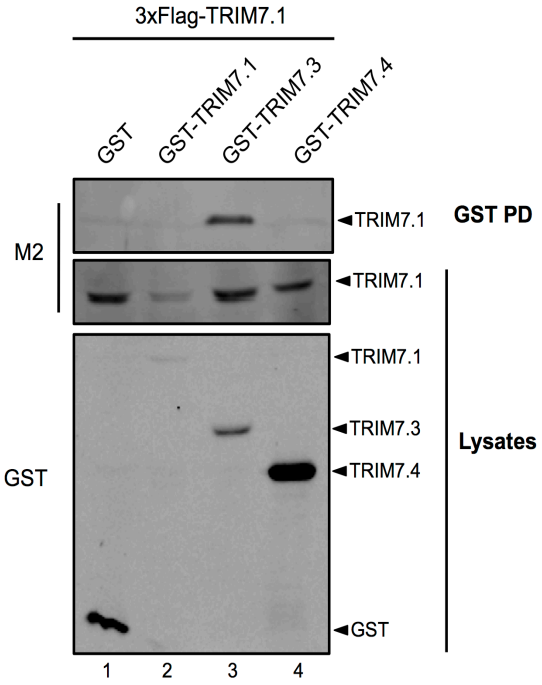


Figure 3.9 **TRIM7 forms homo-oligomers.** Immunoblot showing a GST pull down between GST-TRIM7.1/7.3/7.4 and 3xFlag-TRIM7.1 in HEK293T cells. GST alone was used as a negative control. The top panel shows that TRIM7.1 complexes with TRIM7 isoform 3, but not with isoforms 1 or 4. Both the middle and bottom panels show the expression levels of proteins in the cell lysate using antibodies against the Flag-tag and the GST-tag. However, both expression levels of the 3xFlag-TRIM7.1 and GST-TRIM7.1 are reduced when present together (lane 2).

Having shown the TRIM7 isoforms have the potential to form homo-oligomers, we then tested its ability to form hetero-oligomers with other TRIM family members. To do this, the better characterised TRIM11 and TRIM27 proteins were used as these fall in the same sub-class of TRIM proteins as TRIM7 (Ozato et al. 2008). Co- immunoprecipitation analysis demonstrated that TRIM7.1 was able to interact with both members of its class forming hetero-complexes (Figure 3.10, lanes 2 and 3). These data indicate that TRIM7 is capable of forming homodimers but can also heterodimerise with related TRIM proteins.

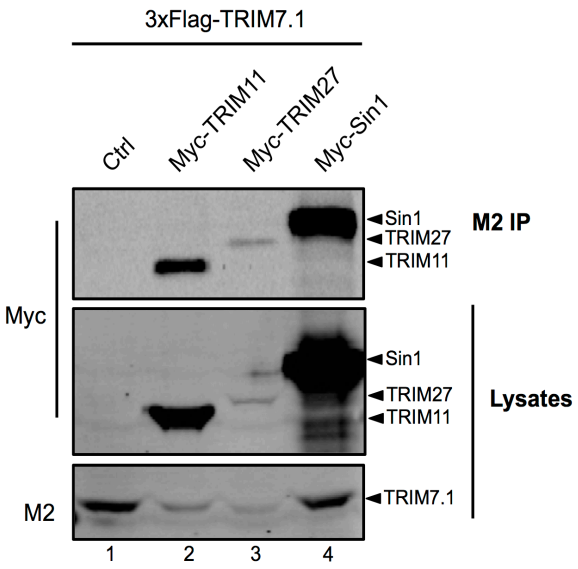


***Figure 3.10* TRIM7 forms hetero-oligomers with TRIM11 and TRIM27.** Immunoblot showing a co-immunoprecipitation between 3xFlag-TRIM7.1 and MycGFP-TRIM11 or TRIM27 in HEK293T cells. An empty vector was used as a negative control, while myc6-hSin1 was used as a positive control for TRIM7.1 binding. The top panel shows that TRIM7.1 complexes with both TRIM11 and TRIM27. Both the middle and bottom panels show the expression levels of proteins using antibodies against the myc-tag and the Flag-tag. TRIM7.1 expression appears to be reduced in the presence of either TRIM11 or TRIM27 *(bottom)* compared to the controls.

Interestingly, a reduction in TRIM7.1 protein levels was observed when it is co-expressed with either TRIM11 or TRIM27 (Figure 3.10, bottom panel). To determine if TRIM11 and TRIM27 were causing a downregulation of TRIM7.1 protein (perhaps via their ubiquitin ligase activity), the transfected cells were treated with MG132, a proteasome inhibitor. MG132 did not rescue the TRIM7.1 protein level but appeared to enhance its loss (Figure 3.11A, lanes 4-6). To test other potential means of TRIM7.1 degradation, another rescue experiment using chloroquine (CQ) was carried out to determine whether TRIM7.1 degradation occurs via the lysosomal pathway. Similarly, no rescue affect was observed upon CQ treatment (Figure 3.11B, lanes 4-6). The conversion of the autophagy marker LC3 from LC3I to LC3II was used as a positive control for CQ treatment (Yoon et al. 2010). It is therefore unclear as to why TRIM11 and TRIM27 affect TRIM7.1 expression.

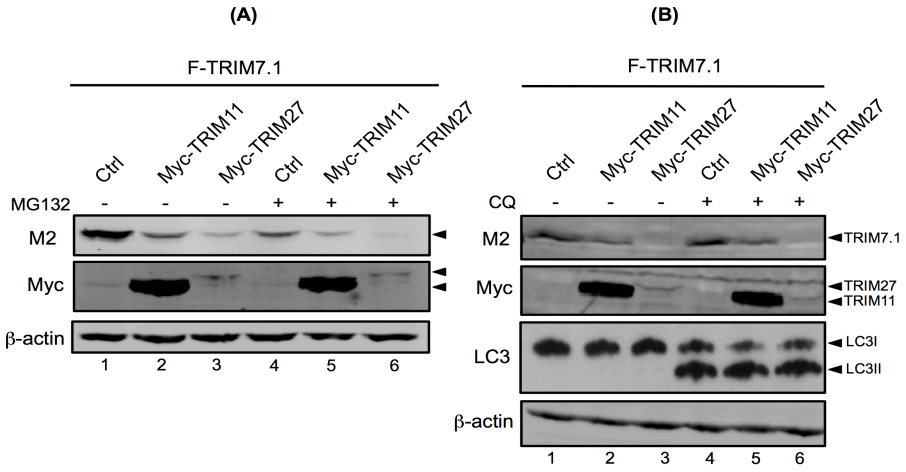


Figure 3.11 **TRIM7.1 is downregulated by TRIM11 and TRIM27. (A)** Immunoblot showing the co-expressed Flag-TRIM7.1 with MycGFP-TRIM11 or TRIM27 in HEK293T cells. An empty vector was used as a negative control. Cells were treated with or without 20 μM MG132 for 4 hours and harvested under normal conditions. Antibodies against the Flag-tag and the myc-tag were used. The top panel shows the expression levels of TRIM7.1 as it decreases in the presence of either TRIM11 or TRIM27. MG132 did not rescue the expression of TRIM7.1. **(B)** The same experiment was repeated using 120 μM chloroquine (CQ) for two hours where no rescue of TRIM7.1 was observed as well. LC3 was used as a positive control for CQ treatment, while β-actin was used as a loading control.

To further investigate the interaction between TRIM7.1 and both TRIM11 and TRIM27, immunofluorescence microscopy was carried out to determine their localisation in cells. Currently little is known about TRIM7.1 localisation, To do this, a construct that expresses GFP fused to the N-terminus of TRIM7.1 was generated which was then transfected into COS7 cells and fluorescence microscopy was performed. The lower panel in Figure 3.12A demonstrates the distribution of the GFP-tagged TRIM7.1 to distinct dot-like structures spread throughout the cytoplasm compared to the negative control that expresses GFP alone (top panel). In order to ensure that the cytoplasmic localisation of TRIM7.1 was not affected by the GFP tagging, COS7 cells were transfected with the Flag-tagged TRIM7.1 instead and stained with the anti-Flag M2 antibody. The localisation appears to be consistent with the results from the GFP-tagging, confirming that TRIM7.1 is cytoplasmic (Figure 3.12B). The experiment was repeated in HeLa cells to demonstrate the consistency of the GFP-TRIM7.1 staining pattern in different cell types and similar results were obtained (Figure 3.12C). It was then determined whether TRIM7 co-localised with TRIM11 or

TRIM27 in cells. The TRIM protein PML was used as a negative control as studies have shown its exclusive localisation to the nucleus (Regad et al. 2001). TRIM7.1 extensively co-localised with TRIM27 and partially with TRIM11, which also displayed some nuclear localization. PML was completely nuclear, thus, did not co-localise with TRIM7.1. These data support the binding experiments that show complex formation between TRIM7.1 and both TRIM11 and TRIM27 (Figure 3.13).

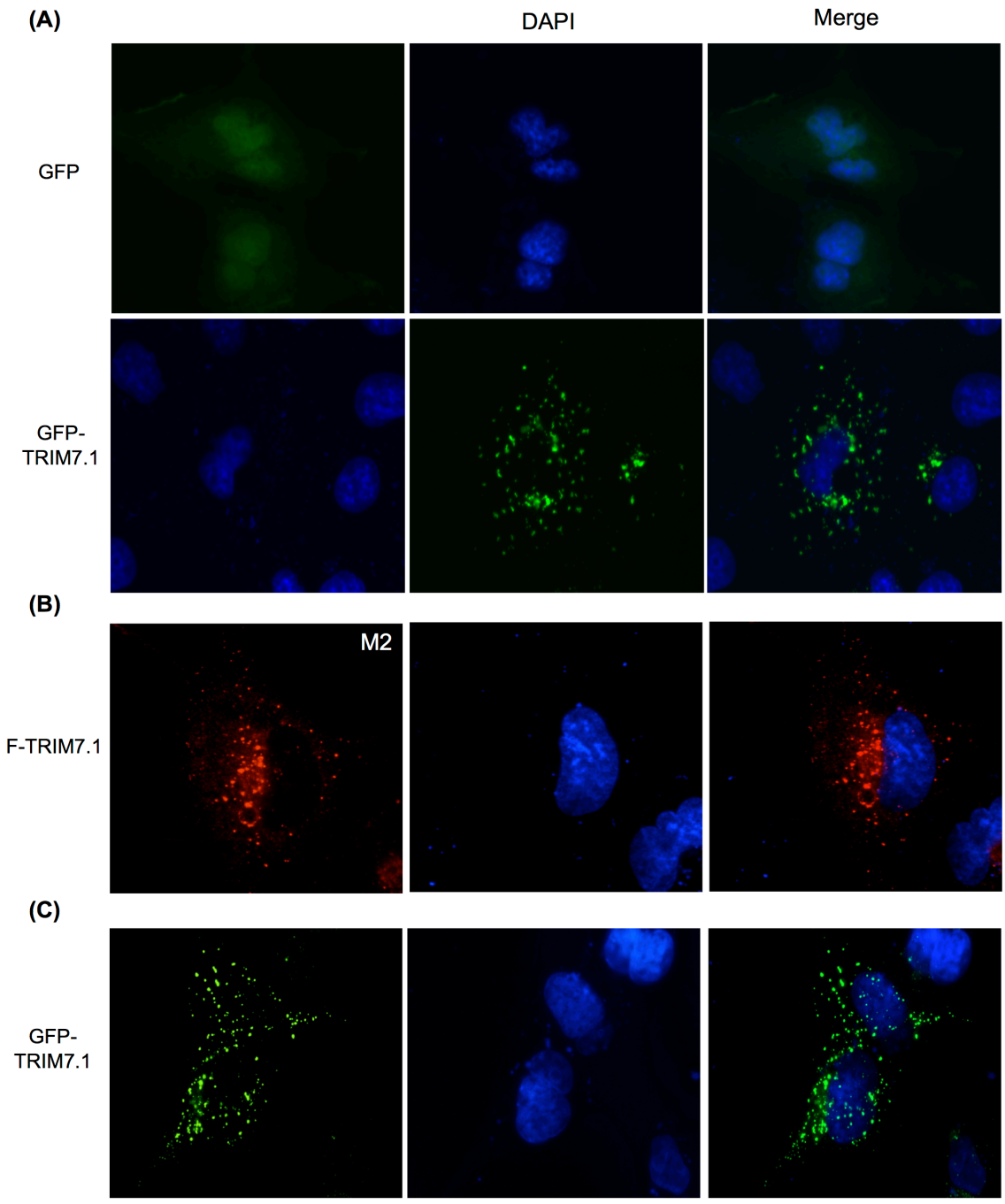


Figure 3.12 **Localisation of TRIM7.1 in COS7 and HeLa cells. (A)** COS7 cells were transfected with pEGFP-C2 and pEGFP-C2-TRIM7.1. The top panel shows the GFP negative control whereas the lower one shows the cytoplasmic localisation of the GFP-tagged TRIM7.1 (green). **(B)** COS7 cells were also transfected with pcDNA3-Flag-TRIM7.1 and stained with the M2 primary antibody (1:500) (red) confirming the cytoplasmic localisation of TRIM7.1. **(C)** GFP-TRIM7.1 was also expressed in HeLa cells to ensure the consistency of TRIM7.1 localisation in different cell lines. DAPI was used for nuclear staining (blue).

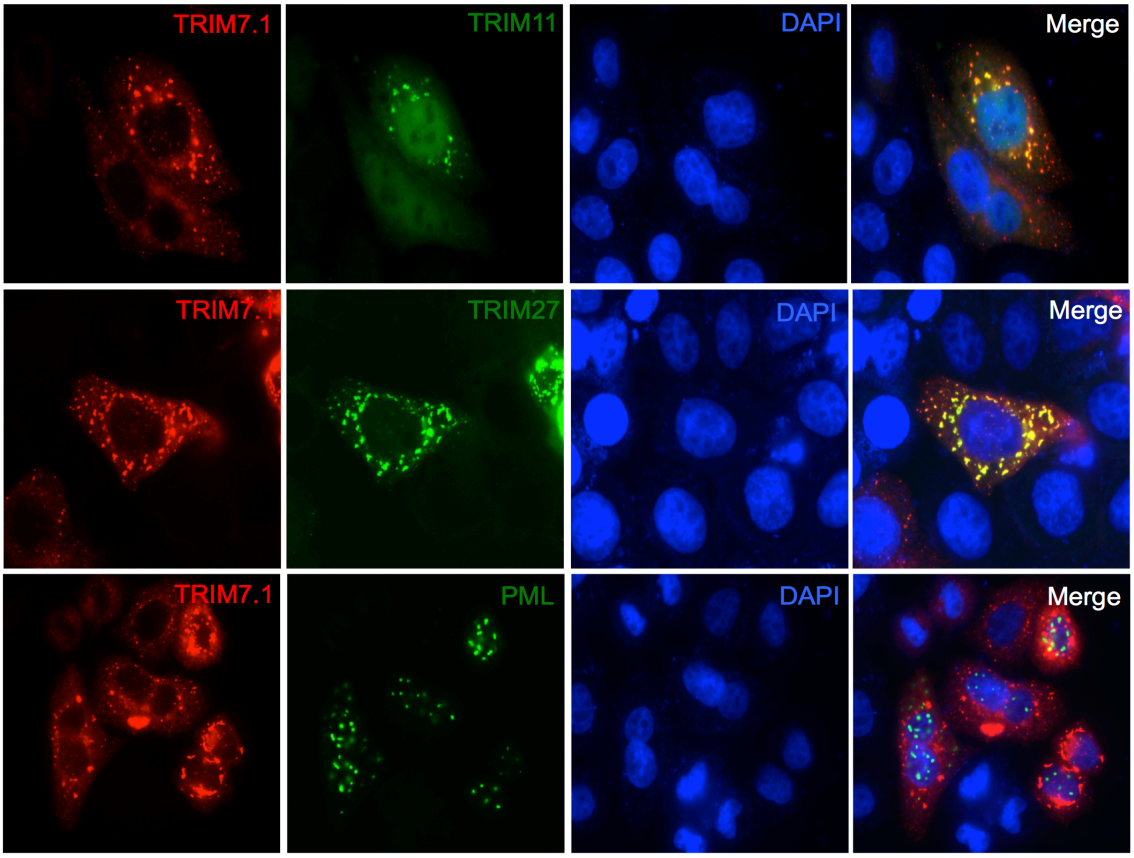


Figure 3.13 **Co-Localisation of TRIM7.1 with TRIM11 and TRIM27.** HeLa cells were co-transfected with pcDNA3-Flag-TRIM7.1, pcDNA3-MycGFP -TRIM11, pcDNA3-MycGFP-TRIM27, and pYFP-PML. The M2 primary primary antibody was used (1:500) to stain TRIM7.1 (red). TRIM11 *(top)* shows a similar cytoplasmic pattern as TRIM7.1 and partially co-localises with it, but also displays some nuclear staining. TRIM27 *(middle)* extensively co-localises with TRIM7.1 into cytoplasmic bodies throughout the cell. PML *(bottom)* displayed a nuclear staining pattern and did not co-localise with TRIM7.1. DAPI was used for nuclear staining (blue).

