Role of eIF3d on the mTOR signalling pathway

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ABSTRACT

The University of Manchester. Submitted by Pablo Antonio Binder Saldaña for the degree of Doctor of Philosophy, and entitled: Role of eIF3d on the mTOR signalling pathway. September 2015.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and participates in sensing and integration of a variety of environmental cues. As a central regulator of cell metabolism, mTOR has gained attention due to its role in many human diseases, including cancer and diabetes. Thus, great efforts are being made to understand signalling via the mTOR pathway as it represents an interesting pharmacological target. In higher eukaryotes, mTOR exists in two evolutionarily conserved multi-protein complexes, TORC1 and TORC2. These complexes contain core components that define each complex and regulate their activities. mTORC1 signalling has been extensively studied but mTORC2 is still poorly understood. The discovery that mTORC2 is a key regulator of the activities of the AGC family kinases AKT, PKC and SGK1, and is involved in cellular processes such as proliferation and cell survival in response to growth factors, has stimulated the study of its activation and regulation.

A core component of mTORC2 is stress-activated protein kinase interacting protein (SIN1), which is essential for the assembly and kinase activity of the complex and has been proposed to act as a scaffold to recruit mTOR substrates to facilitate their phosphorylation. A yeast two-hybrid screen uncovered novel SIN1 binding proteins that may be potential regulators of mTORC2 activity. One of these is subunit D of the Eukaryotic translation initiation factor 3 (eIF3d), a conserved protein involved in protein metabolism and cytoskeletal organization. This interaction has been confirmed by pull-down assays with overexpressed proteins in HEK-293 cells and the C-terminal region of eIF3d has been identified as critical for this interaction. Moreover, it has been confirmed that recombinant and endogenous eIF3d co-precipitate with endogenous SIN1 but not the mTORC2 components RICTOR and mTOR or the mTORC1 component RAPTOR. It is also shown that the interaction of SIN1 and eIF3d is likely to be occurring in the nucleus. In Hela cells, knockdown of eIF3d expression causes a decrease in the phosphorylation of the mTORC2 targets AKT and PKC on their turn motif sites, but also leads to a significant increase in the phosphorylation and activation of the mTORC1 target S6 kinase. This suggests that eIF3d is a regulator of both mTORC1 and mTORC2 signalling. Reduced expression of eIF3d leads to an increase in cell size, decreased cell proliferation and impairment of cell cycle progression, possibly by promotion of mitotic spindle multipolarity. Additionally, eIF3d knockdown leads to a delay in stress granule formation in Hela cells under arsenite stress, indicating that eIF3d might be acting as a negative mTORC1 regulator by contributing to its stress granule mediated inhibition. Thus eIF3d appears to be a regulator of cell size, proliferation and stress responses, in part mediated by modulation of mTOR activity.
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Abbreviations

4EBP1 – eIF4E-binding protein 1
14-3-3 – 14th fraction bovine brain homogenate, electrophoretic position 3.3

A
AA – amino acid
AGC Kinase – PKA/PKG/PKC protein kinase family
AMP – adenosine monophosphate
AMPK – AMP-dependent protein kinase
APS – ammonium persulphate

B
BP – base pair
BSA – bovine serum albumin

C
CDK – cyclin dependent kinase
CREB – cAMP response element-binding protein
CRIM – conserved region in the middle

D
DAPI – 4',6-diamidino-2-phenylindole
DEP domain – Disheveled, Egl-10 and Pleckstrin domain
DEPTOR – DEP-domain containing mTOR-interacting protein
DMEM – Dulbecco’s modified eagle’s medium
DNA – deoxyribonucleic acid
dNTP – deoxyribonucleotide triphosphate
DTT – Dithiothreitol

E
E. coli – *Escherichia coli*
EDTA – ethylenediaminetetraacetic acid
eEF – eukaryotic elongation factor
EGF – epidermal growth factor
EGTA – ethylene glycol tetra acetic acid
eIF – eukaryotic initiation factor
ER – endoplasmic reticulum
ERAD – Endoplasmic-reticulum-associated protein degradation
ERK – extracellular-signal-regulated kinase

F
FBS – fetal bovine serum
FKBP – FK506-binding protein
FOX – forkhead box transcription factor family
Fwd. – forward primer

G
G3BP – Ras GTPase-activating protein-binding protein 1
GAP – GTPase activating protein
GDP – guanosine diphosphate
GEF - Guanine nucleotide exchange factor
GFP – green fluorescent protein
Grb – growth factor receptor-bound protein
GS – glycogen synthase
GSH – glutathione tripeptide
GSK – GS kinase
GST – glutathione-S-transferase
GTP – guanosine triphosphate

H
H – histone
HBSS – Hanks’ Balanced Salt Solution
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF – hypoxia inducible factor
HM – hydrophobic motif
Hsp – heat shock protein

I
Ig – immunoglobulin
IGF – insulin like growth factor
IkB – inhibitor of NFkB
ILR – insulin like receptor
IP – immunoprecipitation
IP3R – Inositol trisphosphate receptor
IRS – insulin receptor substrate

J
JNK – c-Jun N-terminal kinase

K
kDA – kilo Daltons
L
LKB1 – liver kinase B1

M
MAM – mitochondria-associated ER membrane
MAPK – mitogen activated protein kinase
MAPKAPK – MAPK-activated protein kinase
MCS – multiple cloning site
MEF – mouse embryonic fibroblast
MEK – MAPK/ERK kinase
miRNA – micro RNA
mLST8 – mammalian lethal with Sec13 protein 8
mRNA – messenger RNA
mTOR – mammalian target of rapamycin
MTT – thiazoly blue tetrazodium bromide

N
NES – nuclear export sequence
NF1 – neurofibromatosis type 1
NFkB – nuclear factor k-light-chain-enhancer of activated B cells
NLS – nuclear localisation sequence

O
ORF – open reading frame

P
PACS2 – phosphofurin acidic cluster sorting protein 2
PAGE – polyacrylamide gel electrophoresis
PBS – Phosphate Buffered Saline

PDCD4 – programmed cell death protein 4

PCR – polymerase chain reaction

PDK1 – 3-phosphoinositide-dependent kinase 1

PFA – paraformaldehyde

PI(3,4,5)P₃ – phosphatidylinositol-3,4,5-trisphosphate

PI(4,5)P₂ – phosphatidylinositol-4,5-bisphosphate

PI3K – class 1a phosphoinositide-3-kinase

PIKK – PI3K-related kinase

PH – Pleckstrin homology

PKA – cAMP-dependent protein kinase

PKC – protein kinase C

PLK – polo-like kinase

PMSF – phenylmethylsulfonyl fluoride

PNK – polynucleotide kinase

PPAR – peroxisome proliferator-activated receptor

PRAS40 – proline-rich AKT (PKB) substrate 40

PROTOR – protein observed with RICTOR

PTEN – phosphatase and tensin homologue

PVDF – polyvinylidene fluoride

R

Rac – subfamily of Rho GTPases

Raf – v raf-1 murine leukaemia viral oncogene homolog 1

Rag – subfamily Ras-like small GTPases

RAPTOR – regulatory-associated-protein of mTOR

Ras – rat sarcoma

RBD – Raf-like Ras-binding domain
RBS – RNA-binding site
REDD – regulated in development and DNA damage responses
Redox – reduction/oxidation
Rev – reverse primer
Rheb – Ras homology enriched in brain
RICTOR – rapamycin-insensitive companion of mTOR
RNA – ribonucleic acid
RNAi – RNA interference
ROS – reactive oxygen species
RPM – revolutions per minute
rRNA – ribosomal RNA
RSK – p90 ribosomal S6 kinase
RT – room temperature
RTK – receptor tyrosine kinase

S
S6K – p70 ribosomal S6 kinase
S. cerevisiae – Saccharomyces cerevisiae
SAPK – stress-activated protein kinase
SD – standard deviation
SDS – sodium dodecyl sulphate
SEM – standard error of the mean
SGK – serum glucocorticoid induced kinase
shRNA – short hairpin RNA
SIN1 – stress activated protein kinase interacting protein 1
siRNA – small interfering RNA
SKAR – S6K1 Aly/ REF-like target
SREBP – sterol regulatory binding element protein
T
TBC – Tre2-Bub2-Cdc16
TBC1D7 – TBC-1 domain family, member 7
TEL2 – telomere maintenance 2
TEMED – N, N, N’, N’-tetramethylethylenediamine
TLB – Triton lysis buffer
TM – turn motif
TOR – target of rapamycin
Tris – tris(hydroxymethyl)aminomethane
TSC – tuberous sclerosis

U
U – units of specific activity
ULK – Unc-51-like-kinase
Unc – uncoordinated family member
UTR – untranslated region
UV – ultraviolet

W
Wnt – wingless and integration

Y
YAP – Yes-associated protein
Chapter 1: Introduction

Living organisms have developed mechanisms to respond to physical and chemical variation in their environment, allowing them to survive, grow and proliferate when conditions are appropriate. Cells respond through the activation of nutrient-sensing pathways in order to regulate their size, growth and metabolism. In addition to these pathways, the control of such processes is regulated by the action of factors secreted by other cells (Chantranupong et al., 2015). The understanding of how cell growth and proliferation is controlled in response to environmental signals is still an unresolved biological problem. Studies initially performed in yeast have demonstrated that the protein kinase Target of Rapamycin (TOR) participates in regulating the synthesis of macromolecules required for essential cellular processes. It acts as a network integrator of metabolism in response to environmental cues (Howell and Manning, 2011).