The dot-like cytoplasmic pattern of TRIM7.1 seemed to be specific to certain areas of the cells. We were intrigued to determine whether TRIM7.1 might co-localise with cellular organelles that show somewhat similar staining patterns, including mitochondria and endosomes. GFP-TRIM7.1 expressing cells were stained with mitotracker (mitochondrial marker) but there was no overlap in the staining patterns (data not shown). Endosomal staining was also tested for co-localisation with TRIM7.1. There are three main classes of endosomes each with their specific markers; early, recycling, and late endosomes (Sadowski et al. 2009). COS7 cells transfected with a construct expressing GFP-TRIM7.1 were stained with EEA1, an early endosomal marker (Figure 3.14A), transferrin receptor (TR), a recycling endosomal marker (Figure 3.14B), and Lamp1, a late endosomal marker (Figure 3.14C). None of the endosomal markers significantly co-localised with TRIM7.1 as they appeared to be more abundant, smaller in size, and dispersed throughout the cell. Both EEA1 and Lamp1 displayed similar staining patters as they appeared to be spread evenly throughout the cytoplasm, whereas the transferrin receptor staining mainly focused around the nucleus. To confirm the results, the same experiments were repeated in HeLa cells and similar fluorescent staining was obtained (data not shown). It therefore remains unclear as to the nature of the cytoplasmic bodies that contain TRIM7.1.

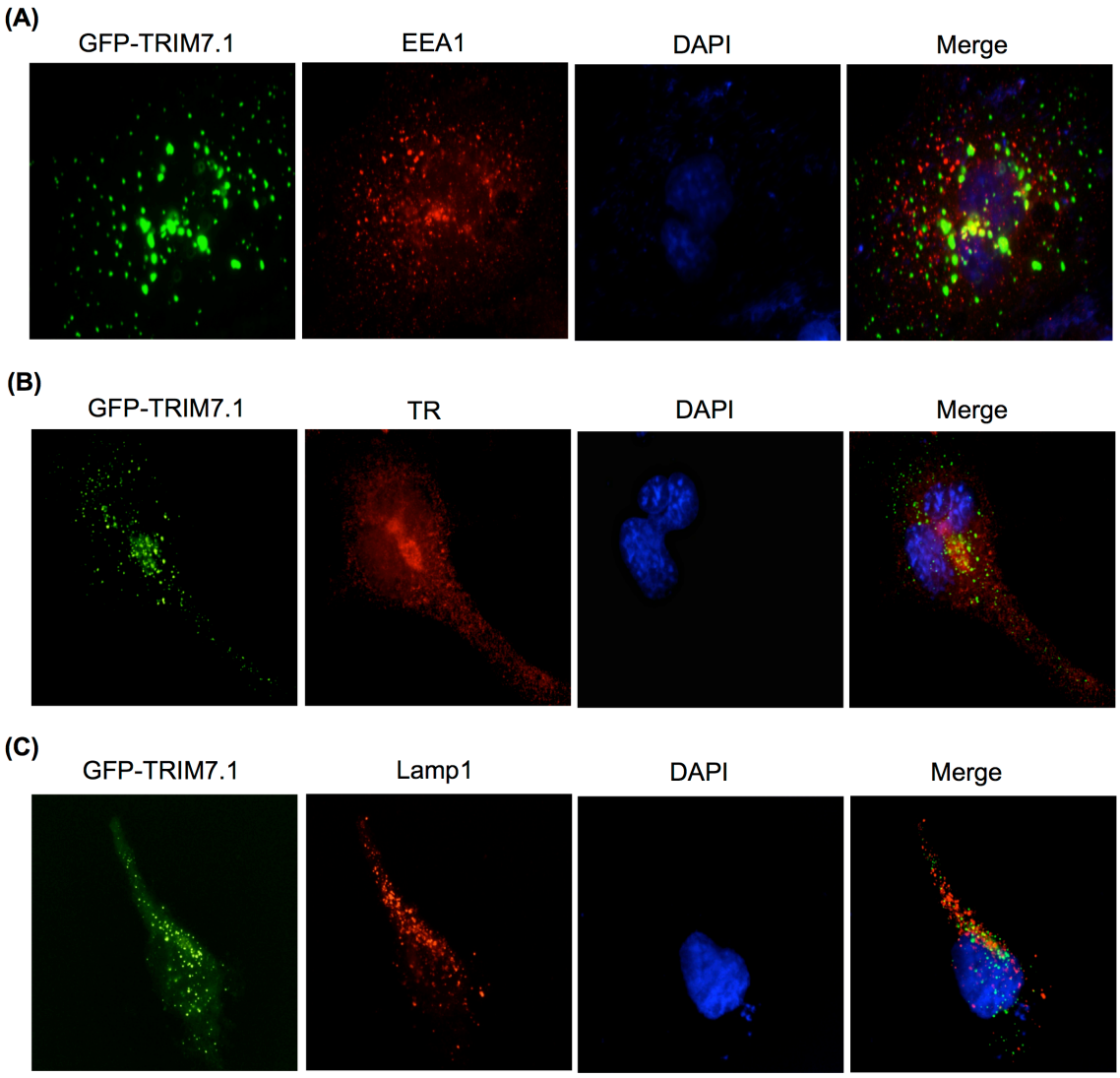


Figure 3.14 **TRIM7.1 does not co-localise with endosomes.** There was no co-localisation between the GFP-tagged TRIM7.1 and the endosomal markers (red) tested in COS7 cells. EEA1 **(A)** and Lamp1 **(C)** staining are dispersed throughout the cytoplasm while transferrin receptor (TR) staining **(B)** is mainly focused around the nucleus. Both EEA1 and Lamp1 were used at a dilution of 1:250 made in 0.1% (v/v) PBS/Triton-X whereas transferrin receptor was used at a dilution of 1:50. Alexa® Fluor^{594}-conjugated secondary antibody was used at a dilution of 1:500 for all markers. DAPI was used for nuclear staining (blue).

3.1.6 TRIM27 binds to Sin1

As TRIM7 can form complexes with TRIM11 and TRIM27, it was determined if, like TRIM7, they could bind to Sin1 as well. To achieve this, 3xFlag-tagged TRIM11 or TRIM27 were co-expressed with myc-Sin1 in HEK293T cells. The 3xFlag-TRIM7.1 was used as a positive control for Sin1 binding. Following immunoprecipitation of the TRIM proteins it was evident that, like TRIM7.1, TRIM27 was able to interact with Sin1 (Figure 3.15, lane 3). Both TRIM7.1 and TRIM27 showed similar binding capabilities towards Sin1. No interaction between TRIM11 and Sin1 was observed. This suggests that Sin1 may be able to bind certain TRIM family members in addition to TRIM7.

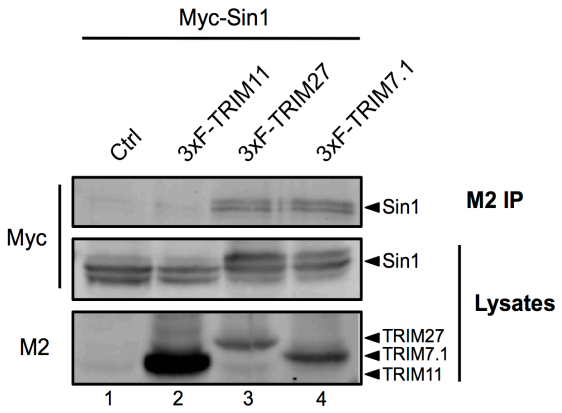


***Figure 3.15* TRIM27 binds to Sin1.** Immunoblot showing a co-immunoprecipitation analysis of myc6-Sin1 binding to 3xFlag-TRIM11 or 3xFlag -TRIM27 in HEK293T cells. An empty vector was used as a negative control, whereas binding of myc6-Sin1 to 3xFlag-TRIM7.1 was used as a positive control. The M2 antibody was used for the immunoprecipitation shown in the top panel, whereas both the middle and bottom panels show the expression levels of both myc-Sin1 and the 3xF-tagged proteins in the cell lysate using antibodies against the myc- and the Flag-tags, respectively. TRIM27, but not TRIM11, binds to Sin1 at levels similar to Sin1 binding to TRIM7.1.

3.1.7 Conclusion

My data demonstrate the binding of TRIM7 to the mTORC2 component Sin1 in mammalian cultured cells (Figure 3.1), which is consistent with the yeast two-hybrid screen data from the Whitmarsh lab (unpublished data). Furthermore, TRIM7 interacted with the C-terminal region of Sin1 that includes the PH domain, but lies outside of the highly conserved central CRIM domain (see Figure 1.7). The CRIM domain has been demonstrated to bind to PKC and has been

proposed to also bind AKT (Cameron et al. 2011), while the N-terminus of Sin1 is required for its recruitment into mTORC2 (Frias et al. 2006). The PH domain of Sin1 is reported to mediate its association with membranes (Schroder et al. 2007) but as Sin1/mTORC2 is found throughout the cell it remains unclear how this association is regulated. The binding of TRIM7 to the C-terminal PH domain-containing region suggests that it could affect membrane recruitment. In addition, as it does not bind to Sin1 regions required for mTORC2 assembly and AGC family kinase recruitment, it suggests that TRIM7 could also be recruited to active mTORC2 complexes. Indeed, mTOR was observed in TRIM7.1 immunoprecipitates (Figure. 3.5). The TRIM7.1-Sin1 binding affinity was not affected by stimuli that are known to regulate mTORC2 signalling. However, the lack of a positive control for both the IGF-1 and H_2O_2 stimulation (e.g. pAkt S473) makes it hard to completely eliminate any possible effects on the TRIM7.1-Sin1 interaction. In addition, not only the full length TRIM7.1 binds Sin1, the N-terminal truncated TRIM7.3 isoform has also been demonstrated to complex with Sin1 suggesting that the C-terminal region of TRIM7 is required for Sin1 binding. This was supported by the fact that no interaction was observed with TRIM7.4 that exhibits a truncated C-terminus (Figure 3.9). Despite Sin1 interacting with both TRIM7.1 and TRIM7.3, we cannot conclude that Sin1 has a higher affinity towards TRIM7.3 as opposed to TRIM7.1 due to them being expressed at different levels and the absence of a loading control. While TRIM7 associates with mTORC2 components and is a phosphoprotein (Figures 3.1 and 3.5A), it was not a direct substrate for any of the main kinases involved in the mTORC2 pathway (i.e. mTOR, Akt1, or PKCα). This suggests that distinct signalling pathways may regulate TRIM7 function and that TRIM7 may integrate these pathways to impact on mTORC2 activity. It is also possible that the third AGC kinase that is targeted by mTORC2, SGK1, might phosphorylate TRIM7, but this was not tested.

Other possible approaches to further characterise the phosphorylation of TRIM7 and potential upstream kinase need to be considered. For instance, performing mass spectrometry on the phosphorylated GST-TRIM7.1 would allow the

determination of potential phosphorylation sites. Using open source phospho peptide databases and kinase panels would further allow the understanding of TRIM7 phosphorylation and potential upstream kinase based on a wide array of screens performed on different proteins and kinases.

Like other better characterised TRIM proteins (e.g. TRIM5 and TRIM27) (Li et al. 2007a, Chu and Yang 2011), TRIM7 is also able to form (i) homo-oligomers with its different isoforms and (ii) hetero-complexes with other members of its class that co-localise into cytoplasmic loci in the cells (Figures 3.10 and 3.13). The nature of these loci is unclear as they did not co-localise with cytoplasmic organelles such as mitochondria or endosomes (Figure 3.14). Interestingly, Sin1 can bind to TRIM27, in addition to TRIM7 (Figure 3.15). Whether this is direct binding or as part of a TRIM7-TRIM27 complex is not clear. However, it indicates that mTORC2 may be targeted by additional class IV TRIM proteins.

3.2 TRIM7.1 Possesses E3 Ligase Activity

3.2 TRIM7.1 Possesses E3 Ligase Activity

3.2.1 Introduction

The TRIM family of proteins are the largest group of proteins that exhibit a RING domain (Reymond et al. 2001). Many of the TRIM proteins that have been studied that possess the RING domain have ubiquitin E3 ligase activity allowing them to either ubiquitinate themselves or target other substrates for degradation or for processes involved in cellular trafficking (Weissman et al. 2011). This chapter aims to shed some light on the potential role of TRIM7 as an ubiquitin E3 ligase since it contains a RING domain. Both *in vivo* and *in vitro* ubiquitination assays were carried out to clarify this and to determine if TRIM7 has mono- or poly-ubiquitinating activity. This is crucial for understanding its role as a modulator for protein trafficking or degradation. Testing TRIM7 as a potential SUMO E3 ligase was also performed as Chu and colleagues have shown recently the potential role of some TRIM proteins in possessing dual ubiquitin and SUMO E3 ligase activities (Chu and Yang 2011).

3.2.2 Autoubiquitination of TRIM7.1 is dependent on its RING domain

RING domains have a consensus sequence (Cys-X_2-Cys-X_{9-39}-Cys-X_{1-3}-His-X_{2-3}-Cys-X_2-Cys-X_{4-48}-Cys-X_2-Cys) that exhibit seven conserved Cystine residues and one conserved Histidine residue that together form two zinc finger motifs (Saurin et al. 1996). Other studies have shown that disrupting the zinc motif affects the overall E3 ligase activity of the protein (Yamauchi et al. 2008). In alignment with other RING-containing TRIM proteins, TRIM7.1 possesses the same RING-consensus sequence between amino acid residues 29 and 81. A double mutant of TRIM7.1 was generated that exhibited two point mutations leading to replacement of the conserved third Cysteine and the Histidine residue of the RING domain with Alanine. This would be predicted to compromise zinc binding and block the E3 ligase activity. A second mutant lacking the entire RING domain was also generated. A Flag-tagged version of this double mutant (C44A/H46A), the RING-deleted mutant, or wild type Flag-TRIM7.1 were expressed in

HEK293T cell in the absence or presence of His-tagged ubiquitin followed by Ni^{+}-NTA pull down under denaturing conditions. Cells were treated with the proteasome inhibitor MG132 to allow the accumulation of ubiquitin-targeted molecules. The denaturing conditions prevent the action of de-ubiquitinases. Figure 3.16 demonstrates that wild-type TRIM7.1 is ubiquitinated in cells as shown by a dark smear of ubiquitinated proteins that is not present in the absence of ubiquitin (lane 4, starting at the 50 kDa marker upwards). A slight reduction in TRIM7.1 ubiquitination was observed with the double mutant (lane 6), whereas a significant depletion in its ubiquitination was shown in the case of the RING-deleted mutant (lane 8). A TRIM7.1 single mutant (C44A) was used as well that showed similar effects as the double mutant (data not shown). The pattern of the TRIM7.1 smear indicates a strong signal for single TRIM7.1 band migrating more slowly than the predicted size of TRIM7.1 and a smear above this. These findings demonstrate that TRIM7.1 can be ubiquitinated and suggest that it may be autoubiquitination that is dependent on its RING finger. However, it was unclear why the double mutant (C44A/H46A) did not significantly affect TRIM7.1's autoubiquitination. It suggests that these mutations have not disabled the RING domain.

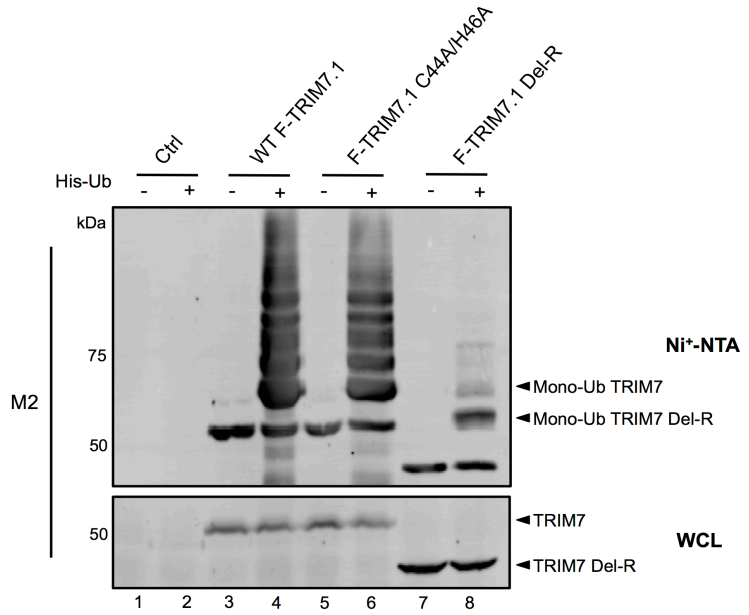


***Figure 3.16* TRIM7.1 ubiquitination in cells.** Immunoblot showing a Ni^{+}-NTA pull down where wild-type, double mutant (C44A/H46A), and deletion-mutant (Del-R) Flag-TRIM7.1 were co-expressed with 6xHis-Ubiquitin in HEK293T cells. An empty vector was used as a negative control (Ctrl). Cells were treated with 20 μM MG132 for 4 hours and harvested under denaturing conditions. The top panel indicates the pull down were a smear of ubiquitinated proteins was observed. The smear was slightly reduced compared to wild-type when the TRIM7.1 double mutant was expressed with ubiquitin, while a significant reduction of the smear was observed in the case of the RING-deleted mutant of TRIM7.1. The arrowheads on the top panel indicated the single TRIM7.1 bands conjugated with ubiquitin (mono-ubiquitin), non-ubiquitinated TRIM7.1 runs just above the 50 kDa marker. The bottom panel shows the whole cell lysate (WCL) protein expression levels using the M2 antibody.

3.2.3 TRIM7.1 is monoubiquitinated in cells and in vitro

The appearance of a strong single TRIM7.1 band that migrates more slowly on a gel in the presence of ubiquitin suggests that TRIM7 may be monoubiquitinated (Figure 3.16). The size difference would fit with a single ubiquitin being conjugated to TRIM7.1. To address this, the experiment was repeated using the wild-type 3xFlag-TRIM7.1 expressed with either wild-type (WT) or mutant His-tagged ubiquitin. For the ubiquitin mutants, both ubiquitin K48R and K7R were used. The ubiquitin K48R mutant prevents the ubiquitin molecule from forming poly-ubiquitin chains on residue 48 as the Lysine has been substituted with an Arginine. The ubiquitin K7R mutant prevents the formation of poly-ubiquitin chains on any of its seven Lysine residues as they have all been mutated to Arginine. Figure 3.17A shows no significant difference in the TRIM7.1-

ubiquitinated smear pattern between wild-type and K48R ubiquitin as the main single ubiquitinated TRIM7.1 band was present in both cases as was the smear (lanes 3 and 4). When the ubiquitin K7R mutant was used (Figure 3.17B), there was a reduction in the smear intensity although there was a similar band pattern as with the wild-type ubiquitin (lanes 3 and 4). The reduction in the smear intensity with the ubiquitin K7R mutant was reproducible. These findings indicate that TRIM7.1 is mainly monoubiquitinated and the smearing pattern suggests that monoubiquitination is occurring at multiple sites. There may also be polyubiquitination but this would not appear to be K48-linked polyubiquitination.

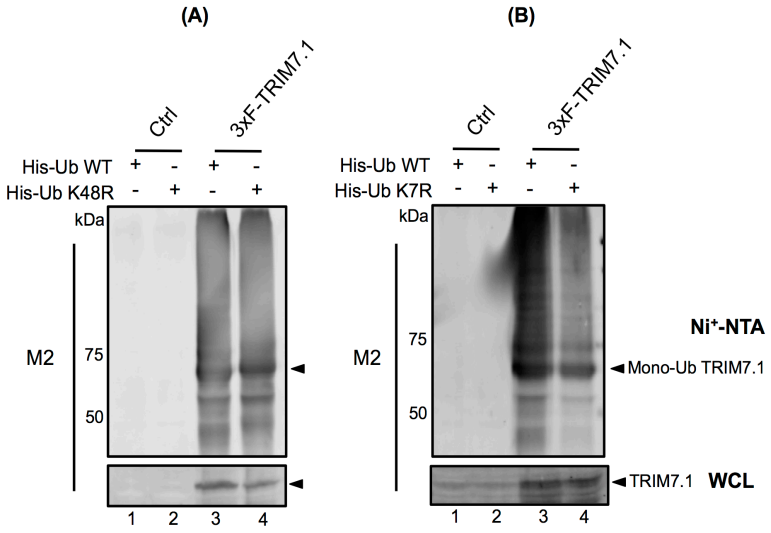


Figure 3.17 **TRIM7.1 is monoubiquitinated in cells.** Immunoblots showing Ni^{+}-NTA pull downs where wild-type (pM107-6xHis-Ubiquitin) and mutant His-ubiquitin (pcDNA3-6xHis-UbiquitinK48R/K7R) were co-expressed with 3xFlag-TRIM7.1 in HEK293T cells. An empty vector was used as a negative control (Ctrl). Cells were treated with 20 μM MG132 for 4 hours and harvested under denaturing conditions. **(A)** The top panel indicates the pull down were a smear of ubiquitinated TRIM7.1 was observed in the presence of both wild-type and ubiquitin K48R mutant. The bottom panel shows the WCL protein expression levels using the M2 antibody. The same experiment was repeated using ubiquitin mutant K7R **(B)**. No significant difference in the ubiquitination pattern of TRIM7.1 was observed with both ubiquitin mutants compared to the wild-type, but a reduction in the smear intensity was observed with the ubiquitin K7R mutant. The arrowheads indicate the monoubiquitinated TRIM7.1 *(top)* and the TRIM7.1 expression levels *(bottom)*.

The monoubiquitination of proteins, unlike K48-linked polyubiquitination, is not thought to target them for degradation (Weissman et al. 2011). To provide further support that TRIM7.1 is monoubiquitinated, wild-type and RING-deleted Flag-tagged TRIM7.1 were expressed in HEK293T cells which were treated with or

without MG132 and their protein levels monitored. We hypothesized that monoubiquitinated TRIM7.1 should not be targeted for proteasomal degradation and that treating with MG132 would have no effect on stabilizing TRIM7.1 since a single ubiquitin molecule acts as a signal for cellular trafficking rather than being targeted for degradation. Indeed, MG132 treatment did not stabilize wild-type TRIM7.1 nor its deletion mutant (Figure 3.18, lanes 4 and 6). However, a slight reduction in their expression levels was observed in the presence of MG132.

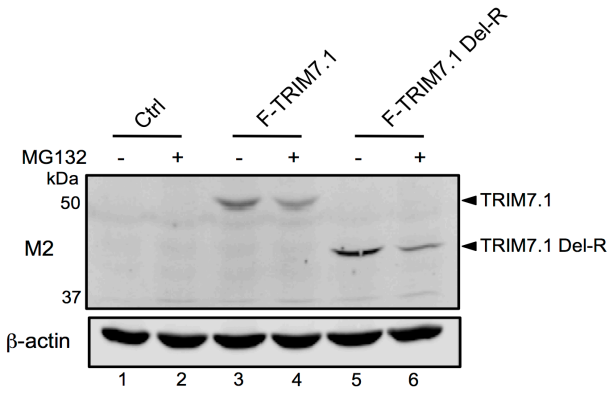


Figure 3.18 **MG132 does not stabilise TRIM7.1.** Immunoblot showing the expression of Flag-TRIM7.1 and its RING-deleted mutant in HEK293T cells. An empty vector was used as a negative control (Ctrl). Cells were treated with 20 μM MG132 for 4 hours and harvested under normal conditions. The top panel shows that in the presence of MG132, both wild-type and Del-R TRIM7.1 expression levels were slightly reduced rather than being stabilized. β-actin was used as a loading control.

Knowing that the RING domain is crucial for ubiquitination of TRIM7.1 we were interested in determining whether the point and deletion mutants had any effect on TRIM7.1 cellular localisation. To clarify this, wild-type, single (C44A) and double (C44A/H46A) mutants, or the RING deleted mutant Flag-TRIM7.1 were expressed in HeLa cells. As shown in Figure 3.19A, the RING mutants exhibited less dot-like structures compared to wild-type TRIM7.1. However, the TRIM7.1 mutants displayed altered peri-nuclear staining patterns rather than the punctate cytoplasmic staining seen with the wild-type. Similar aggregations around the nucleus was observed when wild-type TRIM7.1 was treated with MG132 shown in Figure 3.19B. These findings suggest that mutating or deleting the RING

domain of TRIM7.1, or the addition of a proteasome inhibitor affects the localisation pattern of TRIM7.1

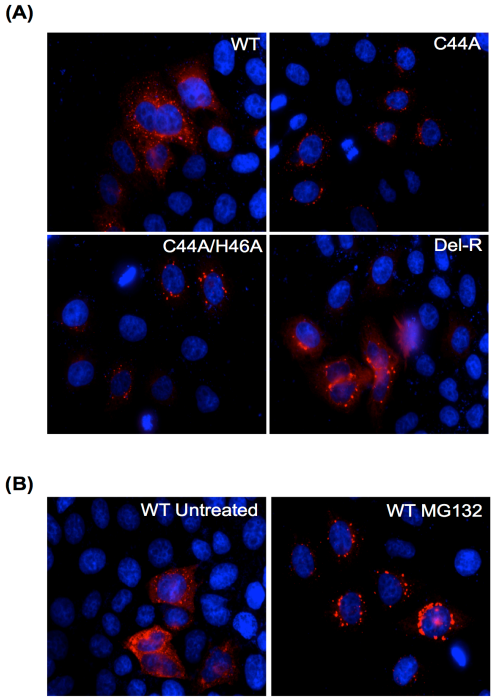


Figure 3.19 **Cellular localisation of TRIM7.1 RING domain mutants. (A)** HeLa cells expressing wild-type Flag-TRIM7.1, single (C44A) or double (C44A/H46A) mutants, or the Del-R mutant are shown. The M2 primary antibody was used (1:500) to stain TRIM7.1 (red). **(B)** When wild-type TRIM7.1 was treated with 20 μM MG132 for 4 hours, TRIM7.1 localisation went from being spread throughout the cytoplasm to peri-nuclear. DAPI was used for nuclear staining (blue).

Having established that TRIM7.1 is ubiquitinated in cells and our data suggesting that this, at least in part, may be being mediated by TRIM7 itself, it was important to perform *in vitro* ubiquitination assays to formally demonstrate that TRIM7 could autoubiquitinate. MBP-TRIM7.1 expressed and purified from bacteria was used for this experiment with or without recombinant E1 and UbcH5A (E2 class D) enzymes in the presence of His-tagged ubiquitin. MBP alone was used as a negative control, while MBP-TRIM11 was used as a positive control as it has been previously shown to autoubiquitinate *in vitro* (Napolitano et al. 2011). Figure 3.20 shows the accumulation of ubiquitin on both TRIM7.1 and TRIM11 when both E1 and UbcH5A were present as indicated by the smear (lanes 4 and 6). This suggests that TRIM7.1 can autoubiquitinate both in cells and *in vitro*.

However, it is difficult to conclude from the *in vitro* assay whether TRIM7.1 monoubiquitinates at multiple sites or polyubiquitinates based on the slow-migrating ubiquitinated smear.

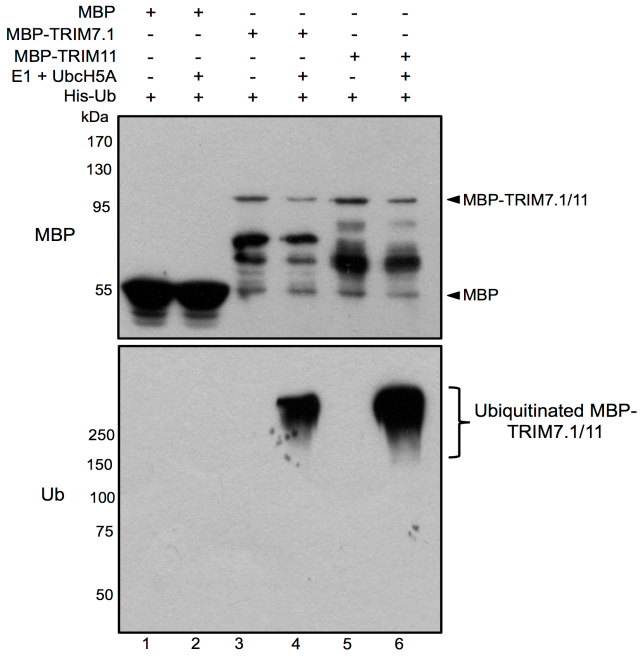


***Figure 3.20 In vitro* autoubiquitination of TRIM7.1.** Immunoblots showing the MBP-TRIM7.1 *in vitro* ubiquitination reaction. MBP was used as a negative control, while MBP-TRIM11 was used as a positive control. The *in vitro* reaction consisted of the MBP-proteins with or without the E1 and E2 (UbcH5A) enzymes in the presence of ubiquitin and was left for 90 minutes at 37^{o}C. The top panel shows an immunoblot against MBP where both MBP-TRIM7.1 and MBP-TRIM11 protein levels were reduced in the presence of the E1 and UbcH5A enzymes. The bottom panel shows the samples blotted against ubiquitin and a smear of autoubiquitinated TRIM7.1 and TRIM11 was observed as indicated migrating slower on the gel.

3.2.4 Modulation of TRIM7.1 ubiquitination

After establishing that TRIM7.1 is an ubiquitin ligase and can form complexes with Sin1, TRIM11, and TRIM27 (see Figures 3.4 and 3.10), we wanted to investigate what effects these binding partners might have on TRIM7.1 ubiquitination. First, Sin1 was tested to monitor its effect on TRIM7.1 ubiquitination in cells. Flag-tagged TRIM7.1 was co-expressed with or without myc-Sin1 in the presence of His-tagged ubiquitin in HEK293T cells. Figure 3.21 shows that no significant changes in the TRIM7.1 ubiquitination pattern was observed in the presence of Sin1 compared to when TRIM7.1 was expressed alone with ubiquitin (first panel, lanes 2 and 5). However, a slight increase in the

smear intensity was observed in the presence of Sin1. Moreover, when the pull down samples were blotted against the myc-tag, Sin1 appeared to be targeted for ubiquitination in the presence of ubiquitin shown by a smear running higher on the gel compared to when expressed alone (second panel, lane 4). The intensity of the Sin1 smear was slightly increased in the presence of TRIM7.1 (second panel, lane 5). These findings suggest that TRIM7.1 might have a potential role in ubiquitinating Sin1.

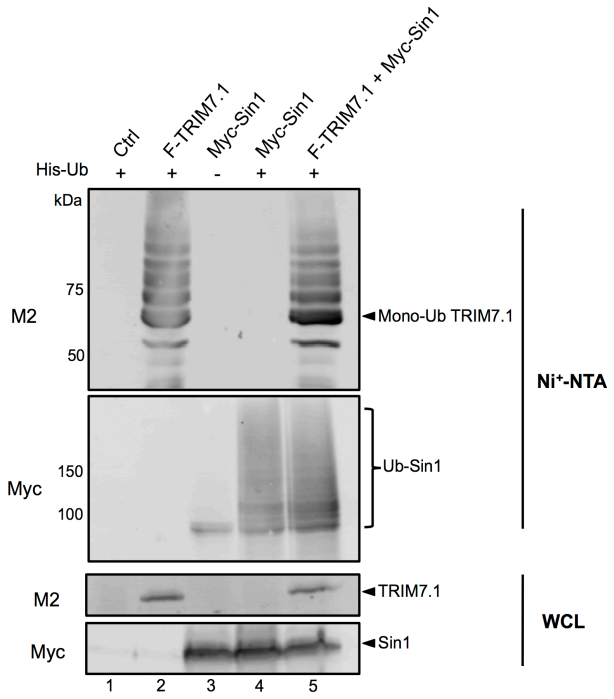


Figure 3.21 **TRIM7.1 may modulate Sin1 ubiquitination.** Immunoblots showing a Ni^{+}-NTA pull down where Flag-TRIM7.1 was co-expressed with or without myc6-hSin1, and 6xHis-Ubiquitin in HEK293T cells. An empty vector was used as a negative control. Cells were treated with 20 μM MG132 for 4 hours and harvested under denaturing conditions. The top pull down panel shows the autoubiquitinated TRIM7.1 smear. No significant change in the smear was observed in the presence of Sin1. However, the second pull down panel shows the ubiquitination of Sin1 in the presence of ubiquitin that was slightly increased in the presence of F-TRIM7.1. The lower two panels show the WCL protein expression levels were both M2 and anti-myc antibodies were used.

A similar experiment was carried out using the myc-tagged TRIM11 and either wild-type or RING-deleted 3xFlag-tagged TRIM7.1 in the presence of ubiquitin. As in the case of Sin1, TRIM11 had no significant effect on TRIM7.1 ubiquitination shown in Figure 3.22 (lane 3). However, TRIM11 slightly increased the ubiquitination levels of the RING-deleted TRIM7.1 mutant (lane 4). In

addition, the protein levels of the RING-deleted TRIM7.1 mutant were reduced in the presence of TRIM11. Similar observations on TRIM7.1ΔR were obtained when TRIM27 was used instead of TRIM11 (data not shown). This suggests that the RING domain of TRIM7.1 may protect it from being targeted for ubiquitination by other TRIM members and that both TRIM11 and TRIM27 can monoubiquitinate the RING-deleted TRIM7.1. However, it is also possible that TRIM11/27 targets TRIM7.1ΔR with poly-ubiquitin chains that might be responsible for the reduction in its protein levels.