1.1 Mammalian Target of Rapamycin

The mammalian target of rapamycin (mTOR), is a large (290 kD) multi-domain protein conserved from yeast to mammals (Figure 1). It is a serine/threonine kinase, that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family and participates in sensing, integrating and responding to a variety of environmental cues, controlling cellular and organismal metabolism, growth, proliferation and survival (Wullschleger et al., 2006). TOR was originally identified by mutations in the Tor1 and Tor2 genes that conferred resistance to the macrolide antibiotic rapamycin in the budding yeast Saccharomyces cerevisiae (Heitman et al, 1991). Rapamycin (or Sirulimus) is produced by the bacteria Streptomyces hygroscopicus and originally gained attention due to its antiproliferative properties. Rapamycin can bind
Figure 1. mTOR kinase. Schematic representation of the general domain structure of the mTOR protein. At the amino terminus, there is a cluster of HEAT (huntingtin, elongation factor 3, a subunit of protein phosphatase 2A and TOR1) repeats. These are followed by a FRAP, ATM and TRRAP (FAT) domain; the FKBP12-rapamycin binding (FRB) domain; the Ser/Thr kinase catalytic domain (KINASE); the regulatory domain (RD); and the carboxy-terminal FATC domain. Indicated are phosphorylation mTOR sites promoting its activity: Ser1261 (phosphorylated by insulin/PI3K), Thr2446 (phosphorylated by AMPK and S6K), Ser2448 (phosphorylated by S6K and AKT), Ser2481 (autophosphorylation site). Amino acid numbers refer to the human mTOR protein. The functions of each domain are indicated. Modified from Watanabe et al., 2011.
and inhibit TOR in a complex with FK506-binding protein 12 (FKBP12) (Fingar and Blenis, 2004).

Unlike yeast, which possesses two Tor genes, all higher eukaryotes possess a single TOR gene. mTOR interacts with several proteins, and exists in two evolutionary conserved multi-protein complexes, mTORC1 and mTORC2 (Figure 2). These two complexes are functionally and structurally distinct and their subunits define their specificity (Wullschleger et al., 2006). Acute exposure to rapamycin inhibits mTORC1 but not mTORC2, thus the activities of the two complexes can be distinguished by their sensitivity to rapamycin. However, prolonged treatment with rapamycin can disrupt mTORC2 assembly as well (Sarbassov et al., 2006). In response to diverse environmental cues, the mTOR signalling pathway impacts on most cellular functions. The major roles of mTOR are exerted through the regulation of members of the AGC family of protein kinases including AKT, Protein Kinase C alpha (PKCα), ribosomal S6 protein Kinase (S6K) and Serum/Glucocorticoid Regulated Kinase 1 (SGK1) (Jacinto and Lorberg, 2008). They are phosphorylated in their kinase activation loop by PDK1 (phosphoinositide-dependent kinase 1) and in the turn and hydrophobic motifs by mTOR (Jacinto and Lorberg, 2008). There is increasing evidence that deregulation of mTOR, resulting in the alteration of the activities of these kinases, occurs in several pathologies such as cancer, type 2 diabetes, and neurodegeneration. Thus, great efforts are being made to understand and pharmacologically target this pathway (Laplante and Sabatini, 2012).

1.1.1 mTOR complexes

The mTOR kinase interacts with several proteins to form two TOR complexes (mTORC1 and mTORC2). These mTOR-containing complexes contain different core components that define each complex and regulate their activities. The two
Figure 2. The mTORC1 and mTORC2 complexes. Schematic representation of the structure, upstream regulators and downstream cellular effects of the protein complex mTORC1 (A) and mTORC2 (B). Modified from Foster and Fingar, 2010.
complexes have different sensitivities to rapamycin and distinct upstream regulators and downstream effectors.

1.1.1.1 mTOR Complex 1

1.1.1.1.1 Signalling via mTORC1

Most of the existing knowledge about mTOR biology relates to cellular functions of mTORC1. It is composed of six core components. The regulatory-associated protein of mTOR (RAPTOR) and proline-rich AKT substrate 40 kDa (PRAS40) are exclusively found in mTORC1, whereas mammalian lethal with Sec13 protein 8 (mLST8, also called GβL), DEP-domain-containing mTOR-interacting protein (DEPTOR), the TTI1/TEL2 complex (Laplante and Sabatini, 2009; Kaizuka et al., 2010) and mTOR itself are shared by both complexes. The association with RAPTOR is the key defining feature of mTORC1. RAPTOR acts as a modulator of mTORC1 activity by stabilising the assembly of the complex and as a scaffold for mTORC1 substrate recruitment (Kim et al., 2002). The TTI1/TEL2 complex appears to be important for mTOR stability and the activity of both mTOR complexes by maintaining their assembly (Kaizuka et al., 2010). PRAS40 and DEPTOR are negative regulators of mTORC1 that impair substrate binding (Wang et al., 2007; Peterson et al., 2009). The role of mLST8 in mTORC1 is less clear (Guertin et al., 2006).

1.1.1.1.2 Regulation of mTORC1

An interesting feature of mTORC1 is the diversity of upstream signals that it senses. It integrates signals from cellular and extracellular cues such as growth factors, stress, energy levels, and oxygen and nutrient availability, in order to control
anabolic processes including protein and lipid synthesis, ribosome biogenesis and autophagy inhibition (Wullschleger et al., 2006). One of the key upstream regulators of mTORC1 is the TSC complex, a heterotrimer of tuberous sclerosis 1 (TSC1, hamartin), TSC2 (tuberin) and TBC1D7 (Dibble et al., 2012). This complex transmits most of the mTORC1 upstream signals by acting as a GTPase-activating protein (GAP) for the GTPase Ras homolog enriched in brain (Rheb). The GTP-bound form of Rheb interacts with mTORC1 and stimulates its kinase activity. Thus, the TSC complex negatively regulates mTORC1 activity by promoting Rheb-GTP hydrolysis (Inoki et al., 2003). Growth factors, including insulin and insulin-like growth factor 1 (IGF1), can regulate mTORC1 activity through the stimulation of the PI3K and Ras pathways. The effector kinases of these pathways, AKT, ERK1/2, and S6K phosphorylate the TSC-TBC complex to inactivate it, consequently activating mTORC1 (Inoki et al., 2002; Ma et al., 2005; Roux et al., 2004). AKT also promotes mTORC1 signalling in a TSC-independent manner by phosphorylating the negative regulator PRAS40, which causes its dissociation from RAPTOR (Sancak et al., 2007; Wang et al., 2007). In addition, mTORC1 also directly phosphorylates PRAS40, resulting in its dissociation from RAPTOR and further activating the complex activity (Oshiro et al., 2007).