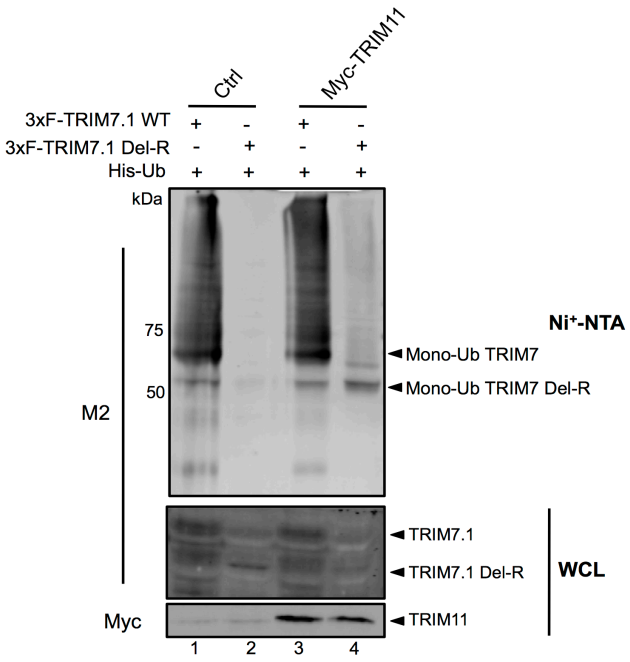


Figure 3.22 **TRIM11 can monoubiquitinate the RING-deleted TRIM7.1.** Immunoblot showing the Ni^{+}-NTA pull down where both wild-type and RING-deleted 3xFlag-TRIM7.1 were co-expressed with MycGFP -TRIM11 and 6xHis-Ubiquitin in HEK293T cells. An empty vector was used as a negative control (Ctrl). Cells were treated with 20 μM MG132 for 4 hours and harvested under denaturing conditions. The top pull down panel shows that no significant difference in TRIM7.1 autoubiquitination in the presence of TRIM11. However, monoubiquitination of TRIM7.1 Del-R was observed in the presence of TRIM11 as indicated. The middle and bottom panels show the WCL protein expression levels using the M2 and anti-myc antibodies.

To determine whether TRIM11 or TRIM27 bind the RING-deleted TRIM7.1 mutants that might potentially affect its ubiquitination, an immunoprecipitation was carried out using the myc-tagged TRIM11/27 co-expressed with the Flag-TRIM7.1ΔR. The immunoprecipitation revealed that both TRIM11 and TRIM27

interact with TRIM7.1ΔR in HEK293T cells (Figure 3.22, lanes 2 and 3). This indicates that both TRIM11 and TRIM27 interact with the C-terminal region of TRIM7.1.

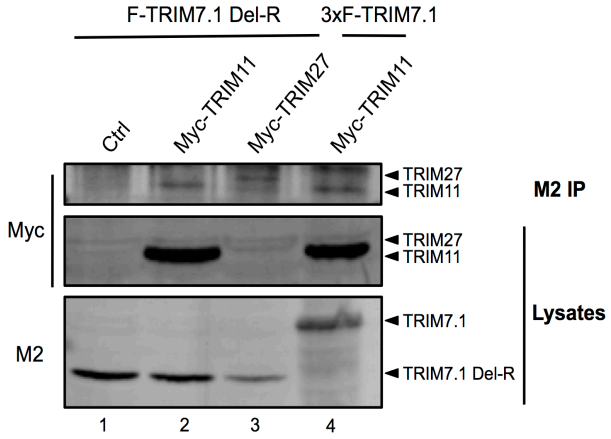


Figure 3.23 **TRIM11 and TRIM27 bind TRIM7.1 Del-R mutant.** Immunoblot showing an immunoprecipitation between MycGFP. -TRIM11/27 and Flag-TRIM7.1 Del-R in HEK293T cells. An empty vector was used as a negative control (Ctrl), while 3xFlag-TRIM7.1 was used as a positive control. Cells were harvested under normal conditions. The top panel shows that both TRIM11 and TRIM27 bind TRIM7.1 Del-R (TRIM27 running slightly higher than TRIM11). The middle and bottom panels show the protein expression levels using the anti-myc and M2 antibodies.

3.2.5 TRIM7.1 as a SUMO E3 ligase

Recently, a study by Chu and colleagues revealed a novel role for TRIM proteins as SUMO E3 ligases. In their study, they showed that TRIM27 acts as a SUMO E3 ligase and can SUMOylate Mdm2 (Chu and Yang 2011). Knowing the structural similarity between TRIM27 and TRIM7.1, we tested whether TRIM7.1 might also have a dual E3 ligase role and if it might possibly target Mdm2 for SUMOylation. To do this, a Ni^{+}-NTA pull down was performed following the co-expression of the 3xFlag-tagged TRIM7.1 in the presence of myc-Mdm2 with or without the His-tagged SUMO1 in HEK293T cells. The GFP-tagged TRIM27 was used as a positive control. Unlike TRIM27, TRIM7.1 was unable to SUMOylate Mdm2 as shown in Figure 3.24 (lane 3). Similar results were obtained using SUMO2 instead of SUMO1 (data not shown). This suggests that TRIM7.1 does not act as a SUMO E3 ligase against Mdm2. However, this does not exclude the

potential role of TRIM7.1 as a SUMO E3 ligase as only Mdm2 was tested as a substrate during our experiments.

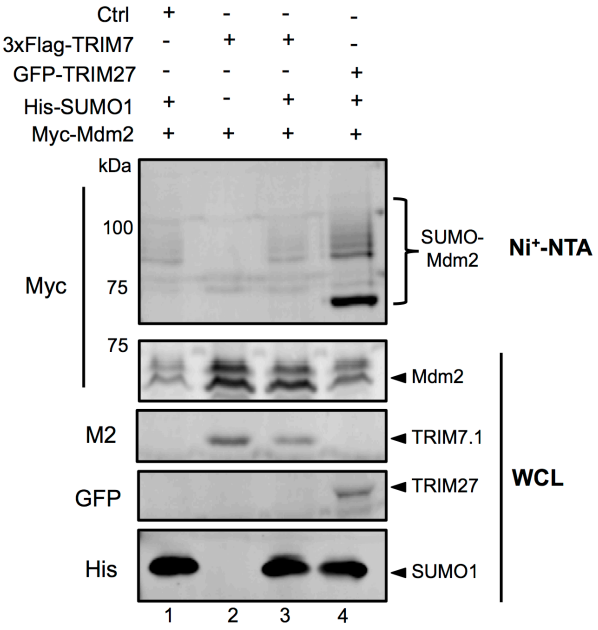


Figure 3.24 **TRIM7.1 does not SUMOylate Mdm2.** Immunoblot showing a Ni^{+}-NTA pull down between 3xFlag-TRIM7.1 and myc-Mdm2 in the presence of His-SUMO1 in HEK293T cells. An empty vector was used as a negative control, while MycGFP -TRIM27 was used as a positive control. Cells were treated with 20 μM MG132 for 4 hours and harvested under denaturing conditions. The first panel shows that unlike TRIM27, TRIM7.1 does not SUMOylate Mdm2. The bottom three panels show the WCL protein expression levels using antibodies against the myc-, Flag-, GFP-, and His-tags.

3.2.6 Conclusion

The data demonstrates a novel role of TRIM7.1 as a ubiquitin E3 ligase that targets itself with ubiquitin, and like many other TRIM members, its autoubiquitination is dependent on its RING domain. This is evident from experiments using a mutant lacking the RING domain (Figure 3.16). However point mutations that should block zinc binding and thus compromise the RING domain do not seem to affect the level of ubiquitination. The reason for this is unclear but it may be possible that the mutated RING domain retains activity or that it recruits other E3 ligases, that would not be recruited if the region containing the RING domain was completely removed. Interestingly, even with the RING deletion mutant there is some residual monoubiquitination of TRIM7, which could support the idea that other E3 ligases may be acting on TRIM7.

Despite having different effects on the levels of TRIM7 ubiquitination, both the point mutants and the RING deletion mutant had altered cytoplasmic localization, which was consistent with findings from previous studies on TRIM5 (another member of class IV TRIMs) (Diaz-Griffero et al. 2006). Similarly, treating cells with MG132 altered TRIM7.1's cellular localisation from cytoplasmic to peri-nuclear aggresomes suggesting that proteasome inhibition affects the cytoplasmic localisation of members of this class of TRIM proteins (Figure 3.19). These aggresomes might possibly have a role in preventing the accumulation of misfolded TRIM7.1 in the cytoplasm in an attempt to allow proper re-folding of the protein to its native form as seen with other TRIMs (Diaz-Griffero et al. 2006).

The ubiquitination of TRIM7.1 does not appear to target it for degradation via the proteasome. This is supported by the fact that a significant proportion of the ubiquitination on TRIM7.1 appears to be monoubiquitination, which does not usually target proteins to the proteasome. However, the experiments with the ubiquitin Lysine mutants suggest that some polyubiquitination can occur, although this is not Lysine 48-linked, which is the major signal for proteasome targeting (Figure 3.17).

The co-expression of Sin1, TRIM11, or TRIM27 had no significant effect on TRIM7.1 autoubiquitination in cells suggesting that TRIM7.1 might form different complexes that are not necessarily required to modulate its levels but might be involved in recruiting it to certain cytoplasmic loci to perform other cellular functions which still remain unclear. However, the ability of TRIM11 and TRIM27 to directly bind and promote monoubiquitination of the RING-deleted TRIM7.1 mutant indicates the importance of the C-terminal region of TRIM7.1 for TRIM11/27 binding. Finally, the ability of co-expressed TRIM7.1 to enhance Sin1 ubiquitination (Figure 3.21) might suggest that it can regulate mTORC2 activity by affecting the stability of its components.

3.3 Functional Roles of TRIM7

3.3 Functional Roles of TRIM7

3.3.1 Introduction

One of the aims of the project was to determine the functional role of TRIM7 and its relevance to mTORC2 signalling. Our findings so far demonstrate the ability of TRIM7.1 to interact with two of the major mTORC2 components, mTOR and Sin1, and its novel role as an ubiquitin E3 ligase. However, binding experiments on their own are not sufficient to ensure the relevance of these interaction to mTORC2 signalling. It was important to demonstrate that loss of TRIM7 could affect mTORC2 signalling. This was achieved by an shRNA approach to knockdown TRIM7 levels in cells. Moreover, cells' behavior was monitored upon knocking down TRIM7 to determine if it might have an affect on cell proliferation. Further characterising TRIM7 would allow us to understand its functional relevance in mTORC2 signalling.

3.3.2 The effect of TRIM7 knockdown on mTORC2 signalling

One crucial step towards understanding the potential role of TRIM7 in mTORC2 signalling was to knockdown TRIM7 and monitor its effects on mTORC2 components, if any. Initially, small interfering RNAs (siRNAs) were used in an attempt to knockdown TRIM7 in HEK293T and HeLa cells but these attempts were shown to be unsuccessful by qPCR analysis (data not shown). Therefore, an shRNA approach was used in transient transfections but again this failed to knockdown TRIM7. Eventually, generating stable HEK293T cell lines expressing either scrambled or TRIM7 shRNAs was successful in knocking down TRIM7. This was shown by (i) Western blot analysis of Flag-TRIM7.1 where reduced levels were demonstrated in the TRIM7 shRNA expressing cells (Figure 3.25A) and (ii) quantification of TRIM7.1 mRNA levels by qPCR which showed an approximate 50% reduction in mRNA levels compared to the control cell line (Figure 3.25B). However, the changes in TRIM7's mRNA transcript levels appeared to be more significant than the changes in its protein levels. Similar

results were obtained with other clones selected from the TRIM7 shRNA stable cells (data not shown).

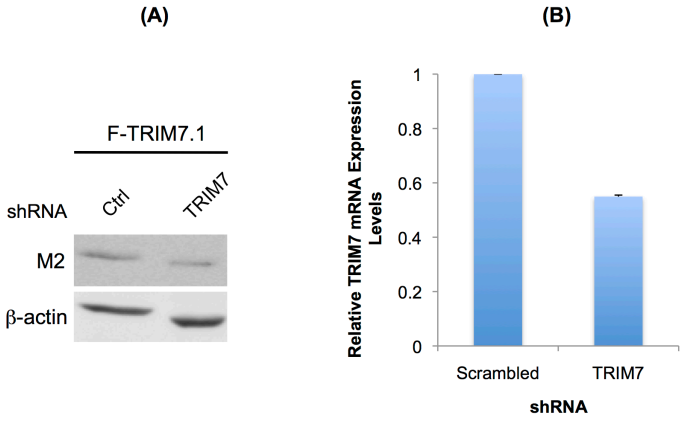


Figure 3.25 **TRIM7 knockdown by shRNA. (A)** Immunoblot showing the expression of Flag-TRIM7.1 in both scrambled and TRIM7 shRNA stable cell lines where a slight reduction in TRIM7.1 expression was observed in TRIM7 knockdown cells. β-actin was used as a loading control. **(B)** qPCR analysis from two repeats of the experiment of RNAs extracted from the shRNA stable cell line showing an approximate reduction of 50% in TRIM7.1 levels. The error bars represent mean ± SD.

After establishing TRIM7 stable knockdown cells, the next step was to monitor whether knocking down TRIM7 affects the individual components of mTORC2 and its downstream target Akt. To do this, both scrambled and TRIM7 stable cell lines were harvested and a Western blot was performed using antibodies against endogenous mTOR, Rictor, Sin1, and Akt. Figure 3.26A shows a clear increase in the levels of the mTORC2 components as well as Akt itself in the TRIM7 shRNA cells compared to the scrambled control (Figure 3.26A). A quantitative analysis of the protein levels was carried out shown by the bar chart in Figure 3.26B. Sin1, mTOR, Rictor and Akt all showed a significant increase in protein levels when the levels of TRIM7 were reduced.

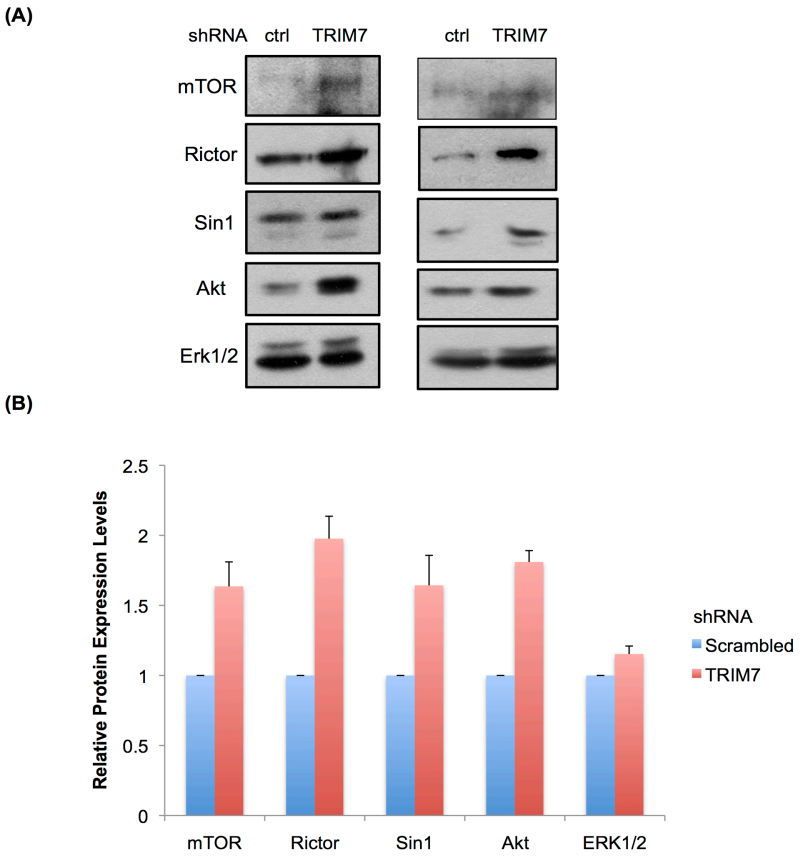


Figure 3.26 **TRIM7 knockdown leads to increased protein levels of mTORC2 components.** **(A)** The left panel shows an immunoblot demonstrating the expression levels of endogenous mTORC2 components and Akt in both scrambled and TRIM7 shRNA stable cell lines. An increase of mTORC2 signalling components was observed in the TRIM7 knockdown cells compared to the scrambled control. ERK1/2 was used as a loading control. A repetition of the same experiment is shown *(right)*. **(B)** A bar chart showing the protein quantification (using Image J) of three repeats of the experiment. The error bars represent mean ± SD with p-values of 0.02, 0.008, 0.03, and 0.01 for mTOR, Rictor, Sin1, and Akt, respectively.

3.3.3 TRIM7 knockdown increases cell proliferation

One striking observation upon knocking down TRIM7 in cells was a noticeable increase in cell proliferation compared to the scrambled cells. This observation was also noted in the other selected clones of the TRIM7 shRNA stable cell line. To determine the proliferative capabilities of both scrambled and TRIM7 shRNA cell lines, a MTT cell proliferation assay was carried out on a three-day time course. Equal numbers of both cell lines were plated on 96-well plates in triplicates and left to grow for three days; Days 1, 2, and 3 where Day 1 is 24 hours after plating cells. Each day the plates were treated with 5 mg/ml MTT for 3

hours and their absorbance was measures at 570 nm. There was approximately a 2-fold increase in cell proliferation in the TRIM7 shRNA cells compared to the scrambled control cells on Day 3.