One of the main functions of mTORC1 is to sense changes in nutrient availability. Amino acids, particularly leucine and arginine, are essential for the activation of the complex (Hara et al., 1998). The amino acid-dependent activation of mTOR requires the Rag GTPases (Kim et al., 2008). In mammals there are four Rag proteins, RagA to RagD, that associate in heterodimers of Rag A or B with Rag C or D. When amino acid levels are low, RagA/B is loaded with GDP and RagC/D with GTP in an inactive complex. In response to amino acid stimulation, in a process dependent on v-ATPase, the lysosomal membrane-associated complex, Ragulator (Sancak et al.,
promotes the recruitment of the Rag GTPases to the lysosome. At the lysosome surface, Ragulator exerts GEF activity towards RagA/B (Bar-Peled et al., 2012) promoting their loading with GTP. The RagA/B-GTP - RagC/D-GDP heterodimer interacts with RAPTOR (Sancak et al., 2008) resulting in the recruitment mTORC1 to the lysosome surface. Translocation of mTORC1 to the lysosome brings it into proximity with its activator Rheb which, in its GTP-bound form, directly stimulates mTORC1 kinase activity. Upon amino acid or growth factor withdrawal, the Rag GTPases in their inactive RagA/B-GDP - RagC/DGTP configuration are unable to recruit mTORC1 but are able to bind and recruit the TSC complex to the lysosome, thereby promoting the inhibition of Rheb and mTORC1 signalling (Demetriades et al., 2014; Menon et al., 2014). In response to insulin stimulation, the TSC complex dissociates from the lysosomal surface and is no longer able to suppress Rheb activity. This couples the nutrient sensing mechanism with growth factor stimulation in regulating mTOR signalling (Menon et al., 2014).

The exact mechanism by which the nutrient-dependent mTORC1 activation machinery senses amino acids is not fully understood. It has been proposed that this requires the presence of amino acids in the lysosomal lumen and involves the participation of the lysosomal amino acid transporter SLC38A9 which, through interaction with the Ragulator-Rag GTPases complex, mediates mTORC1 activation in response to arginine levels (Rebsamen et al., 2015; Wang and Holst, 2015). In addition, Leucyl-tRNA synthetase participates in the sensing of intracellular leucine concentration, acting as a GTPase-activating protein (GAP) towards RagD and, contributing to mTORC1 activation (Han et al., 2012).

In addition to requiring the availability of amino acids, protein synthesis is a major energy-consuming process and cells need to be able to regulate the activation of
translation when the energy is scarce. In response to hypoxia or energy deprivation, adenosine monophosphate-activated protein kinase (AMPK) acts as a sensor and regulates mTORC1 through the TSC complex. AMPK activates TSC2 by direct phosphorylation, increasing its GAP activity toward Rheb (Inoki et al., 2003b). In response to energy stress, AMPK also can directly phosphorylate RAPTOR which promotes its binding to 14-3-3 and leads to mTORC1 inhibition (Gwinn et al., 2008). In addition, the protein REDD1 (Regulation of DNA Damage Response 1) binds 14-3-3 in response to hypoxia and releases TSC2 from its 14-3-3 mediated inhibition resulting in mTORC1 inactivation when energy levels are low (DeYoung et al., 2008).

1.1.1.1.3 Non-classical mTORC1 regulators

In addition to growth factors, nutrient availability and energy status, mTOR also responds to other recently described inputs such as the Hippo, WNT and Notch signalling pathways (Shimobayashi and Hall, 2014). The Hippo pathway negatively regulates mTOR activity through phosphorylation and inhibition of the Yes-associated protein (YAP). Active YAP stimulates transcription of the microRNA miR-29, which in turn inhibits the translation of PTEN, thus activating PI3K signalling and both mTORC1 and mTORC2. The canonical WNT signalling pathway promotes mTORC1 activity by preventing the phosphorylation and induction of the TSC complex by GSK3β whilst hyperactivation of Notch signalling increases RAPTOR protein expression and its interaction with mTOR (Shimobayashi and Hall, 2014).

It has also been shown that micro RNAs target the mTOR pathway at several levels, either by directly affecting the expression of mTOR kinase, as is the case of miR-7 (Wang et al., 2013), miR-99a (Jin et al., 2013) miR-100 (Nagaraja et al., 2010) and
miR-101 (Chen et al., 2012), or by targeting key genes within the pathway including those expressing upstream regulators of mTOR, such as IGF-R, IRS1, PI3K, AKT and PTEN (AlQurashi et al., 2013).

1.1.1.4 mTORC1 effectors

The main cellular process associated with mTORC1 activity is protein synthesis. Specific active-site inhibitors of mTOR significantly reduce overall rates of protein synthesis in proliferating cultured cells (Beretta et al., 1996). The best-characterised downstream targets of mTORC1 are ribosomal S6 kinase (S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding protein (4E-BP1), both of which regulate mRNA translation (Foster and Fingar, 2010) (Figure 3). mTORC1-dependent phosphorylation of S6K1 and 4E-BP1 is facilitated by the eIF3 complex that functions as a scaffold to localise it to the ribosome and promote its interaction with its substrates (Holz et al., 2005). The phosphorylation of 4E-BP1 at multiple sites by mTOR prevents its binding to the cap-binding protein eIF4E, enabling eIF4E to associate with eIF4G to form the eIF4F complex that is required for the initiation of mRNA translation (Dennis et al., 2012).

S6K1 participates in the regulation of translation initiation, growth and ribosome biogenesis through the phosphorylation of various effectors. The main target of S6K is ribosomal protein S6, a component of the 40S ribosome subunit that is essential for protein synthesis, and ribosomal biogenesis (Chauvin et al., 2014). Other S6K targets that stimulate protein synthesis are eIF4B (eukaryotic translation initiation factor 4B, PDCD4 (programmed cell death protein 4), eEF2K (eukaryotic elongation factor-2 kinase and SKAR (S6K1 Aly/ REF-like target) (Magnuson et al., 2011).
Figure 3. mTORC1 pathway. General schematic representation of the structure, upstream regulators, downstream targets and cellular effects of the protein complex mTORC1. Modified from Wataya-Kaneda, 2015. For detailed information see section 1.1.1.1.1.
mTORC1 also controls other anabolic pathways such as the synthesis of lipids. It participates in the activation of peroxisome proliferator-activated receptor γ (PPAR-γ), a known master regulator of adipogenesis (Zhang et al., 2009). mTORC1 also regulates, via the phosphorylation of S6K and Lipin-1, the activity of sterol regulatory element-binding protein 1/2 (SREBP1/2), a transcription factor controlling the expression lipogenic genes (Li et al., 2011 and Peterson et al., 2011).

A further function of mTORC1 that contributes to cell growth is the negative regulation of autophagy. Autophagy is required for the recycling of organelles as a form of adaptation to nutrient deprivation (Boya et al., 2013). mTORC1 directly phosphorylates the autophagy initiator ULK1 (unc-51-like kinase 1) which results in the suppression of its activity and consequently the inhibition of autophagy (Jung et al., 2009).

1.1.1.2 mTOR Complex 2

1.1.1.2.1 Signalling via mTORC2

mTORC2 is composed of seven different proteins, some of them being common to mTORC1, and all appear to be structurally and functionally conserved in eukaryotes from yeast to mammalian cells. These are mTOR, rapamycin-insensitive companion of mTOR (RICTOR), stress-activated protein kinase interacting protein (SIN1), protein observed with RICTOR-1 (PROTOR-1), mLST8, DEPTOR and the TTI1/TEL2 complex (Laplante and Sabatini, 2009; Kaizuka et al., 2010). The stability and functional integrity of mTORC2 is dependent on the presence of the core subunits RICTOR and SIN1 (Frias et al., 2006). RICTOR interacts with PROTOR-1 (Woo et al., 2007) which appears to play a role in mTORC2 mediated activation of SGK1, but not AKT or PKCα, in kidney cells (Pearce et al., 2011). mLST8 is
essential for maintaining the RICTOR-mTOR association and its disruption prevents
the mTORC2 mediated phosphorylation of AKT and PKCα (Guertin et al, 2006).
DEPTOR, as with mTORC1, acts as a negative regulator of mTORC2 activity
(Peterson et al, 2009).

Acute exposure to rapamycin inhibits mTORC1 but not mTORC2, thus the activities
of the two complexes can be distinguished by their sensitivity to rapamycin. However, prolonged treatment with rapamycin can disrupt mTORC2 assembly and activity as well (Sarbassov et al., 2006). Its later discovery, relative resistance to rapamycin (as it is resistant only to acute exposure), and the absence of mTORC2 specific inhibitors has made mTORC2 regulation more difficult to address. Deletion of the mTORC2 components RICTOR, SIN1 or mLST8 causes early embryonic lethality in mice which has also complicated the study of mTORC2 function (Jacinto et al., 2006).