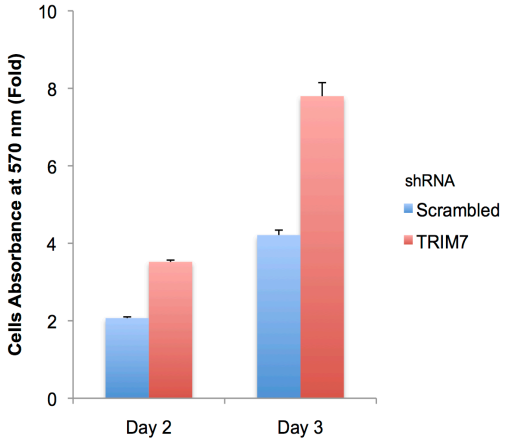


***Figure 3.27* TRIM7 knockdown enhances cell proliferation.** A quantitative analysis of an MTT cell proliferation assay comparing the scrambled and TRIM7 shRNA stable cell lines. Cells were treated with 5 mg/ml MTT for 3 hours. The experiment was carried out throughout three days in triplicates where an increase of approximately 2-fold in cell proliferation was observed in the TRIM7 knockdown cells compared to the scrambled control cells at Day 3. The absorbance of cells from Days 2 and 3 were normalized to those of Day 1 as no difference between the absorbance of control and TRIM7 shRNA cells was observed 24 hours post-plating. The error bars represent mean ± SD.

3.3.4 Conclusion

To address the functional relevance of TRIM7 in mTORC2 signalling, stable shRNA cell lines were generated in HEK293T cells. This achieved a 50% reduction in TRIM7 mRNA levels, however, it was not possible to determine the effect on the endogenous protein level of TRIM7 due to the lack of commercial antibodies that are capable of recognizing endogenous protein. TRIM7 appears to maintain the expression of the mTORC2 components as its reduced level promoted an increase in the protein levels of mTOR, Rictor, Sin1 and Akt (Figure 3.26). This correlated with an increase in the proliferative capabilities of cells suggesting that enhanced mTORC2 signalling may contribute to an increase in cell growth/division. It will be particularly important to confirm these results by re-expressing TRIM7 into the knockdown cells and see if it rescues the changes.

This could be achieved by generating a TRIM7 mutant that will not be recognized by the shRNA and at the same time not disrupt TRIM7's three-dimensional conformation and maintain its functional role in the cell. Re-expressing a RING-mutant TRIM7 into the knockdown cells can also be used to determine whether the observed affects are dependent on its ubiquitin E3 ligase activity or not. However, achieving a better knockdown of both TRIM7's transcript and protein levels is crucial to further elucidate its potential role in mTORC2 signalling and cell proliferation. To do this, more clones expressing TRIM7 shRNA need to be screened and further characterised.

Chapter Four

Discussion

4. Discussion

Compared to many other members of the TRIM family, TRIM7 is one of the least studied. Apart from the importance of its B30.2 domain for glycogenin interaction (Zhai et al. 2004), its functional role in the cell has remained elusive. So far, only the different splice variants and structural domains of TRIM7 have been established in the literature indicating that it possesses a RING domain (Zhai et al. 2004), the most characterised of all of its domains. Many proteins that possess a RING domain exhibit E3 ligase activities and the TRIM family of proteins are the largest group exhibiting RING domains (Reymond et al. 2001). TRIM proteins are involved in many cellular processes and play important roles in human diseases including cancers (Meroni and Diez-Roux 2005). This project aimed to determine the relevance of the interaction between TRIM7 and the mTORC2 component Sin1. In addition, we aimed to further characterise TRIM7 to understand its potential role in cells. It was initially confirmed that the novel interaction between TRIM7 and Sin1 occurred in mammalian cells and that TRIM7 could be found in complexes containing mTOR (Figures 3.1 and 3.5A). The interaction between TRIM7 and Sin1 did not show any significant changes following stimulation with two known activators of mTORC2, IGF-1 and hydrogen peroxide. While this does not eliminate the possibility that other stimuli that activate mTORC2 may modulate the TRIM7.1-Sin1 interaction, it suggests that the interaction may be constitutive or perhaps regulated by other signalling pathways in the cell. However, the absence of a positive control for the stimuli possibly suggests that mTORC2 signalling might not have been activated as expected, and therefore, no changes in the TRIM7.1-Sin1 interaction was observed. Sin1 demonstrated a differential ability to bind to the different TRIM7 isoforms by binding to TRIM7.1 and TRIM7.3, but not TRIM7.4 (Figure 3.4). This indicated the importance of the C-terminal region of TRIM7 for its binding to Sin1 as both TRIM7 isoforms 1 and 3 have an identical C-terminus, that is lacking in isoform 4. The significance of this differential binding is unclear at the moment as specific functional differences between the TRIM7 isoforms have yet to be

uncovered. It would be interesting to re-express the different isoforms in the TRIM7 knockdown cell line to determine which have an effect on the levels of mTORC2 proteins and cell proliferation. It might be expected that TRIM7.4 does not complement these changes as it is unable to bind to Sin1.

The presence of mTOR in TRIM7 immunoprecipitates (Figure 3.5A) suggests that TRIM7 may actually be recruited to mTORC2. However, from this experiment it can't be distinguished whether mTOR can directly bind to TRIM7 or if the binding is being mediated by Sin1. Experiments where Sin1 is knocked-down by siRNA could be used to help distinguish these possibilities. mTOR appeared to exert a negative effect on TRIM7.1 protein levels, but this was not dependent on mTOR activity (Figure 3.5B). This was not followed up further but it is possible it could be due to an experimental artifact whereby the promoters of the transfected TRIM7 expression plasmids is being affected by co-expression of the mTOR expressing plasmid. It was further shown that, despite TRIM7 being a phosphoprotein, it was not a direct mTOR substrate or a substrate for the downstream kinases Akt1 and PKCα. An initial failed attempt with mass spectrometry was also carried out to determine the potential phosphorylated residues on TRIM7. Further optimization of the experiment is required in order for it to be repeated. Other approaches such as In-Gel kinase assays can be performed to determine the potential kinase(s) for TRIM7.1 and to identify the phosphorylation sites. Determining the kinase(s) of TRIM7.1 will be crucial to understand which signalling pathway TRIM7.1 may part of, and will help in elucidating the potential cellular roles it may be involved in.

Although the RING domain is the most characterised compared to the other domains within TRIM proteins, many studies have shown the importance of the CC domain to allow TRIM proteins to form complexes with each other by either forming homo- or hetero-oligomers (Li et al. 2011). TRIM5 and TRIM24 are two examples where they have been extensively studied and have been demonstrated to form hetero-oligomers with other members of their class (Li et al. 2007a). Similarly, our data demonstrated the ability of TRIM7 to form both

homo-oligomers, by forming a TRIM7.1-TRIM7.3 complex, and hetero-oligomers with both TRIM11 and TRIM27. However, we were not able to demonstrate the ability of TRIM7 to form a homo-oligomer complex between two TRIM7.1 proteins due to them being downregulated when expressed together (Figure 3.9). One possible explanation could that the decreased protein levels may be due to TRIM7.1's ubiquitin E3 ligase activity that targets itself for ubiquitination, and thereby, degradation via the proteasome. In addition, both TRIM11 and TRIM27 complexing with TRIM7.1 and downregulating its levels may also be explained by the possibility of either TRIM11 or TRIM27 targeting TRIM7.1 for degradation. However, TRIM7.1 levels are not rescued by proteasomal or lysosomal inhibitors suggesting some other mode of regulation. Again there is the caveat that co-transfecting expression plasmids may affect their transcription. At the moment, the significance of TRIM7.1 complexing with itself, TRIM11, or TRIM27 is not clear nor is its co-localisation into specific cytoplasmic loci. Nevertheless, these findings were consistent with other studies that have demonstrated the ability of TRIM proteins of the same class to complex into distinct cytoplasmic bodies (Li et al. 2007a). This suggests that TRIM proteins with structural similarities may form more than one complex and possibly regulate each other's cellular expression or localisation. The finding that Sin1 is also in complexes with TRIM27 might suggest that a TRIM7-TRIM27 complex may be recruited to mTORC2. Unlike ubiquitination that mainly targets proteins for proteasomal degradation (Weissman 1997), SUMOylation has been shown to promote protein stabilization, localisation, and facilitates protein-protein interactions (Johnson 2004, Hay 2005, Geiss-Friedlander and Melchior 2007). Since TRIM27 has been recently shown to exhibit SUMO E3 ligase activity (Chu and Yang 2011), it would be interesting to determine whether the TRIM7-Sin1 or TRIM7-mTOR interaction could be facilitated by TRIM27's SUMO E3 activity, and whether it plays a role in recruiting TRIM7 to either Sin1 or mTOR. In addition, understanding if the expression of TRIM27 complements the changes observed in TRIM7 knockdown cells, or whether it plays a distinct role from TRIM7 would be crucial as it possesses both ubiquitin and SUMO E3 ligase activities (Chu and Yang 2011).

The finding that TRIM7 displays ubiquitin E3 ligase activity is the first molecular function that has been assigned to TRIM7. However, the autoubiquitination of TRIM7.1 mainly appeared to be monoubiquitination based on the use of ubiquitin mutants that can't form polyubiquitin chains. TRIM7.1's E3 activity was dependent on the presence of its RING domain although residual ubiquitination was still present when the RING domain was deleted (Figure 3.16). Other studies on TRIM proteins have also demonstrated similar findings where deleting the RING domain does not completely abolish their autoubiquitination in cells (Napolitano et al. 2011). Despite a main monoubiquitinated TRIM7.1 band appearing in the ubiquitination smear, we cannot eliminate the possibility of TRIM7.1 also polyubiquitinating itself as the intensity of the ubiquitinated smear in the presence of the ubiquitin K7R mutant was reduced compared to wild-type ubiquitin (Figure 3.17B). This reduction can be explained by the fact that mutating all Lysine residues of the ubiquitin molecule could prevent possible di- or multi-ubiquitination of TRIM7.1 as many TRIM proteins exhibiting ubiquitin E3 ligase activity have been shown to self-ubiquitinate in more than one way by either mono-, di-, multi-, or poly-ubiquitinating themselves (Diaz-Griffero et al. 2004). Nevertheless, we can eliminate the fact that TRIM7.1 forms K48-linked poly-ubiquitin chains as the autoubiquitination of TRIM7.1 was not affected when this residue was mutated on ubiquitin (Figure 3.17A). It is possible that K63-linked polyubiquitination is occurring which plays a role in aiding protein-protein interactions or lysosomal delivery (Sun and Chen 2004). Thus, it would be helpful to determine what effect mutating K63 would have on TRIM7.1 autoubiquitination.

Disruption or deletion of TRIM7.1's RING domain affected its expression levels and altered its cellular localisation to peri-nuclear bodies (Figure 3.19A). This phenotype has also been demonstrated in previous studies on TRIM5. It has been shown that disrupting the RING domain of TRIM5 affects its expression levels and prevents proper folding of the protein allowing it to translocate to peri-nuclear regions via the microtubule-organizing center (MOTC). These peri-nuclear structures can further be seen upon proteasome inhibition forming larger

aggresomes that contain chaperone proteins allowing proper re-folding of TRIM5 to its native form (Diaz-Griffero et al. 2006). These aggresomes are similar to what we have shown with wild-type TRIM7.1 upon MG132 treatment (Figure 3.19B). In the case of nuclear TRIMs such as PML, disruption of either the RING or B-box domains prevented the formation of nuclear bodies suggesting that the RBCC domain as an integral unit is important for proper protein folding despite each domain exerting separate functions (Borden et al. 1995, Borden et al. 1996). It has been suggested that TRIM5 levels are maintained by continuous synthesis and rapid degradation controlling its turnover. The distinct cytoplasmic dot-like structures appear when the overall levels of TRIM5 synthesis overwhelms the cell's ability to degrade it (Diaz-Griffero et al. 2006). Since both TRIM5 and TRIM7.1 fall in the same class of TRIM proteins, it is possible that this may also apply to TRIM7.1. It may act as an aggresomal precursor linked to the microtubules, and that disrupting its RING domain may affect its interdomain interactions leading to aggresomal formation. The translocation of TRIM7.1 to peri-nuclear loci may be an attempt to re-fold TRIM7.1 to its native form (Figure 4.1). This suggests that members of sub-classes TRIM proteins behave similarly and might potentially have similar cellular functionalities.

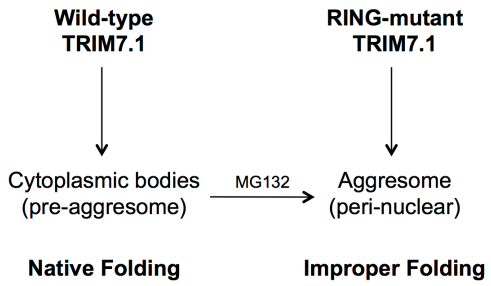


Figure 4.1 **The importance of TRIM7.1's RING domain integrity.** A hypothesis showing the potential importance of TRIM7.1 RING domain for its folding and how it affects its cellular localisation.

Previous studies on TRIM27 have demonstrated a novel role of TRIM proteins as SUMO E3 ligases where TRIM27 was able to SUMOylate many targets including Mdm2 (Chu and Yang 2011). Unlike TRIM27, our data failed to show the ability of TRIM7.1 to SUMOylate Mdm2 suggesting that TRIM7.1 does not act as a

SUMO E3 ligase against Mdm2. However, this does not exclude the potential role of TRIM7.1 as a dual E3 ligase as only Mdm2 was tested as a substrate during our experiments and further targets need to be examined. It is not clear whether the dual E3 activities of TRIM proteins take place simultaneously or not, but this demonstrates the various roles TRIM proteins can play in post-translational modifications.

So far, there have been few studies linking TRIM protein to either of the two mTOR complexes. The data presented here potentially shows a novel role where TRIM7 could affect mTORC2 signalling, and thereby, cell proliferation. We show that the presence of TRIM7 is important for maintaining the levels of the mTORC2 components. Our stable cell lines have shown a significant increase in cell proliferation upon a reduction in TRIM7 levels (Figure 3.27). This could possibly be explained by our finding that levels of mTORC2 proteins increase in these cells (Figure 3.26). We cannot rule out the possibility that the increase in cell proliferation could be due to off-target effects of the shRNA's integration with the cells' genome. However, two different clones were isolated for each shRNA (both scrambled and TRIM7) but only one clone was used for the Western blots and MTT assays. Therefore, more clones need to be thoroughly tested to show consistency of our findings and the changes need to be rescued by adding back TRIM7 to the cells. Nevertheless, the slight increase in Sin1 autoubiquitination levels in the presence of TRIM7.1 allows us to hypothesize a potential role for TRIM7.1 in modulating mTORC2 components. Studies have shown that the absence of Sin1 or Rictor prevents the assembly of the mTORC2 complex, therefore, reducing its activity (Frias et al. 2006). If TRIM7 could ubiquitinate Sin1 and target it for degradation this would affect the overall assembly of mTORC2 and potentially lead to reduced protein levels of the components. Knocking down TRIM7 would prevent Sin1 degradation leading to the stabilization of the mTORC2 complex thereby promoting cell proliferation (Figure 4.2). It is also possible that Sin1 may not be the key target of TRIM7 within mTORC2, but is simply required to recruit TRIM7 to the complex. TRIM7 could then target other components of the complex.

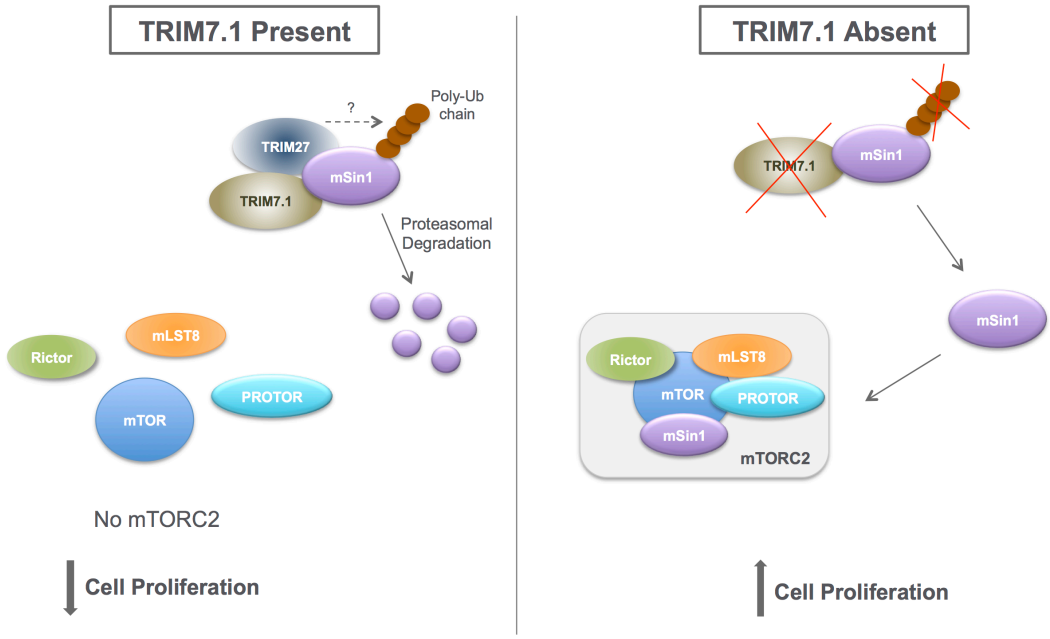


***Figure 4.2* A hypothesis showing the potential role of TRIM7.1 on cell proliferation.** When TRIM7.1 is present, it is able to target Sin1 for degradation via the ubiquitin-proteasome pathway preventing mTORC2 assembly, and therefore, reducing the rate of cellular proliferation. The role of TRIM27/Sin1 binding remains illusive. In the absence of TRIM7.1, Sin1 is free from TRIM7.1-mediated degradation allowing the formation of mTORC2 promoting cell proliferation.

It will be especially important to determine whether the changes in the levels of mTORC2 components and cell proliferation are dependent upon the E3 ligase activity of TRIM7. This can be tested by re-expressing the TRIM7 mutant lacking the RING domain in the knockdown cell line.

Although TRIM proteins have not been directly linked to mTOR signalling before, recent studies have shown the involvement of a few TRIM proteins with key downstream targets of mTORC2, Akt and PKC. TRIM13, which is deleted in many human tumors, has been shown to interact with both Mdm2 and Akt, and target them for degradation via its ubiquitin E3 ligase activity thereby inducing apoptosis (Joo et al. 2011). TRIM17, which is also a novel ubiquitin E3 ligase, has been shown to trigger apoptosis in neurons via the PI3K-Akt-GSK3 pathway (Lassot et al. 2010), whereas TRIM41 has been implicated in binding and modulating PKC turnover levels by targeting it with ubiquitin for degradation (Chen et al. 2007). Taken together, these findings support the idea of TRIM

proteins' role in regulating key players in major signalling pathways that can lead to cancer. Since both TRIM17 and TRIM41 fall in the same class as TRIM7 (i.e. class IV), it is possible that, like TRIM11 and TRIM27, they may form hetero-oligomeric complexes with TRIM7. This would suggest that diverse TRIM complexes may exhibit distinct E3 ligase activities to regulate multiple signalling pathways in cells. Our findings suggest that there might be a possible link between TRIM7 and cancer. Loss of TRIM7 could potentially promote tumourigenesis by triggering mTORC2 signalling, and thereby, increase the rate of cell proliferation allowing tumor growth. However, it is still not clear whether TRIM7's E3 ligase activity and its ability to from larger complexes with other family members contributes to the increase in cell proliferation or not.

Summary

The research presented in this thesis has uncovered a number of original findings. It has uncovered a potentially new role of TRIM proteins in regulating mTORC2 signalling, in particular that the previously poorly characterized TRIM family member, TRIM7, binds to the Sin1 component of mTORC2 and displays ubiquitin E3 ligase activity. Furthermore, it is demonstrated that TRIM7 may be required for controlling the protein levels of the mTORC2 components and for regulating cell proliferation. There are a number of key areas for future research. These include determining whether the E3 ligase activity of TRIM7.1 is critical for its regulation of mTORC2, and if so, if Sin1 or some of the other components are the key target. It will also be important to identify the pathways regulating the phosphorylation of TRIM7 and how this affects its functions. Further work is also needed to shed light on the roles of related TRIM family members in regulating mTORC2. TRIM7 is also likely to have roles independent of mTORC2 signalling, as also occurs with the case of Sin1 (Schroder et al. 2005) and Rictor that is able to form an E3 ligase complex targeting SGK1 for degradation (Gao et al. 2010).

References

5. References

Allton, K., Jain, A. K., Herz, H. M., Tsai, W. W., Jung, S. Y., Qin, J., Bergmann, A., Johnson, R. L. and Barton, M. C. (2009) 'Trim24 targets endogenous p53 for degradation', *Proc Natl Acad Sci U S A,* 106(28), 11612-6.

Alonso, M. D., Lomako, J., Lomako, W. M. and Whelan, W. J. (1995) 'A new look at the biogenesis of glycogen', *Faseb Journal,* 9(12), 1126-1137.

Alsheich-Bartok, O., Haupt, S., Alkalay-Snir, I., Saito, S., Appella, E. and Haupt, Y. (2008) 'PML enhances the regulation of p53 by CK1 in response to DNA damage.', *Oncogene,* 27(26), 3653-61.

Aronova, S., Wedaman, K., Aronov, P. A., Fontes, K., Ramos, K., Hammock, B. D. and Powers, T. (2008) 'Regulation of ceramide biosynthesis by TOR complex 2', *Cell Metab,* 7(2), 148-58.

Bayascas, J. R., Wullschleger, S., Sakamoto, K., Garcia-Martinez, J. M., Clacher, C., Komander, D., van Aalten, D. M. F., Boini, K. M., Lan, F., Lipina, C., Logie, L., Sutherland, C., Chudek, J. A., van Diepen, J. A., Voshol, P. J., Lucocq, J. M. and Alessi, D. R. (2008) 'Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance', *Molecular and Cellular Biology,* 28(10), 3258-3272.

Bernardi, R., Guernah, I., Jin, D., Grisendi, S., Alimonti, A., Teruya-Feldstein, J., Cordon-Cardo, C., Simon, M. C., Rafii, S. and Pandolfi, P. P. (2006) 'PML inhibits HIF-1 alpha translation and neoangiogenesis through repression of mTOR', *Nature,* 442(7104), 779-785.

Bernardi, R. and Pandolfi, P. P. (2007) 'Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies', *Nat Rev Mol Cell Biol,* 8(12), 1006-16.

Bhaskar, P. T. and Hay, N. (2007) 'The Two TORCs and Akt', *Developmental Cell,* 12(4), 487-502.

Blagosklonny, M. V. (2006) 'Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition', *Cell Cycle,* 5(18), 2087-102.

Borden, K. L., Boddy, M. N., Lally, J., O'Reilly, N. J., Martin, S., Howe, K., Solomon, E. and Freemont, P. S. (1995) 'The solution structure of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML', *EMBO J,* 14(7), 1532-41.

Borden, K. L., Lally, J. M., Martin, S. R., O'Reilly, N. J., Solomon, E. and Freemont, P. S. (1996) 'In vivo and in vitro characterization of the B1 and B2 zinc-binding domains from the acute promyelocytic leukemia protooncoprotein PML', *Proc Natl Acad Sci U S A,* 93(4), 1601-6.

Bornancin, F. and Parker, P. J. (1996) 'Phosphorylation of threonine 638 critically controls the dephosphorylation and inactivation of protein kinase C alpha', *Current Biology,* 6(9), 1114-1123.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J. and Greenberg, M. E. (1999) 'Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor', *Cell,* 96(6), 857-68.

Brunet, A., Park, J., Tran, H., Hu, L. S., Hemmings, B. A. and Greenberg, M. E. (2001) 'Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a)', *Molecular and Cellular Biology,* 21(3), 952-965.

Burgering, B. M. T. and Coffer, P. J. (1995) 'Protein-kinase-b (c-Akt) in phosphatidylinositol-3-oh inase signal-transduction', *Nature,* 376(6541), 599-602.

Cameron, A. J., Linch, M. D., Saurin, A. T., Escribano, C. and Parker, P. J. (2011) 'mTORC2 targets AGC kinases through Sin1-dependent recruitment', *Biochem J,* 439(2), 287-97.

Cantley, L. C. and Neel, B. G. (1999) 'New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway', *Proc Natl Acad Sci U S A,* 96(8), 4240-5.

Carson, R. P., Fu, C., Winzenburger, P. and Ess, K. C. (2013) 'Deletion of Rictor in neural progenitor cells reveals contributions of mTORC2 signaling to tuberous sclerosis complex', *Hum Mol Genet,* 22(1), 140-52.

Chen, C. C., Jeon, S. M., Bhaskar, P. T., Nogueira, V., Sundararajan, D., Tonic, I., Park, Y. and Hay, N. (2010) 'FoxOs inhibit mTORC1 and activate Akt by inducing the expression of Sestrin3 and Rictor', *Dev Cell,* 18(4), 592-604.

Chen, C. H. and Sarbassov, d. D. (2011) 'The mTOR (mammalian target of rapamycin) kinase maintains integrity of mTOR complex 2', *J Biol Chem,* 286(46), 40386-94.

Chen, D., Gould, C., Garza, R., Gao, T., Hampton, R. Y. and Newton, A. C. (2007) 'Amplitude control of protein kinase C by RINCK, a novel E3 ubiquitin ligase', *J Biol Chem,* 282(46), 33776-87.

Chu, Y. and Yang, X. (2011) 'SUMO E3 ligase activity of TRIM proteins', *Oncogene,* 30(9), 1108-16.

Chueh, S. C. J. and Kahan, B. D. (2005) 'Clinical application of sirolimus in renal transplantation: an update', *Transplant International,* 18(3), 261-277.

Crino, P. B., Nathanson, K. L. and Henske, E. P. (2006) 'The tuberous sclerosis complex', *N Engl J Med,* 355(13), 1345-56.

Dada, S., Demartines, N. and Dormond, O. (2008) 'mTORC2 regulates PGE(2)-mediated endothelial cell survival and migration', *Biochemical and Biophysical Research Communications,* 372(4), 875-879.

Dagert, M. and Ehrlich, S. D. (1979) 'Prolonged incubation in calcium chloride improves the competence of Escherichia coli cells', *Gene,* 6(1), 23-8.

Davis, R. J. (1994) 'MAPKs - New JNK expands the group', *Trends in Biochemical Sciences,* 19(11), 470-473.

de Thé, H., Lavau, C., Marchio, A., Chomienne, C., Degos, L. and Dejean, A. (1991) 'The PML-RAR alpha fusion mRNA generated by the t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR', *Cell,* 66(4), 675-84.

Deshaies, R. J. and Joazeiro, C. A. (2009) 'RING domain E3 ubiquitin ligases', *Annu Rev Biochem,* 78, 399-434.

Diaz-Griffero, F., Li, X., Javanbakht, H., Song, B., Welikala, S., Stremlau, M. and Sodroski, J. (2006) 'Rapid turnover and polyubiquitylation of the retroviral restriction factor TRIM5', *Virology,* 349(2), 300-15.

Dibble, C. C., Asara, J. M. and Manning, B. D. (2009) 'Characterization of Rictor Phosphorylation Sites Reveals Direct Regulation of mTOR Complex 2 by S6K1', *Molecular and Cellular Biology,* 29(21).

Dibble, C. C., Elis, W., Menon, S., Qin, W., Klekota, J., Asara, J. M., Finan, P. M., Kwiatkowski, D. J., Murphy, L. O. and Manning, B. D. (2012) 'TBC1D7 is a third subunit of the TSC1-TSC2 complex upstream of mTORC1', *Mol Cell,* 47(4), 535-46.

Emoto, K., He, Y., Ye, B., Grueber, W. B., Adler, P. N., Jan, L. Y. and Jan, Y. N. (2004) 'Control of dendritic branching and tiling by the Tricornered-kinase/Furry signaling pathway in Drosophila sensory neurons', *Cell,* 119(2), 245-56.

Facchinetti, V., Ouyang, W. M., Wei, H., Soto, N., Lazorchak, A., Gould, C., Lowry, C., Newton, A. C., Mao, Y. X., Miao, R. Q., Sessa, W. C., Qin, J., Zhang, P. M., Su, B. and Jacinto, E. (2008) 'The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C', *Embo Journal,* 27(14), 1932-1943.

Fan, Q. W., Cheng, C. K., Nicolaides, T. P., Hackett, C. S., Knight, Z. A., Shokat, K. M. and Weiss, W. A. (2007) 'A dual phosphoinositide-3-kinase alpha/mTOR inhibitor cooperates with blockade of epidermal growth factor receptor in PTEN-mutant glioma', *Cancer Research,* 67(17), 7960-7965.

Foufelle, F. and Ferré, P. (2002) 'New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c', *Biochem J,* 366(Pt 2), 377-91.

Fox, J. D., Routzahn, K. M., Bucher, M. H. and Waugh, D. S. (2003) 'Maltodextrin-binding proteins from diverse bacteria and archaea are potent solubility enhancers', *FEBS Lett,* 537(1-3), 53-7.

Frias, M. A., Thoreen, C. C., Jaffe, J. D., Schroder, W., Sculley, T., Carr, S. A. and Sabatini, D. M. (2006) 'mSin1 Is Necessary for Akt/PKB Phosphorylation, and Its Isoforms Define Three Distinct mTORC2s', *Current Biology,* 16(18), 1865-1870.

Gangloff, Y. G., Mueller, M., Dann, S. G., Svoboda, P., Sticker, M., Spetz, J. F., Um, S. H., Brown, E. J., Cereghini, S., Thomas, G. and Kozma, S. C. (2004) 'Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development', *Molecular and Cellular Biology,* 24(21), 9508-9516.

Gao, D., Wan, L., Inuzuka, H., Berg, A. H., Tseng, A., Zhai, B., Shaik, S., Bennett, E., Tron, A. E., Gasser, J. A., Lau, A., Gygi, S. P., Harper, J. W., DeCaprio, J. A., Toker, A. and Wei, W. (2010) 'Rictor forms a complex with Cullin-1 to promote SGK1 ubiquitination and destruction', *Mol Cell,* 39(5), 797-808.

Gao, F. B. (2007) 'Molecular and cellular mechanisms of dendritic morphogenesis', *Curr Opin Neurobiol,* 17(5), 525-32.

García-Martínez, J. and Alessi, D. (2008) 'mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1).', *Biochem J,* 416(3), 375-85.

Geiss-Friedlander, R. and Melchior, F. (2007) 'Concepts in sumoylation: a decade on', *Nat Rev Mol Cell Biol,* 8(12), 947-56.

Gems, D. and Doonan, R. (2009) 'Antioxidant defense and aging in C. elegans: is the oxidative damage theory of aging wrong?', *Cell Cycle,* 8(11), 1681-7.

Ghosh, D., Srivastava, G., Xu, D., Schulz, L. and Roberts, R. (2008) 'A link between SIN1 (MAPKAP1) and poly(rC) binding protein 2 (PCBP2) in counteracting environmental stress.', *Proc Natl Acad Sci U S A,* 105(33), 11673-8.

Glidden, E. J., Gray, L. G., Vemuru, S., Li, D., Harris, T. E. and Mayo, M. W. (2012) 'Multiple site acetylation of Rictor stimulates mammalian target of rapamycin complex 2 (mTORC2)-dependent phosphorylation of Akt protein', *J Biol Chem,* 287(1), 581-8.

Grütter, C., Briand, C., Capitani, G., Mittl, P. R., Papin, S., Tschopp, J. and Grütter, M. G. (2006) 'Structure of the PRYSPRY-domain: implications for autoinflammatory diseases', *FEBS Lett,* 580(1), 99-106.

Guertin, D., Stevens, D., Thoreen, C., Burds, A., Kalaany, N., Moffat, J., Brown, M., Fitzgerald, K. and Sabatini, D. (2006) 'Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1.', *Dev Cell,* 11(6), 859-71.

Guertin, D. A. and Sabatini, D. M. (2005) 'An expanding role for mTOR in cancer', *Trends in Molecular Medicine,* 11(8), 353-361.

Guertin, D. A. and Sabatini, D. M. (2007) 'Defining the Role of mTOR in Cancer', *Cancer Cell,* 12(1), 9-22.

Guertin, D. A., Stevens, D. M., Saitoh, M., Kinkel, S., Crosby, K., Sheen, J.-H., Mullholland, D. J., Magnuson, M. A., Wu, H. and Sabatini, D. M. (2009) 'mTOR Complex 2 Is Required for the Development of Prostate Cancer Induced by Pten Loss in Mice', *Cancer Cell,* 15(2), 148-159.

Guertin, D. A., Stevens, D. M., Thoreen, C. C., Burds, A. A., Kalaany, N. Y., Moffat, J., Brown, M., Fitzgerald, K. J. and Sabatini, D. M. (2006) 'Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC alpha but not S6K1', *Developmental Cell,* 11(6), 859-871.

Gwinn, D. M., Shackelford, D. B., Egan, D. F., Mihaylova, M. M., Mery, A., Vasquez, D. S., Turk, B. E. and Shaw, R. J. (2008) 'AMPK

phosphorylation of raptor mediates a metabolic checkpoint', *Mol Cell,* 30(2), 214-26.

Hagiwara, A., Cornu, M., Cybulski, N., Polak, P., Betz, C., Trapani, F., Terracciano, L., Heim, M. H., Rüegg, M. A. and Hall, M. N. (2012) 'Hepatic mTORC2 activates glycolysis and lipogenesis through Akt, glucokinase, and SREBP1c', *Cell Metab,* 15(5), 725-38.

Hahn-Windgassen, A., Nogueira, V., Chen, C. C., Skeen, J. E., Sonenberg, N. and Hay, N. (2005) 'Akt activates the mammalian target of rapamycin by regulating cellular ATP level and AMPK activity', *Journal of Biological Chemistry,* 280(37), 32081-32089.

Han, J. M., Jeong, S. J., Park, M. C., Kim, G., Kwon, N. H., Kim, H. K., Ha, S. H., Ryu, S. H. and Kim, S. (2012) 'Leucyl-tRNA synthetase is an intracellular leucine sensor for the mTORC1-signaling pathway', *Cell,* 149(2), 410-24.

Hara, K., Maruki, Y., Long, X. M., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J. and Yonezawa, K. (2002) 'Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action', *Cell,* 110(2), 177-189.