1.1.1.2.2 Regulation of mTORC2

mTORC2 responds primarily to growth factors in a PI3K-dependent manner,
participating as a key regulator in processes such as cell survival, metabolism,
proliferation and cytoskeletal organisation (Oh and Jacinto, 2011). However, the mechanism leading to mTORC2 activation is not well understood. A recent report showed that in response to insulin, PDK1 phosphorylation of AKT at Thr308, increases its kinase activity sufficiently to mediate SIN1 phosphorylation at Thr86. Phosphorylation of SIN1 results in enhanced mTORC2 kinase activity and AKT Ser473 phosphorylation leading to full activation of AKT (Yang et al., 2015). Growth factors can also regulate mTORC2 activity through the TSC complex. Unlike their negative regulation of mTORC1, they positively regulate mTORC2 activity in a GAP-independent manner by physically associating with the complex (Huang et al, 2008).
1.1.1.2.3 mTORC2 effectors

mTORC2 is the kinase responsible for phosphorylating the turn motif (TM) and the hydrophobic motif (HM) of the AGC kinases (Jacinto and Lorberg, 2008). The best characterised mTORC2 substrate is AKT, which regulates cellular processes such as cell survival, growth, and proliferation through the phosphorylation of several effectors (Oh and Jacinto, 2011). In addition to AKT, mTORC2 directly phosphorylates PKCα (Sarbassov et al., 2004), and SGK1, a kinase controlling ion transport and cell growth (García-Martínez and Alessi, 2008) (Figure 4).

Active AKT promotes cell survival through the phosphorylation of FoxO1/3a, nuclear factor-kB (NF-kB), and cAMP response element-binding protein (CREB), which leads to the transcription of anti-apoptotic genes. On the other hand, AKT promotes cell proliferation by phosphorylation of p21, p27 and by cyclin D1 up-regulation via GSK3β phosphorylation (Liang and Slingerland, 2003). AKT activation is highly sensitive to growth factor stimulation. In response to insulin stimulation, IRS-1 recruits PI3K to the plasma membrane to phosphorylate phosphatidylinositol-4,5-bisphosphate (PI(4,5)P$_2$) to phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P$_3$), which recruits both PDK1 and AKT to the membrane allowing PDK1 to phosphorylate AKT on Thr 308 (Williams et al., 2000). Full activation of AKT requires mTORC2-dependent phosphorylation on its hydrophobic motif Ser 473 (Sarbassov et al., 2005). It has been suggested that mTORC2 does not control all the cellular functions of AKT. Knockout of mTORC2 components in mouse fibroblasts impairs the phosphorylation of AKT at Ser 473, but not Thr 308, resulting in reduced phosphorylation of the transcription factor FoxO1/3a while other AKT substrates are minimally affected (Guertin et al., 2006). Thus, phosphorylation on the HM site could be part of the mechanism modulating AKT substrate specificity.
Figure 4. mTORC2 pathway. General schematic representation of the structure, upstream regulators, downstream targets and cellular effects of the protein complex mTORC2. Modified from Wataya-Kaneda, 2015. For detailed information see section 1.1.1.2.1
In addition to its functions in cell survival and proliferation through modulation of AKT, the first function defined for mTORC2, was the regulation of the actin cytoskeleton (Oh and Jacinto, 2011). In mouse fibroblasts, disruption of the core components of mTORC2 leads to actin polymerization and cell spreading (Jacinto et al., 2004), and in human neutrophils, knockdown of RICTOR leads to defects in cell polarity (Liu et al., 2010). In HeLa cells, knockdown of mTORC2 components leads to an increase in stress fibres and cytoplasmic paxillin patch formation (Sarbassov et al., 2004). Although the precise mechanism of how mTORC2 controls the actin cytoskeleton is not fully understood, PKCα along with other effectors, such as paxillin and Rho GTPases, have been implicated in the regulation of cell size and shape by affecting the actin cytoskeleton in an mTORC2 dependent manner (Sarbassov et al., 2004). Dysregulation of mTORC2 activity can result in altered cell motility in different cell types, including cancer cells where migration plays a key role in metastasis (Masri et al., 2007).

Studies examining the subcellular localisation of mTORC2 have found a high abundance of the complex in the endoplasmic reticulum (ER) (Boulbés et al., 2011) and mTORC2 activity has been shown to be highly dependent on ribosome association. By physically associating with ribosomes, mTORC2 mediates the co-translational phosphorylation of AKT at its TM site (Thr450) (Oh et al., 2010). Growth factors, acting via PI3K, promote mTORC2 association with ribosomes and the post-translational phosphorylation of AKT at its HM site (Ser473) (Zinzalla et al., 2011). Additionally this mTORC2-ribosome interaction correlates with mTORC2 activity in both melanoma and colon cancer cells exhibiting elevated PI3K signalling (Zinzalla et al., 2011). This suggests that the role of mTORC2 as the regulatory kinase of AKT and related kinases occurs on the surface of the ER. Furthermore, it was recently reported that active mTORC2 localises to the ER-subcompartment termed mitochondria-associated ER membrane (MAM) in a growth factor
stimulation-dependent manner. At this location, mTORC2 controls MAM integrity and mitochondrial function via AKT phosphorylation of IP3R, Hexokinase2 and phosphofurin acidic cluster sorting protein 2 (PACS2), contributing to mitochondrial metabolism and cell survival (Betz et al., 2013).

1.1.1.3 Crosstalk between mTORC1 and mTORC2

Interestingly, the two mTOR complexes intercommunicate by distinct feedback regulatory mechanisms (Figure 5). High mTORC1 activity limits mTORC2 signalling by impairing the PI3K-mediated activation of mTORC2. mTORC1 directly phosphorylates and stabilises the growth factor receptor adaptor protein Grb10 which negatively regulates growth factor signalling by binding to the insulin and IGF-1 receptors (Yu et al., 2011). In addition, mTORC1 through the activation of S6K, participates in the phosphorylation of Insulin receptor substrate 1 (IRS-1), preventing the recruitment and activation of PI3K (Um et al., 2004). In addition, the two core mTORC2 components RICTOR and SIN1 are directly phosphorylated by S6K1, resulting in inhibition of the mTORC2-dependent phosphorylation of AKT on S473 (Dibble et al., 2009 and Liu et al., 2014). On the other hand, active AKT can phosphorylate TSC2 and inhibit its GAP activity towards Rheb resulting in the subsequent activation of mTORC1 (Yoshida et al., 2011). Similarly, the inhibitory activity of PRAS40 towards mTORC1 is relieved by AKT phosphorylation (Wang et al., 2007). Thus, mTORC2 activation appears to positively regulate mTORC1 activity.
Figure 5. mTORC1 and mTORC2 Crosstalk. Overview of the regulatory crosstalk between mTORC1 and mTORC2. For a details see section 1.1.1.3.
1.1.2 mTOR Signalling in Cancer

The participation of mTOR in critical cellular growth and survival processes supports its importance in cancer pathogenesis. This has generated significant interest in the targeting of the pathway for cancer therapy (Advani, 2010). Oncogenic activation of mTOR signalling supports cancer cell growth, survival, and proliferation (Laplante and Sabatini 2012). For example, the hyperactivation of the PI3K/mTORC2/AKT and mTORC1/S6K signalling pathways has been reported to promote tumor formation (Sridharan and Basu, 2011; Georgescu et al., 2010). Genes encoding components of the PI3K signalling pathway including TSC1/2, serine threonine kinase 11 (LKB1), PTEN, and neurofibromatosis type 1 (NF1) are all upstream of the mTOR complexes and are often mutated in human cancers (Laplante and Sabatini 2012). Loss of p53, a common event in cancer, also promotes mTORC1 activation by impairing the p53-dependent transcriptional regulation of tensin homolog deleted on chromosome 10 (PTEN) (Feng et al., 2005). Overexpression of the mTORC2 subunit RICTOR has been reported in glioma, breast and prostate cancers, and has been associated with increased proliferative and invasion potential in cell lines (Hietakangas and Cohen, 2008 and Masri et al., 2007). RICTOR is also required in PTEN-deficient prostate cancer cell lines to form tumours in xenograft mice (Guertin et al., 2009). Similarly, overexpression of SIN1 has been associated with AKT hyperactivation in aggressive thyroid carcinoma (Moraitis et al., 2014) underpinning the role of mTORC2 in cancer pathogenesis.
1.2 Stress-Activated Protein Kinase-Interacting 1 (SIN1)

In addition to RICTOR, the second defining component of mTORC2 is the SAPK-interacting protein 1 (SIN1). SIN1 is a 522-amino acid protein in humans and is a member of a widely conserved family (Schroder et al., 2004). SIN1 contains a conserved region in the middle (CRIM) domain and most of its orthologues contain a Ras-binding domain (RBD) and a pleckstrin homology (PH) domain (Cameron et al., 2011) (Figure 6 A). The CRIM is the signature sequence of the SIN1 family with no significant similarity to other known protein domains, so is likely to have a specific function (Schroder et al., 2004). Fission yeast strains lacking the SIN1 gene are sterile, sensitive to stress, and have a delayed cell cycle. Interestingly, SIN1 function appears to be evolutionary conserved as these defects can be rescued by expressing human SIN1 (Wang and Roberts, 2005). Transcripts from the human SIN1 gene are alternatively spliced generating five isoforms, three of which assemble into mTORC2 (Frias et al., 2006).

SIN1 is essential for mTORC2 integrity and kinase activity (Jacinto, 2006). RICTOR and SIN1 form a very stable complex and the knockdown of either protein results in the destabilisation of the other and the loss of integrity of the mTORC2 complex (Yang et al., 2006). SIN1 has been proposed to act as a scaffold to recruit AKT and PKCα to the TORC2 complex to facilitate their phosphorylation (Cameron et al., 2011). In addition, SIN1 may localise the TORC2 complex to membranes via its PH domain or help integrate TORC2 with other pathways, for example Ras via its RBD (Schroder et al., 2007).

The deletion of the SIN1 gene results in embryonic lethality in mice, making its study in vivo difficult (Jacinto et al., 2006). Ablation of SIN1 in cellular models makes them more sensitive to stress-induced apoptosis, consistent with a decrease in
Figure 6. The domain organisation of SIN1 and eIF3d. A Schematic representation of the domain structure human SIN1 protein: **CRIM**: Conserved region in the middle, **RBD**: Ras binding domain **PH**: pleckstrin homology domain. Indicated are the regulatory phosphorylation sites: **Thr86** (phosphorylated by AKT and S6K), **Thr398** (phosphorylated by S6K). B Schematic representation of the domain structure human eIF3d protein: **RBS**: RNA binding site, **NLS**: Nuclear localisation site, **C-Terminal**: C-terminal region of the protein. The respective general functions of each domain are indicated.
AKT-dependent FoxO1/3a phosphorylation. In Hela cells, loss of SIN1 expression increases actin fibers in the cytoplasm, no longer restricting them to the cell cortex. This is a similar actin fibre organisation phenotype as that observed following knockdown of RICTOR (Yang et al., 2006). Furthermore, consistent with SIN1 interaction with members of the MAPK pathway (Cheng et al., 2005), SIN1 binds to p38, and stimulates the osmotic stress-induced phosphorylation of ATF-2 by p38 (Makino et al., 2006). Recently it has been reported that SIN1 plays a key role in the feedback regulation between mTORC1 and mTORC2 (Liu et al., 2013). Direct phosphorylation of SIN1 at Thr 86 and Thr 398 by S6K or AKT, suppresses the mTORC2 kinase activity by dissociating SIN1 from mTORC2. This promotes the destabilisation of the complex and inhibits the growth factor-induced AKT phosphorylation by mTORC2. Interestingly, a SIN1-Arg81Thr mutation has been found in an ovarian cancer patient. This leads to disruption of the S6K phosphorylation motif and a significant reduction in phosphorylation at Thr86, thereby promoting hyper-activation of mTORC2 by bypassing the negative feedback regulation (Liu et al., 2013). This suggests a potential mechanism by which mutations in the mTORC1–S6K–SIN1 signalling axis can cause aberrant mTORC2 activity found in tumours.

1.3 Eukaryotic Translation Initiation Factor 3 Subunit D (eIF3d)

Interaction between the subunit D of the Eukaryotic translation initiation factor 3 (eIF3d, Figure 6 B) and the mTORC2 component SIN1 has been observed in a two hybrid yeast assay and in cultured human cells (Whitmarsh, A., unpublished results), making this protein an interesting candidate for studying the regulation of SIN1 function in the context of mTORC2. This finding could be indicative of a functional role of the interaction of SIN1 with the translation machinery, which would
be supported by the observed location of mTORC2 on ribosomes in the ER (Boulbés et al., 2011). However, whether the interaction of eIF3d with SIN1 possesses a biological role in the mTORC2 signalling pathway by acting as a substrate or forming part of the mTORC2 complex remains to be elucidated.

1.3.1 The eIF3 complex

The eukaryotic translation initiation factor 3 (eIF3) is the largest multi-protein complex among the eIFs. eIF3 plays a role in the formation of the 43S preinitiation complex by interacting with the ternary complex of eIF2/GTP/methionyl initiator tRNA, and by promoting mRNA recruitment and scanning (Hinnebusch, 2006). eIF3 also participates in the recycling of eukaryotic post-termination complexes by promoting splitting of ribosomes into their 60S subunits and tRNA- and mRNA-bound 40S subunits after translational termination (Pisarev et al., 2007). eIF3 associates with mTORC1 in a growth-factor-dependent manner and this promotes S6K1 phosphorylation. Activated S6K1 dissociates from the eIF3 complex and phosphorylates downstream targets implicated in translation such as S6 and eIF4B promoting protein synthesis (Holz et al., 2005).

Mammalian eIF3 contains 13 non-identical subunits, designated eIF3a to eIF3m (Goh et al., 2011). eIF3 from budding yeast is simpler and contains the orthologs of the five mammalian eIF3 subunits, eIF3a, eIF3b, eIF3c, eIF3g and eIF3i. These form the core complex, being essential for translation initiation in yeast. In addition to these core subunits, fission yeast contains homologues of four other mammalian eIF3 subunits absent in budding yeast, eIF3d, eIF3e, eIF3f and eIF3h. These seem not to be essential for the formation of an active mammalian eIF3 complex (Masutani et al., 2007). Since the basic translation machinery is highly conserved
between yeast and mammals this raises the question of what specific functions these additional eIF3 subunits may play.

1.3.2 eIF3d as part of the translational machinery.

Most of the knowledge of eIF3d comes from studies performed on the fission yeast homologue Moe1. Moe1 physically associates with the eIF3 core subunits as well as with 40S ribosomal particles (Bandyopadhyay et al., 2002). Moe1 and Yin6 (eIF3e yeast ortholog) are required for the stable association of eIF3 subunits in fission yeast, but only when the complex is dissociated from ribosomes by KCl treatment. (Bandyopadhyay et al., 2002). Strains lacking Moe1 are viable and have a reduced rate of translation by about 30-40% compared with wild type cells, and also display a reduced growth rate (Bandyopadhyay et al, 2002). This suggests that even if Moe1 is not essential for translational initiation, it could be required for either the optimal rate of translation or for the translation of a specific subset of mRNAs.

1.3.3 eIF3d as regulator of cytoskeletal organization.

In addition of its participation as a component of the eIF3 complex, the activity of Moe1 has been linked to changes in cytoskeletal organisation that affect growth and morphology. Yeast lacking Moe1 have more microtubule bundles compared with wild type cells, with abnormalities in the spindle (Chen et al., 1999). Consistent with the idea that Moe1 could negatively affect the assembly and stability of microtubules, deletion of the MOE1 gene renders cells more resistant microtubule depolymerisation (Chen et al., 1999). Moreover, both Δmoe1 and Δyin6 in combination with defects in the yeast Ras pathway have been reported to cause defects in spindle formation and chromosome segregation (Yen and Chang, 2000).
A further mechanism by which Moe1 may regulate growth is through its cooperative interaction with Yin6 and the chaperone Cdc48 (Otero et al., 2010). Interestingly, Cdc48, Yin6 and Moe1 co-localise at the ER, consistent with Moe1 and Yin6 being part of the eIF3 complex. Furthermore, the interaction between Cdc48 and the Yin6-Moe1 complex is required for proper regulation of Endoplasmatic-reticulum-associated protein degradation (ERAD) and chromosome segregation in yeast cells, implying a potential role in mitosis regulation (Otero et al., 2010).