Harrington, L. S., Findlay, G. M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N. R., Cheng, S., Shepherd, P. R., Gout, I., Downes, C. P. and Lamb, R. E. (2004) 'The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins', *Journal of Cell Biology,* 166(2), 213-223.

Hauge, C., Antal, T. L., Hirschberg, D., Doehn, U., Thorup, K., Idrissova, L., Hansen, K., Jensen, O. N., Jorgensen, T. J., Biondi, R. M. and Frodin, M. (2007) 'Mechanism for activation of the growth factor-activated AGC kinases by turn motif phosphorylation', *Embo Journal,* 26(9), 2251-2261.

Haupt, S., di Agostino, S., Mizrahi, I., Alsheich-Bartok, O., Voorhoeve, M., Damalas, A., Blandino, G. and Haupt, Y. (2009) 'Promyelocytic leukemia protein is required for gain of function by mutant p53.', *Cancer Res,* 69(11), 4818-26.

Hay, R. T. (2005) 'SUMO: a history of modification', *Mol Cell,* 18(1), 1-12.

He, W., Dorn, D. C., Erdjument-Bromage, H., Tempst, P., Moore, M. A. and Massagué, J. (2006) 'Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway', *Cell,* 125(5), 929-41.

Helliwell, S. B., Wagner, P., Kunz, J., Deuterreinhard, M., Henriquez, R. and Hall, M. N. (1994) 'TOR1 and TOR2 are structurally and functionally similar but

not identical phosphatidylinositol kinase homologs in yeast', *Molecular Biology of the Cell,* 5(1), 105-118.

Henry, J., Mather, I. H., McDermott, M. F. and Pontarotti, P. (1998) 'B30.2-like domain proteins: update and new insights into a rapidly expanding family of proteins', *Mol Biol Evol,* 15(12), 1696-705.

Herquel, B., Ouararhni, K., Khetchoumian, K., Ignat, M., Teletin, M., Mark, M., Béchade, G., Van Dorsselaer, A., Sanglier-Cianférani, S., Hamiche, A., Cammas, F., Davidson, I. and Losson, R. (2011) 'Transcription cofactors TRIM24, TRIM28, and TRIM33 associate to form regulatory complexes that suppress murine hepatocellular carcinoma', *Proc Natl Acad Sci U S A,* 108(20), 8212-7.

Hickey, M. M. and Simon, M. C. (2006) 'Regulation of angiogenesis by hypoxia and hypoxia-inducible factors', *Curr Top Dev Biol,* 76, 217-57.

Horton, J. D., Goldstein, J. L. and Brown, M. S. (2002) 'SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver', *J Clin Invest,* 109(9), 1125-31.

Hresko, R. C. and Mueckler, M. (2005) 'mTOR center dot RICTOR is the Ser(473) kinase for Akt/protein kinase B in 3T3-L1 adipocytes', *Journal of Biological Chemistry,* 280(49), 40406-40416.

Huang, J. X., Dibble, C. C., Matsuzaki, M. and Manning, B. D. (2008) 'The TSC1-TSC2 complex is required for proper activation of mTOR complex 2', *Molecular and Cellular Biology,* 28(12), 4104-4115.

Ikeda, K., Morigasaki, S., Tatebe, H., Tamanoi, F. and Shiozaki, K. (2008) 'Fission yeast TOR complex 2 activates the AGC-family Gad8 kinase essential for stress resistance and cell cycle control', *Cell Cycle,* 7(3), 358-64.

Inoki, K., Ouyang, H., Zhu, T. Q., Lindvall, C., Wang, Y., Zhang, X. J., Yang, Q., Bennett, C., Harada, Y., Stankunas, K., Wang, C. Y., He, X., MacDougald, O. A., You, M., Williams, B. O. and Guan, K. L. (2006) 'TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth', *Cell,* 126(5), 955-968.

Inoki, K., Zhu, T. and Guan, K. L. (2003) 'TSC2 mediates cellular energy response to control cell growth and survival', *Cell,* 115(5), 577-90.

Ivanov, A. V., Peng, H., Yurchenko, V., Yap, K. L., Negorev, D. G., Schultz, D. C., Psulkowski, E., Fredericks, W. J., White, D. E., Maul, G. G., Sadofsky, M. J., Zhou, M. M. and Rauscher, F. J. (2007) 'PHD domain-mediated E3

ligase activity directs intramolecular sumoylation of an adjacent bromodomain required for gene silencing', *Mol Cell,* 28(5), 823-37.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S., Huang, Q., Qin, J. and Su, B. (2006) 'SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity.', *Cell,* 127(1), 125-37.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M. A., Hall, A. and Hall, M. N. (2004) 'Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive', *Nature Cell Biology,* 6(11), 1122-U30.

Jaworski, J., Spangler, S., Seeburg, D. P., Hoogenraad, C. C. and Sheng, M. (2005) 'Control of dendritic arborization by the phosphoinositide-3'-kinase-Akt-mammalian target of rapamycin pathway', *J Neurosci,* 25(49), 11300-12.

Jiang, Y., Gram, H., Zhao, M., New, L., Gu, J., Feng, L., Di Padova, F., Ulevitch, R. J. and Han, J. (1997) 'Characterization of the structure and function of the fourth member of p38 group mitogen-activated protein kinases, p38delta', *J Biol Chem,* 272(48), 30122-8.

Johnson, E. S. (2004) 'Protein modification by SUMO', *Annu Rev Biochem,* 73, 355-82.

Jones, K. T., Greer, E. R., Pearce, D. and Ashrafi, K. (2009) 'Rictor/TORC2 regulates Caenorhabditis elegans fat storage, body size, and development through sgk-1', *PLoS Biol,* 7(3), e60.

Joo, H. M., Kim, J. Y., Jeong, J. B., Seong, K. M., Nam, S. Y., Yang, K. H., Kim, C. S., Kim, H. S., Jeong, M., An, S. and Jin, Y. W. (2011) 'Ret finger protein 2 enhances ionizing radiation-induced apoptosis via degradation of AKT and MDM2', *Eur J Cell Biol,* 90(5), 420-31.

Kaizuka, T., Hara, T., Oshiro, N., Kikkawa, U., Yonezawa, K., Takehana, K., Iemura, S., Natsume, T. and Mizushima, N. (2010) 'Tti1 and Tel2 are critical factors in mammalian target of rapamycin complex assembly', *J Biol Chem,* 285(26), 20109-16.

Kakizuka, A., Miller, W. H., Umesono, K., Warrell, R. P., Frankel, S. R., Murty, V. V., Dmitrovsky, E. and Evans, R. M. (1991) 'Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR alpha with a novel putative transcription factor, PML', *Cell,* 66(4), 663-74.

Kamada, Y., Fujioka, Y., Suzuki, N. N., Inagaki, F., Wullschleger, S., Loewith, R., Hall, M. N. and Ohsumi, Y. (2005) 'Tor2 directly phosphorylates the AGC

kinase Ypk2 to regulate actin polarization', *Molecular and Cellular Biology,* 25(16), 7239-7248.

Khetchoumian, K., Teletin, M., Tisserand, J., Mark, M., Herquel, B., Ignat, M., Zucman-Rossi, J., Cammas, F., Lerouge, T., Thibault, C., Metzger, D., Chambon, P. and Losson, R. (2007) 'Loss of Trim24 (Tif1alpha) gene function confers oncogenic activity to retinoic acid receptor alpha', *Nat Genet,* 39(12), 1500-6.

Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., Tempst, P. and Sabatini, D. M. (2002) 'MTOR interacts with Raptor to form a nutrient-sensitive complex that signals to the cell growth machinery', *Cell,* 110(2), 163-175.

Kim, S. H., Kowalski, M. L., Carson, R. P., Bridges, L. R. and Ess, K. C. (2013) 'Heterozygous inactivation of tsc2 enhances tumorigenesis in p53 mutant zebrafish', *Dis Model Mech*.

Klugbauer, S. and Rabes, H. M. (1999) 'The transcription coactivator HTIF1 and a related protein are fused to the RET receptor tyrosine kinase in childhood papillary thyroid carcinomas', *Oncogene,* 18(30), 4388-93.

Kobayashi, T. and Cohen, P. (1999) 'Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2', *Biochemical Journal,* 339, 319-328.

Koike-Kumagai, M., Yasunaga, K., Morikawa, R., Kanamori, T. and Emoto, K. (2009) 'The target of rapamycin complex 2 controls dendritic tiling of Drosophila sensory neurons through the Tricornered kinase signalling pathway', *EMBO J,* 28(24), 3879-92.

Kota, R. S. and Runge, K. W. (1999) 'Tel2p, a regulator of yeast telomeric length in vivo, binds to single-stranded telomeric DNA in vitro', *Chromosoma,* 108(5), 278-90.

Kunz, J. and Hall, M. N. (1993) 'Cyclosporin A, FK506 and rapamycin: more than just immunosuppression', *Trends Biochem Sci,* 18(9), 334-8.

Lamouille, S., Connolly, E., Smyth, J. W., Akhurst, R. J. and Derynck, R. (2012) 'TGF-β-induced activation of mTOR complex 2 drives epithelial-mesenchymal transition and cell invasion', *J Cell Sci,* 125(Pt 5), 1259-73.

Land, S. C. and Tee, A. R. (2007) 'Hypoxia-inducible factor 1alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif', *J Biol Chem,* 282(28), 20534-43.

Lassot, I., Robbins, I., Kristiansen, M., Rahmeh, R., Jaudon, F., Magiera, M. M., Mora, S., Vanhille, L., Lipkin, A., Pettmann, B., Ham, J. and Desagher, S. (2010) 'Trim17, a novel E3 ubiquitin-ligase, initiates neuronal apoptosis', *Cell Death Differ,* 17(12), 1928-41.

Le Douarin, B., Nielsen, A. L., You, J., Chambon, P. and Losson, R. (1997) 'TIF1 alpha: a chromatin-specific mediator for the ligand-dependent activation function AF-2 of nuclear receptors?', *Biochem Soc Trans,* 25(2), 605-12.

Li, X., Gold, B., O'hUigin, C., Diaz-Griffero, F., Song, B., Si, Z., Li, Y., Yuan, W., Stremlau, M., Mische, C., Javanbakht, H., Scally, M., Winkler, C., Dean, M. and Sodroski, J. (2007a) 'Unique features of TRIM5alpha among closely related human TRIM family members', *Virology,* 360(2), 419-33.

Li, X., Song, B. W., Xiang, S. H. and Sodroski, J. (2007b) 'Functional interplay between the B-box 2 and the B30.2(SPRY) domains of TRIM5 alpha', *Virology,* 366, 234-244.

Li, Y., Inoki, K., Vacratsis, P. and Guan, K. L. (2003) 'The p38 and MK2 kinase cascade phosphorylates tuberin, the tuberous sclerosis 2 gene product, and enhances its interaction with 14-3-3', *J Biol Chem,* 278(16), 13663-71.

Ling, L. U., Tan, K. B., Lin, H. and Chiu, G. N. (2011) 'The role of reactive oxygen species and autophagy in safingol-induced cell death', *Cell Death Dis,* 2, e129.

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J. L., Bonenfant, D., Oppliger, W., Jenoe, P. and Hall, M. N. (2002) 'Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control', *Molecular Cell,* 10(3), 457-468.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K. and Avruch, J. (2005) 'Rheb binds and regulates the mTOR kinase', *Current Biology,* 15(8), 702-713.

Long, X. M., Spycher, C., Han, Z. S., Rose, A. M., Muller, F. and Avruch, J. (2002) 'TOR deficiency in C-elegans causes developmental arrest and intestinal atrophy by inhibition of mRNA translation', *Current Biology,* 12(17), 1448-1461.

Louria-Hayon, I., Grossman, T., Sionov, R., Alsheich, O., Pandolfi, P. and Haupt, Y. (2003) 'The promyelocytic leukemia protein protects p53 from Mdm2-mediated inhibition and degradation.', *J Biol Chem,* 278(35), 33134-41.

Lovejoy, C. A. and Cortez, D. (2009) 'Common mechanisms of PIKK regulation', *DNA Repair (Amst),* 8(9), 1004-8.

Ma, X. J. M. and Blenis, J. (2009) 'Molecular mechanisms of mTOR-mediated translational control', *Nature Reviews Molecular Cell Biology,* 10(5), 307-318.

Mah, A. S., Jang, J. and Deshaies, R. J. (2001) 'Protein kinase Cdc15 activates the Dbf2-Mob1 kinase complex', *Proc Natl Acad Sci U S A,* 98(13), 7325-30.

Makino, C., Sano, Y., Shinagawa, T., Millar, J. B. and Ishii, S. (2006) 'Sin1 binds to both ATF-2 and p38 and enhances ATF-2-dependent transcription in an SAPK signaling pathway', *Genes Cells,* 11(11), 1239-51.

Mammucari, C., Milan, G., Romanello, V., Masiero, E., Rudolf, R., Del Piccolo, P., Burden, S. J., Di Lisi, R., Sandri, C., Zhao, J., Goldberg, A. L., Schiaffino, S. and Sandri, M. (2007) 'FoxO3 controls autophagy in skeletal muscle in vivo', *Cell Metab,* 6(6), 458-71.

Manning, B. D. and Cantley, L. C. (2007) 'AKT/PKB signaling: Navigating downstream', *Cell,* 129(7), 1261-1274.

Marshall, C. J. (1994) 'MAP kinase kinase kinase, MAP kinase kinase and MAP kinase', *Current Opinion in Genetics and Development,* 4(1), 82-89.

Martens, J. H., Brinkman, A. B., Simmer, F., Francoijs, K. J., Nebbioso, A., Ferrara, F., Altucci, L. and Stunnenberg, H. G. (2010) 'PML-RARalpha/RXR Alters the Epigenetic Landscape in Acute Promyelocytic Leukemia', *Cancer Cell,* 17(2), 173-85.

Masoro, E. J. (2000) 'Caloric restriction and aging: an update', *Exp Gerontol,* 35(3), 299-305.

Medvedik, O., Lamming, D. W., Kim, K. D. and Sinclair, D. A. (2007) 'MSN2 and MSN4 link calorie restriction and TOR to sirtuin-mediated lifespan extension in Saccharomyces cerevisiae', *PLoS Biol,* 5(10), e261.

Meroni, G. and Diez-Roux, G. (2005) 'TRIM/RBCC, a novel class of 'single protein RING finger' E3 ubiquitin ligases.', *Bioessays,* 27(11), 1147-57.

Millar, J. B. A., Buck, V. and Wilkinson, M. G. (1995) 'PYP1 and PYP2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell-size at division in fission yeast', *Genes & Development,* 9(17), 2117-2130.

Minucci, S., Maccarana, M., Cioce, M., De Luca, P., Gelmetti, V., Segalla, S., Di Croce, L., Giavara, S., Matteucci, C., Gobbi, A., Bianchini, A., Colombo, E., Schiavoni, I., Badaracco, G., Hu, X., Lazar, M. A., Landsberger, N.,

Nervi, C. and Pelicci, P. G. (2000) 'Oligomerization of RAR and AML1 transcription factors as a novel mechanism of oncogenic activation', *Molecular Cell,* 5(5), 811-820.

Mondesire, W. H., Jian, W. G., Zhang, H. X., Ensor, J., Hung, M. C., Mills, G. B. and Meric-Bernstam, F. (2004) 'Targeting mammalian target of rapamycin synergistically enhances chemotherapy-induced cytotoxicity in breast cancer cells', *Clinical Cancer Research,* 10(20), 7031-7042.

Moustakas, A. and Heldin, C. H. (2007) 'Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression', *Cancer Sci,* 98(10), 1512-20.

Murakami, M., Ichisaka, T., Maeda, M., Oshiro, N., Hara, K., Edenhofer, F., Kiyama, H., Yonezawa, K. and Yamanaka, S. (2004) 'mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells', *Molecular and Cellular Biology,* 24(15), 6710-6718.

Murray, J. T., Campbell, D. G., Morrice, N., Auld, G. C., Shpiro, N., Marquez, R., Peggie, M., Bain, J., Bloomberg, G. B., Grahammer, F., Lang, F., Wulff, P., Kuhl, D. and Cohen, P. (2004) 'Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3', *Biochemical Journal,* 384, 477-488.

Nakashima, N., Noguchi, E. and Nishimoto, T. (1999) 'Saccharomyces cerevisiae putative G protein, Gtr1p, which forms complexes with itself and a novel protein designated as Gtr2p, negatively regulates the Ran/Gsp1p G protein cycle through Gtr2p', *Genetics,* 152(3), 853-67.

Napolitano, L. M., Jaffray, E. G., Hay, R. T. and Meroni, G. (2011) 'Functional interactions between ubiquitin E2 enzymes and TRIM proteins', *Biochem J,* 434(2), 309-19.

Napolitano, L. M. and Meroni, G. (2012) 'TRIM family: Pleiotropy and diversification through homomultimer and heteromultimer formation', *IUBMB Life,* 64(1), 64-71.

Ozato, K., Shin, D., Chang, T. and Morse, H. r. (2008) 'TRIM family proteins and their emerging roles in innate immunity.', *Nat Rev Immunol,* 8(11), 849-60.