1.3.4 eIF3d in Cancer

Aberrant expression of several eIF3 subunits is found in a variety of human cancers (Dong and Zhang, 2006) suggesting a link between eIF3 dependent regulation of translational initiation and neoplastic transformation. The eIF3 core components eIF3a, eIF3b, eIF3c, and eIF3i are reported to promote malignant transformation (Zhang et al., 2007) and overexpression of these subunits has been found in human breast (Bachman et al., 1997) oesophageal (Chen and Burger, 1999), lung (Lin et al., 2001), colon (Haybaeck et al., 2010 and Wang et al., 2012) and testicular cancers (Rothe et al., 2000). In addition, the overexpression of eIF3h serves as a prognostic marker of local recurrence, invasiveness, and metastasis in breast and prostate cancers (Nupponen et al., 2000 and Savinainen et al., 2004), whereas knockdown of this subunit inhibits the growth of prostate cancer cells (Savinainen et al., 2006). Decreased expression of eIF3e is frequently seen in human breast and lung carcinomas and appears to be linked to poor prognosis (Buttitta et al., 2005 and Marchetti et al., 2001). eIF3e suppression also affects cell proliferation and survival in glioblastoma cells (Sesen et al., 2014), while eIF3d knockdown causes decreased cell proliferation and colony formation as well as defects in cell cycle progression in prostate (Gao et al., 2015), melanoma (Li et al., 2015), glioma (Ren
et al., 2015), colon (Yu et al., 2014) and lung (Lin et al., 2015) cancer cell lines. These data suggest a role of eIF3d in cancer progression by modulating cell proliferation. The down-regulation of eIF3d in colon and lung cancer cell lines results in changes in phosphorylation of effector proteins from signalling pathways involved in cell proliferation, growth, and survival (Yu et al., 2014 and Lin et al., 2015), suggesting a role for eIF3d in the modulating these pathways.

1.4 Hypothesis

In the context of elucidating the role of essential components of mTOR signalling, it is important to determine how these integrate with other cellular regulators. The mTORC2 component SIN1, appears to link the mTOR pathway to other proteins and represents a potential target for cancer therapy.

The interaction observed between SIN1 and eIF3d suggests that eIF3d might have a role in mTORC2 related functions in the cell. The participation of eIF3d in cytoskeleton organisation, its location on the ribosome and its participation in anabolic processes make it an interesting candidate for study in the context of regulating mTOR activity.

My hypothesis is that eIF3d participates in the regulation of the mTOR signalling pathway by direct interaction with the mTORC2 core component SIN1. eIF3d could potentially (i) act as a scaffold for the recruitment of novel mTOR regulators or substrates, (ii) determine the localisation of mTORC2, or (iii) provide a point of communication between mTORC1 and mTORC2.
1.5 Aims Of The Project

1.5.1 To determine whether eIF3d is a component of mTORC2 via its recruitment by SIN1.

The interaction between eIF3d and SIN1 could suggest that it is a component of mTORC2 and that this association could have a regulatory role. Therefore it will be tested whether eIF3d interacts with other components of mTOR complexes by immunoprecipitation and immunolocalisation assays. It will also be evaluated whether this association is influenced by stimuli known to alter mTOR signalling.

1.5.2 To determine the role of eIF3d in regulating mTOR activity.

The RAPTOR-mTOR complex and S6K have been shown to interact with the eIF3 complex in an insulin-stimulation dependent manner (Holz et al., 2005). Thus, eIF3d could be modulating mTOR activity by interacting with this complex as part of eIF3. In order to evaluate the biological relevance of the interaction between eIF3d and SIN1 on mTOR signalling, a cellular model of eIF3d knockdown by RNAi will be used. It will be determined whether eIF3d disruption affects (i) the phosphorylation of mTOR targets, including S6K, AKT, PKCα and SGK1, (ii) cellular processes associated with mTORC2 activity, and (iii) the subcellular localisation of mTORC2

1.5.3 To determine the role of eIF3d in mammalian cells.

The eIF3d yeast ortholog Moe1 has been implicated in translation initiation, cell growth, morphology, microtubule stability and organization (Bandyopadhyay et al, 2002; Yen and Chang, 2000). It will be tested whether modulation of the expression of eIF3d by knockdown or overexpression could have phenotypic effects similar to those observed in yeast, and whether these relate to its interaction with SIN1/mTORC2.
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Reagents

General laboratory reagents and chemicals, unless otherwise stated, were purchased from Sigma-Aldrich, Bio-Rad, Invitrogen, Promega, QIAgen, Amersham Biosciences and Fisher. For a list of antibodies used see Table 1.

2.1.2 Constructs

The expression vectors used encode for human proteins, except for protein tags, pCDNA3, pCDNA3-Flag-eIF3d, pCDNA3-Myc6-Sin1, pEBG, pEBG-eIF3d, pEBG-eIF3d(482-548), pEBG-eIF3d(482-529), pCMV-Flag-RICTOR, pRK5-HA-RAPTOR, pEGFP-C2, pEGFP-C2-eIF3d, pKLO.1-puro and pKLO.1-puro Non-Target shRNA were kindly provided by A. Whitmarsh. For primers used in cloning and generation of mutants see Table 2.

2.1.3 siRNAs

The siRNAs used were purchased from Dharmacon, Eurofins Genomics, and MWG-Biotech AG. For sequences of the siRNAs used see Table 3.
Table 1: Antibodies.

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Table 2: Mutagenesis and Cloning Primers

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### Table 4: eIF3d targeting shRNAs Oligos

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2.2 Methods

2.2.1 Cell Cultures

Hek293T and HeLa cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco), supplemented with 10% Fetal bovine serum, 1% antibiotics penicillin/streptomycin (100 U/mL) and 1X GlutaMAX™. Cells were kept in liquid nitrogen, then thawed and grown in T-75 cultures flasks until confluent. Cells were incubated at 37 °C and 5% CO₂ atmosphere.

To perform experiments, cells were washed in Hanks’ Balanced Salt Solution (HBSS) and detached from the flasks using Trypsin-EDTA at 37 °C for 5 minutes. Trypsin was inactivated by adding complete growth medium and the cell suspension was transferred to 6 well, 12 well or 10 cm cell culture dishes. For Insulin-like growth factor (IGF-1) stimulation, cells were serum starved for 6 hours and 100 ng/ml of IGF-1 in 10% BSA was directly added to the growth media and incubated for 20 minutes before lysis and sample collection. For H₂O₂ treatment, cells were incubated for 30 minutes with 500μM H₂O₂ before cell lysis and sample collection. For arsenite treatment, cells were incubated for 30 minutes in growth media containing 500μM of Sodium (meta)arsenite before cell lysis and sample collection.

2.2.2 Plasmid DNA transfection

Transfection of all plasmid DNA was performed on growing cells at 60%-70% confluence using the JetPEI™ transfection reagent (Polyplus Transfection) following the manufacturer’s forward transfection protocol. 3 μg of total plasmid DNA were used for transfection and cells were grown for 48 hours prior to harvesting unless otherwise stated.
2.2.3 RNAi Transfection

Small interfering RNAs were transfected into growing cells at 60%-70% confluence using Lipofectamine® RNAiMAX (Invitrogen) transfection reagent with the manufacturer’s forward transfection protocol. All siRNA were used at a final concentration of 20 nM. Cells were incubated at 37°C for 48 hours before they were treated and harvested for further analysis. The sequences of the siRNA used are indicated in Table 3.

2.2.4 Preparation of cell lysates

Growth media was aspirated and cells were rinsed twice with 1x PBS to remove residual growth medium and cell debris. Cells were then lysed in Triton lysis buffer (TLB) containing: 20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA pH 7.4, 1% Triton X-100 (alternatively lysis buffer containing 0.3% CHAPS instead of Triton), 25 mM β-glycerophosphate, 1 mM Na₃Va₄, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 ug/ml aprotinin and 10 ug/ml leupeptin. Cells were scraped and transferred to 1.5 mL micro-centrifuge tubes and centrifuged at 4 °C for 10 min at 14000 rpm. The supernatant was collected in a new tube and used immediately or kept at -80°C.

2.2.5 Determination of protein concentration.

Protein concentration in TLB cell lysates was measured by dilution of each sample lysate in water at a 1:4 ratio and adding it to 200 μL of 1:4 diluted Bradford reagent (BioRad). The reaction was incubated for 5 minutes at room temperature, and the absorbance for each sample was measured at a wavelength of 595 nm. To
calculate the protein concentration of the samples a standard curve obtained from bovine serum albumin standards was used.

2.2.6 Immunoblotting.

Proteins in cell lysates were resolved by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Equal amounts of lysate samples in SDS loading buffer (60 mM Tris pH 6.8, 2% (w/v) SDS, 1.85 (w/v) DTT, 5% (v/v) glycerol, 0.003% (w/v) bromophenol blue) were boiled for 5 minutes to denature proteins. Samples were briefly spun and loaded onto 10% polyacrylamide gels. Resolving gels were prepared by mixing Tris-HCl pH 8.8 buffer (0.375 M), Acrylamide (Protogel), SDS (0.1%), Ammonium persulfate (APS, 0.1%), and N, N', N'-tetramethylethylenediamine (TEMED, 30 mM), stacking gels were prepared similarly using Tris-HCl pH 6.8 buffer (0.25 M) instead. Gel electrophoresis was performed at 150 V for 1.5 hours in buffer containing 25 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS. Precision Plus Protein™ (Bio-Rad) was used as standard for molecular weight estimation. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride (PVDF, Millipore) membranes by semidry transfer in buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% methanol (v/v) at 15 V for 2 hours or by wet transfer in buffer containing 25 mM Tris-HCl, 192 mM glycine, 20% methanol (v/v) and 0.05% SDS for blotting higher molecular weight proteins. Membranes were incubated 1 hour at room temperature in blocking solution (5% (w/v) skimmed milk powder diluted in Tris-buffered saline-Tween (TBST) containing 15 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween-20 (v/v), followed by overnight incubation at 4°C with primary antibody diluted in blocking solution. Membranes were then washed two times for 10 minutes in TBST and incubated with the secondary antibody diluted 1:10000 in blocking solution for 1 hour at room temperature. Afterwards membranes were
washed four times for 5 minutes in TBST and once for 5 minutes with TBS and
developed using either enhanced chemiluminescent reagent (ECL™ Prime western
blotting detection reagent, Amersham™) according to manufacturer’s instructions or
using the LI-COR Odyssey infrared imaging system.

2.2.7 Co-Immunoprecipitation and GST pull-down assay

For immunoprecipitations, cleared lysates were incubated with 2.0 µg of antibody at
4°C overnight on a rotating platform in a 1.5 mL microcentrifuge tube. 30 µl of a
50% slurry of Protein G or Protein A-Dynabeads® Magnetic Beads (Invitrogen) were
added and incubated for 3 more hours at 4°C. The beads were washed four times
with 1.0 mL of lysis buffer and collected each time by magnetic separation for 1
minute prior to aspiration of the buffer. For GST pull-down assays, cleared lysates
were incubated overnight at 4°C after directly adding 30 µl of a 50% slurry of
SuperGlu resin (Generon). The beads were washed four times with 1.0 mL of lysis
buffer and collected by pulse centrifugation for 5 seconds followed by aspiration of
the buffer. Samples were then resuspended directly in SDS loading buffer and kept
on ice or at -20°C until ready to run SDS-PAGE. Before loading, the samples were
boiled at 100°C for 5 minutes and pulse centrifuged to collect the beads at the
bottom of the tube.

2.2.8 Immunofluorescence

Hela cells were seeded into 6 well dishes containing sterile coverslips. Cells grown
on coverslips were fixed for 20 minutes at room temperature in 4%
paraformaldehyde (PFA; solution in PBS, pH 7.4). After washing 3 times with PBS,
cell membranes were permeabilised for 20 minutes at room temperature with PBS
containing 0.2% (v/v) Triton X-100. Samples were washed 3 times with PBS containing 0.1% (v/v) TritonX-100 and blocked for 1 hour in blocking solution (3% (w/v) BSA in PBS) at room temperature. The samples were then incubated with the respective primary antibodies diluted in blocking solution at 4°C overnight. The next day, the coverslips were washed 3 times with PBS containing 0.1% (v/v) TritonX-100, and incubated with Alexa Fluor®-conjugated secondary antibody (diluted 1:1000 in blocking solution) for 2 hours in darkness at room temperature. After 3 washes with PBS containing 0.1% (v/v) TritonX-100, the coverslips were washed in PBS and dipped once in water, then mounted onto glass microscope slides using ProLong® Mountant with DAPI (Molecular Pobes). Images were collected on an Olympus BX51 upright microscope using a 10x/ 0.30 Plan Fln objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Specific band pass filter sets for DAPI, FITC and Texas red were used to prevent bleed through from one channel to the next. Images were then processed and analysed using ImageJ (http://rsb.info.nih.gov/ij).

2.2.9 Plasmid DNA purification

Plasmids were propagated in competent DH5α E. coli grown overnight in either 2 ml (mini-preparations) or 300ml (maxi-preparations) LB broth medium with 100 μg/mL ampicillin at 37 °C under agitation. Bacteria were collected by centrifugation at 5000 rpm for 5 minutes, the supernatant was discarded and the pellet was resuspended and purified using QIAgen® plasmid preparation kits following the manufacturer’s instructions. Quantification of the samples was performed at 260/280 nm absorbance with a Nanodrop® spectrophotometer. The integrity of the plasmids were confirmed by restriction digestion assays. Agarose gels (1%) were made and run with TBE buffer (90mM Tris, 90mM boric acid, 2mM EDTA) with various DNA
ladders (Bioline) for identification of fragment size, and visualized by UV light using the BioRad Gel Doc™ EZ System.

2.2.10 Generation of eIF3d deletion mutants

Deletion mutants were generated by PCR using the QuikChange site-directed mutagenesis protocol (Stratagene) and inserting stop codons in the corresponding protein coding sequence. The PCR mixture for site-directed mutagenesis of the different mutations was composed of 1X reaction buffer, 50ng DNA template (pEBG-eIF3d(482-548), pCDNA-flag-eIF3d and pEGFP2-C2-eIF3d vectors), 100 pmol of each forward and reverse primer, 1μL of dNTP mix provided, and distilled water to a total volume of 50μL. 2.5 U of Pfu Ultra DNA polymerase (Stratagene) was subsequently added. The PCR programme was as follows: 1 cycle at 95°C for 30 seconds, 18 cycles at 95°C for 30 seconds, 55°C for 1 minute and 68°C for 7 minutes. Following the PCR, 10 U of DpnI enzyme were added directly to each amplification reaction, mixed gently and incubated at 37°C for 1 hour in order to digest the parental DNA. XL10-Gold Ultracompetent Cells (Agilent Technologies) cells were then transformed with DNA constructs by heat shock. 1μL of Plasmid DNA was added directly to 50μL of competent XL10-Gold cells and incubated for 30 minutes on ice. Heat pulse was then performed for 45 seconds at 42°C and then the cells were placed on ice for 2 minutes. Cells were then incubated at 37°C for 1 hour with shaking, plated on agar with ampicillin or kanamycin (100 μg/mL) and incubated overnight at 37 °C. Colonies were picked and grown in LB Broth with 100 μg/mL ampicillin or kanamycin and plasmid DNA was purified using QIAgen® plasmid preparation kits. Generation of the mutants was corroborated by DNA sequencing using the BigDye Terminator v3.1 (Applied Biosystems) using 1μg of plasmid DNA and 0.2μM sequencing primer following the manufacturer’s
instructions. Automated Sanger sequencing was performed using an ABI 3730 Genetic analyser. The primers used to generate the different mutants are indicated in Table 2.

2.2.11 Cell fractionation

Cells were harvested in ice cold PBS and pelleted at 1,500 rpm for 5 minutes. The pellet was resuspended by pipetting in 400 μL of Ice cold Buffer A (10mM HEPES pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT and freshly adding 10 μg/ml aprotinin and 10 μg/ml leupeptin and incubated on ice for 15 minutes to allow cells to swell. After the incubation, 25 μL of 10% NP-40 were added and the samples were vigorously vortexed for 10 seconds and centrifuged at 4°C, 14000 rpm for 30 seconds. Then the supernatant, containing the cytoplasmic fraction, was collected and stored at -80°C. The pellet was resuspended in 50μl ice-cold Buffer B (20mM HEPES pH 7.9, 0.4M NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT, freshly adding 10 μg/ml aprotinin and 10 μg/ml leupeptin), and vigorously vortexed for 45 minutes at 4°C. The samples were then centrifuged at 14,000 rpm at 4°C for 5 minutes. The supernatant, containing the nuclear fraction and the pellet containing the insoluble fraction were separated and stored at -80°C.