Parada, L. A., Hallén, M., Tranberg, K. G., Hägerstrand, I., Bondeson, L., Mitelman, F. and Johansson, B. (1998) 'Frequent rearrangements of chromosomes 1, 7, and 8 in primary liver cancer', *Genes Chromosomes Cancer,* 23(1), 26-35.

Park, J., Leong, M. L. L., Buse, P., Maiyar, A. C., Firestone, G. L. and Hemmings, B. A. (1999) 'Serum and glucocorticoid-inducible kinase (SGK) is a target of the PI 3-kinase-stimulated signaling pathway', *Embo Journal,* 18(11), 3024-3033.

Pearce, L. R., Sommer, E. M., Sakamoto, K., Wullschleger, S. and Alessi, D. R. (2011) 'Protor-1 is required for efficient mTORC2-mediated activation of SGK1 in the kidney', *Biochem J,* 436(1), 169-79.

Peterson, R. T. and Schreiber, S. L. (1999) 'Kinase phosphorylation: Keeping it all in the family', *Current Biology,* 9(14), R521-R524.

Peterson, T. R., Laplante, M., Thoreen, C. C., Sancak, Y., Kang, S. A., Kuehl, W. M., Gray, N. S. and Sabatini, D. M. (2009) 'DEPTOR Is an mTOR Inhibitor Frequently Overexpressed in Multiple Myeloma Cells and Required for Their Survival', *Cell,* 137(5), 873-886.

Pickart, C. M. (2001) 'Mechanisms underlying ubiquitination', *Annu Rev Biochem,* 70, 503-33.

Pickart, C. M. and Eddins, M. J. (2004) 'Ubiquitin: structures, functions, mechanisms', *Biochim Biophys Acta,* 1695(1-3), 55-72.

Puigserver, P., Rhee, J., Donovan, J., Walkey, C. J., Yoon, J. C., Oriente, F., Kitamura, Y., Altomonte, J., Dong, H., Accili, D. and Spiegelman, B. M. (2003) 'Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction', *Nature,* 423(6939), 550-5.

Quy, P. N., Kuma, A., Pierre, P. and Mizushima, N. (2013) 'Proteasome-dependent activation of mammalian target of rapamycin complex 1 (mTORC1) is essential for autophagy suppression and muscle remodeling following denervation', *J Biol Chem,* 288(19), 13639.

Raab-Graham, K. F., Haddick, P. C., Jan, Y. N. and Jan, L. Y. (2006) 'Activity- and mTOR-dependent suppression of Kv1.1 channel mRNA translation in dendrites', *Science,* 314(5796), 144-8.

Regad, T., Saib, A., Lallemand-Breitenbach, V., Pandolfi, P. P., de Thé, H. and Chelbi-Alix, M. K. (2001) 'PML mediates the interferon-induced antiviral state against a complex retrovirus via its association with the viral transactivator', *EMBO J,* 20(13), 3495-505.

Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E., Messali, S., Cainarca, S., Guffanti, A., Minucci, S., Pelicci, P. G. and Ballabio, A. (2001) 'The tripartite motif family identifies cell compartments', *Embo Journal,* 20(9), 2140-2151.

Rhodes, D. A., de Bono, B. and Trowsdale, J. (2005) 'Relationship between SPRY and B30.2 protein domains. Evolution of a component of immune defence?', *Immunology,* 116(4), 411-417.

Sabile, A., Meyer, A. M., Wirbelauer, C., Hess, D., Kogel, U., Scheffner, M. and Krek, W. (2006) 'Regulation of p27 degradation and S-phase progression by Ro52 RING finger protein', *Molecular and Cellular Biology,* 26(16), 5994-6004.

Saci, A., Cantley, L. C. and Carpenter, C. L. (2011) 'Rac1 regulates the activity of mTORC1 and mTORC2 and controls cellular size', *Mol Cell,* 42(1), 50-61.

Sancak, Y., Bar-Peled, L., Zoncu, R., Markhard, A. L., Nada, S. and Sabatini, D. M. (2010) 'Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids', *Cell,* 141(2), 290-303.

Sancak, Y., Peterson, T. R., Shaul, Y. D., Lindquist, R. A., Thoreen, C. C., Bar-Peled, L. and Sabatini, D. M. (2008) 'The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1', *Science,* 320(5882), 1496-1501.

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

Sarbassov, D. D., Ali, S. M. and Sabatini, D. M. (2005a) 'Growing roles for the mTOR pathway', *Current Opinion in Cell Biology,* 17(6), 596-603.

Sarbassov, D. D., Guertin, D. A., Ali, S. M. and Sabatini, D. M. (2005b) 'Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex', *Science,* 307(5712), 1098-1101.

Sarbassov, D. D. and Sabatini, D. M. (2005) 'Redox regulation of the nutrient-sensitive raptor-mTOR pathway and complex', *Journal of Biological Chemistry,* 280(47), 39505-39509.

Saurin, A. J., Borden, K. L., Boddy, M. N. and Freemont, P. S. (1996) 'Does this have a familiar RING?', *Trends Biochem Sci,* 21(6), 208-14.

Schroder, W., Bushell, G. and Sculley, T. (2005) 'The human stress-activated protein kinase-interacting 1 gene encodes JNK-binding proteins.', *Cell Signal,* 17(6), 761-7.

Schroder, W., Cloonan, N., Bushell, G. and Sculley, T. (2004) 'Alternative polyadenylation and splicing of mRNAs transcribed from the human Sin1 gene', *Gene,* 339, 17-23.

Schroder, W. A., Buck, M., Cloonan, N., Hancock, J. F., Suhrbier, A., Sculley, T. and Bushell, G. (2007) 'Human Sin1 contains Ras-binding and pleckstrin homology domains and suppresses Ras signalling', *Cell Signal,* 19(6), 1279-89.

Schürmann, A., Brauers, A., Massmann, S., Becker, W. and Joost, H. G. (1995) 'Cloning of a novel family of mammalian GTP-binding proteins (RagA, RagBs, RagB1) with remote similarity to the Ras-related GTPases', *J Biol Chem,* 270(48), 28982-8.

Shiozaki, K. and Russell, P. (1995) 'cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast', *Nature,* 378(6558), 739-743.

Short, K. M. and Cox, T. C. (2006) 'Subclassification of the RBCC/TRIM superfamily reveals a novel motif necessary for microtubule binding', *Journal of Biological Chemistry,* 281(13), 8970-8980.

Silver, N., Proctor, G. B., Arno, M. and Carpenter, G. H. (2010) 'Activation of mTOR coincides with autophagy during ligation-induced atrophy in the rat submandibular gland', *Cell Death Dis,* 1, e14.

Skurat, A. V., Dietrich, A. D., Zhai, L. M. and Roach, P. J. (2002) 'GNIP, a novel protein that binds and activates glycogenin, the self-glucosylating initiator of glycogen biosynthesis', *Journal of Biological Chemistry,* 277(22), 19331-19338.

Soukas, A. A., Kane, E. A., Carr, C. E., Melo, J. A. and Ruvkun, G. (2009) 'Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in Caenorhabditis elegans', *Genes Dev,* 23(4), 496-511.

Sulis, M. L. and Parsons, R. (2003) 'PTEN: from pathology to biology', *Trends in Cell Biology,* 13(9), 478-483.

Sun, L. and Chen, Z. J. (2004) 'The novel functions of ubiquitination in signaling', *Curr Opin Cell Biol,* 16(2), 119-26.

Tamaskovic, R., Bichsel, S. J., Rogniaux, H., Stegert, M. R. and Hemmings, B. A. (2003) 'Mechanism of Ca2+-mediated regulation of NDR protein kinase through autophosphorylation and phosphorylation by an upstream kinase', *J Biol Chem,* 278(9), 6710-8.

Tang, S. J., Reis, G., Kang, H., Gingras, A. C., Sonenberg, N. and Schuman, E. M. (2002) 'A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus', *Proc Natl Acad Sci U S A,* 99(1), 467-72.

Thiery, J. P. and Sleeman, J. P. (2006) 'Complex networks orchestrate epithelial-mesenchymal transitions', *Nat Rev Mol Cell Biol,* 7(2), 131-42.

Thoreen, C. C., Kang, S. A., Chang, J. W., Liu, Q. S., Zhang, J. M., Gao, Y., Reichling, L. J., Sim, T. B., Sabatini, D. M. and Gray, N. S. (2009) 'An ATP-competitive Mammalian Target of Rapamycin Inhibitor Reveals Rapamycin-resistant Functions of mTORC1', *Journal of Biological Chemistry,* 284(12), 8023-8032.

Tsai, W. W., Wang, Z., Yiu, T. T., Akdemir, K. C., Xia, W., Winter, S., Tsai, C. Y., Shi, X., Schwarzer, D., Plunkett, W., Aronow, B., Gozani, O., Fischle, W., Hung, M. C., Patel, D. J. and Barton, M. C. (2010) 'TRIM24 links a non-canonical histone signature to breast cancer', *Nature,* 468(7326), 927-32.

Tucci, P. (2012) 'Caloric restriction: is mammalian life extension linked to p53?', *Aging (Albany NY),* 4(8), 525-34.

Tullet, J. M. A., Hertweck, M., An, J. H., Baker, J., Hwang, J. Y., Liu, S., Oliveira, R. P., Baumeister, R. and Blackwell, T. K. (2008) 'Direct inhibition of the longevity-promoting factor SKN-1 by insulin-like signaling in C. elegans', *Cell,* 132(6), 1025-1038.

van de Loosdrecht, A. A., Beelen, R. H., Ossenkoppele, G. J., Broekhoven, M. G. and Langenhuijsen, M. M. (1994) 'A tetrazolium-based colorimetric MTT assay to quantitate human monocyte mediated cytotoxicity against leukemic cells from cell lines and patients with acute myeloid leukemia', *J Immunol Methods,* 174(1-2), 311-20.

van Wijk, S. J. and Timmers, H. T. (2010) 'The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins', *FASEB J,* 24(4), 981-93.

Vander Haar, E., Lee, S., Bandhakavi, S., Griffin, T. J. and Kim, D. H. (2007) 'Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40', *Nature Cell Biology,* 9(3), 316-U126.

Verheij, M., Ruiter, G. A., Zerp, S. F., van Blitterswijk, W. J., Fuks, Z., Haimovitz-Friedman, A. and Bartelink, H. (1998) 'The role of the stress-activated protein kinase (SAPK/JNK) signaling pathway in radiation-induced apoptosis', *Radiotherapy and Oncology,* 47(3), 225-232.

Walker, K. S., Deak, M., Paterson, A., Hudson, K., Cohen, P. and Alessi, D. R. (1998) 'Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with protein kinase B alpha', *Biochemical Journal,* 331, 299-308.

Wang, L., Heidt, D. G., Lee, C. J., Yang, H., Logsdon, C. D., Zhang, L., Fearon, E. R., Ljungman, M. and Simeone, D. M. (2009) 'Oncogenic function of ATDC in pancreatic cancer through Wnt pathway activation and beta-catenin stabilization', *Cancer Cell,* 15(3), 207-19.

Wang, S. Y., Gao, J., Lei, Q. Y., Rozengurt, N., Pritchard, C., Jiao, J., Thomas, G. V., Li, G., Roy-Burman, P., Nelson, P. S., Liu, X. and Wu, H. (2003) 'Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer', *Cancer Cell,* 4(3), 209-221.

Waskiewicz, A. J. and Cooper, J. A. (1995) 'Mitogen and stress-response pathways - MAP kinase cascades and phosphatase regulation in mammals and yeast', *Current Opinion in Cell Biology,* 7(6), 798-805.

Weissman, A. M. (1997) 'Regulating protein degradation by ubiquitination', *Immunol Today,* 18(4), 189-98.

Weissman, A. M., Shabek, N. and Ciechanover, A. (2011) 'The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation', *Nat Rev Mol Cell Biol,* 12(9), 605-20.

Wilkinson, M., Pino, T., Tournier, S., Buck, V., Martin, H., Christiansen, J., Wilkinson, D. and Millar, J. (1999) 'Sin1: an evolutionarily conserved component of the eukaryotic SAPK pathway.', *EMBO J,* 18(15), 4210-21.

Wong, N., Lai, P., Pang, E., Leung, T. W., Lau, J. W. and Johnson, P. J. (2000) 'A comprehensive karyotypic study on human hepatocellular carcinoma by spectral karyotyping', *Hepatology,* 32(5), 1060-8.

Wu, X. N., Wang, X. K., Wu, S. Q., Lu, J., Zheng, M., Wang, Y. H., Zhou, H., Zhang, H. and Han, J. (2011) 'Phosphorylation of Raptor by p38beta participates in arsenite-induced mammalian target of rapamycin complex 1 (mTORC1) activation', *J Biol Chem,* 286(36), 31501-11.

Wu, Y. T., Ouyang, W., Lazorchak, A. S., Liu, D., Shen, H. M. and Su, B. (2011) 'mTOR complex 2 targets Akt for proteasomal degradation via phosphorylation at the hydrophobic motif', *J Biol Chem,* 286(16), 14190-8.

Wullschleger, S., Loewith, R., Oppliger, W. and Hall, M. N. (2005) 'Molecular organization of target of rapamycin complex 2', *Journal of Biological Chemistry,* 280(35), 30697-30704.

Xu, J., Lamouille, S. and Derynck, R. (2009) 'TGF-beta-induced epithelial to mesenchymal transition', *Cell Res,* 19(2), 156-72.

Yamauchi, K., Wada, K., Tanji, K., Tanaka, M. and Kamitani, T. (2008) 'Ubiquitination of E3 ubiquitin ligase TRIM5 alpha and its potential role', *FEBS J,* 275(7), 1540-55.

Yang, J., Cron, P., Thompson, V., Good, V. M., Hess, D., Hemmings, B. A. and Barford, D. (2002) 'Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation', *Mol Cell,* 9(6), 1227-40.

Yang, Q., Inoki, K., Ikenoue, T. and Guan, K. (2006) 'Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity.', *Genes Dev,* 20(20), 2820-32.

Yao, Y., Suraokar, M., Darnay, B. G., Hollier, B. G., Shaiken, T. E., Asano, T., Chen, C. H., Chang, B. H., Lu, Y., Mills, G. B., Sarbassov, D., Mani, S. A., Abbruzzese, J. L. and Reddy, S. A. (2013) 'BSTA Promotes mTORC2-Mediated Phosphorylation of Akt1 to Suppress Expression of FoxC2 and Stimulate Adipocyte Differentiation', *Sci Signal,* 6(257), ra2.

Ye, Y. and Rape, M. (2009) 'Building ubiquitin chains: E2 enzymes at work', *Nat Rev Mol Cell Biol,* 10(11), 755-64.

Yilmaz, M. and Christofori, G. (2009) 'EMT, the cytoskeleton, and cancer cell invasion', *Cancer Metastasis Rev,* 28(1-2), 15-33.

Yonemoto, W., McGlone, M. L., Grant, B. and Taylor, S. S. (1997) 'Autophosphorylation of the catalytic subunit of cAMP-dependent protein kinase in Escherichia coli', *Protein Engineering,* 10(8), 915-925.

Yoon, Y. H., Cho, K. S., Hwang, J. J., Lee, S. J., Choi, J. A. and Koh, J. Y. (2010) 'Induction of lysosomal dilatation, arrested autophagy, and cell death by chloroquine in cultured ARPE-19 cells', *Invest Ophthalmol Vis Sci,* 51(11), 6030-7.

Yuan, Z., Villagra, A., Peng, L., Coppola, D., Glozak, M., Sotomayor, E. M., Chen, J., Lane, W. S. and Seto, E. (2010) 'The ATDC (TRIM29) protein binds p53 and antagonizes p53-mediated functions', *Mol Cell Biol,* 30(12), 3004-15.

Zarubin, T. and Han, J. (2005) 'Activation and signaling of the p38 MAP kinase pathway', *Cell Res,* 15(1), 11-8.

Zha, X., Hu, Z., He, S., Wang, F., Shen, H. and Zhang, H. (2011) 'TSC1/TSC2 inactivation inhibits AKT through mTORC1-dependent up-regulation of STAT3-PTEN cascade', *Cancer Lett,* 313(2), 211-7.

Zhai, L. M., Dietrich, A., Skurat, A. V. and Roach, P. J. (2004) 'Structure-function analysis of GNIP, the glycogenin-interacting protein', *Archives of Biochemistry and Biophysics,* 421(2), 236-242.

Zhao, J., Brault, J. J., Schild, A., Cao, P., Sandri, M., Schiaffino, S., Lecker, S. H. and Goldberg, A. L. (2007) 'FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells', *Cell Metab,* 6(6), 472-83.

Zheng, M., Wang, Y. H., Wu, X. N., Wu, S. Q., Lu, B. J., Dong, M. Q., Zhang, H., Sun, P., Lin, S. C., Guan, K. L. and Han, J. (2011) 'Inactivation of Rheb by PRAK-mediated phosphorylation is essential for energy-depletion-induced suppression of mTORC1', *Nat Cell Biol,* 13(3), 263-72.

Zinzalla, V., Stracka, D., Oppliger, W. and Hall, M. N. (2011) 'Activation of mTORC2 by association with the ribosome', *Cell,* 144(5), 757-68.