2.2.12 shRNA generation and cloning into the pKLO.1 vector

shRNA oligos were designed using Dharmacon siDESIGN Center software (http://www.dharmacon.com/designcenter/designcenterpage.aspx) using human eIF3d coding region (NM_003753) as the target mRNA nucleotide sequence. The oligos used are indicated in Table 4. The oligos were annealed, using 5 μL of each primer (100 pmol/μL), 5 μL of Pfu PCR-buffer (Agilent), and 35 μl H₂O. The mix was
incubated at 95°C for 1 minute, 65°C for 10 min, and then cooled down slowly at room temperature. Then 4 µL of the annealed oligos were phosphorylated with 1 µl of Polynucleotide kinase (PNK, NEB), 5uL PNK buffer (NEB), 5 µl ATP (10mM) and H₂O added up to 50 µl. The samples were then incubated at 37 °C for 1 hour and then stored at -20 °C until use.

In parallel, 10 µg of the pKLO.1 vector was digested with 2 µL AgeI (NEB) in a 50 µL reaction volume according to manufacturer’s instructions. The digested product was then purified with the QIAquick® PCR purification kit and eluted in 45 µl H₂O. The purified product was further digested with 2 µl EcoRI (NEB) in a reaction volume of 50 µl and then loaded on a 2 % agarose gel. Following electrophoresis, the digested plasmid was purified using the QIAquick® gel extraction kit and eluted in 30µL of H₂O.

To ligate the annealed oligos into the digested vector a ligation reaction was performed using insert: vector ratios of 20:1 and 50:1 and adding 2 µL of T4 ligase buffer 10X (NEB), 1 µl of T4 ligase (NEB) and H₂O up to 20 µl. Samples were then incubated for 4 hours at room temperature and DH5α cells were directly transformed with 5 µL of the ligation products, plated on agar with ampicillin (100 µg/mL) and incubated overnight at 37 °C. Colonies were picked and grown in LB Broth with 100 µg/mL ampicillin and plasmid DNA was purified using the QIAGen® plasmid preparation kit. Correct insertion of the shRNA sequences into the pKLO.1 vector was determined by restriction assays and further corroborated by automated sequencing.
2.2.13 Cell size analysis

Cells were harvested by washing once with PBS and then collected with 400μL of PBS and centrifuged for 5 minutes at 3,500 rpm. The supernatant was aspirated and the cell pellet was resuspended in 400 μL ice cold PBS. The samples were then directly analysed in a Cyan ADP from Beckman Coulter cytometer. Relative size among the samples was assessed by comparing the amount of a 488nm laser scattered by each cell. Data were analysed with the Summit analysis software.

2.2.14 Cell cycle analysis

For the cell cycle analysis, HeLa cells were grown in complete DMEM and synchronised using double thymidine block. Cells were washed twice with HBSS medium and incubated in complete media containing 2 mM thymidine for 12 hours. Cells were then washed twice with HBSS medium and incubated with complete DMEM media for 12 hours. After the incubation, cells were washed twice with HBSS medium and incubated again with 2 mM thymidine containing media for additional 12 hours. Cells are finally washed twice with HBSS, incubated in complete DMEM media and harvested at the indicated time points. Cells were harvested by washing once with PBS and then collected with 400μL of PBS and centrifuged for 5 minutes at 3500 rpm. The supernatant was aspirated and the cell pellet was resuspended in 100 μL ice cold PBS. 1 mL of ice cold 70% ethanol was added directly to the cell suspension while vortexing the tube to fix the cells. Samples were kept at 4°C. At the time of measurement, cells were centrifuged at 3500 rpm for 5 minutes to remove the ethanol and were resuspended in 400 μL PBS. 50μL of RNase (1mg/mL in PBS) and Propidium Iodide (400μg/mL in water) were directly added and the samples were incubated at 37 °C for 30 minutes. The samples were then analysed by flow cytometry in a Cyan ADP from Beckman Coulter cytometer.
Excitation of propidium iodide was done by a 488nm laser and the fluorescent emission was measured using a 613/20 bandpass filter. Data were analysed with the ModFit DNA analysis software.

2.2.15 Cell Proliferation Assays

Cells were seeded at 35,000 cells per well in 12-well plates. 24 hours later the cells were transfected with siRNA or plasmid DNA. Cells were washed twice in PBS and fixed in 4% paraformaldehyde in PBS for 15 minutes. Cells were then washed twice with water and stained with 1 mL of a solution of 0.1% Crystal Violet in 10% ethanol for 20 minutes. After staining, the crystal violet solution was aspirated and cells were washed three times with water and air dried at room temperature. To perform the measurements 1 mL of pure methanol was added to each well and incubated for 20 minutes with shaking. 500 μL of each sample was collected and diluted 1:4 in water. Absorbance was measured in a plate reader at 590nm.

2.2.16 Statistical analysis

Two-way ANOVA followed by Bonferonni corrected post hoc t test was used to verify whether the differences among multiple groups were statistically significant. Student’s t-test was used to verify whether the difference between the means of two independent populations were statistically significant. It was assumed that the populations followed a two-tailed normal distribution and had equal variances. Probability values below 0.05 are considered statistically significant. Data are expressed as mean ± SEM.
Chapter 3: eIF3d is a novel interacting partner of SIN1

3.1 Introduction

mTOR has a critical role in various cellular processes including cell migration, growth, proliferation, metabolic homeostasis, and survival, among others (Schmelzle and Hall, 2000). Thus, the elucidation of signalling via the mTOR pathway will have a broad impact on our understanding of these processes, as well as providing potential targets for the development of novel therapeutic approaches. To date, most of the existing knowledge related to the mTOR pathway has come from the study of mTORC1, with the regulation of mTORC2 still poorly understood owing to its later discovery, its relative resistance to rapamycin and the absence of mTORC2 specific inhibitors. mTORC2 responds to growth factors, such as insulin, and participates as a key regulator in processes such as cellular survival, metabolism, proliferation and cytoskeleton organization, but the signalling pathways leading to mTORC2 activation are still not well characterised (Oh and Jacinto, 2011). The mTOR-interacting protein, SIN1 is critical for mTORC2 stability and catalytic activity (Jacinto et al., 2006). Interestingly, SIN1 participates in mTORC2 substrate recruitment (Cameron et al., 2011) and might link to other proteins that regulate mTORC2 activity. Thus, the identification of novel SIN1 interacting partners will help us gain a better understanding on how mTORC2 signalling can be regulated. eIF3d was found to interact with SIN1 in a yeast two-hybrid screen performed previously in our laboratory (Whitmarsh, unpublished data). Twenty independent eIF3d clones were isolated which represented the largest number for a single protein in the screen. Additional two-hybrid analysis identified the C-terminal region of eIF3d as the region that interacts with SIN1 (Whitmarsh, unpublished data). However, whether endogenous eIF3d interacts with SIN1 in mammalian cells and whether it interacts with other components of mTORC2 is unknown. The aim of this chapter is to characterise the interaction of eIF3d with SIN1 and other
components of the mTOR complexes by immunoprecipitation and immunolocalisation assays. It is also evaluated whether this association is influenced by stimuli known to regulate mTOR signalling.

3.2 Results

3.2.1 eIF3d interacts with SIN1

To test the interaction between eIF3d and SIN1 in mammalian cell lines, GST pull down experiments were performed. Hek293T cells were transfected with constructs expressing Myc-tagged SIN1 and GST or GST fused to either full length eIF3d or the C-terminal region comprising residues 482-548. Consistent with previous observations (Whitmarsh, unpublished results), both full length and truncated eIF3d proteins were able to interact with overexpressed SIN1 (Figure 7A). The amount of SIN1 associated with the C-terminal region of eIF3d appeared greater than what was observed for the full length protein, even when taking into account their expression levels (compare GST-eIF3d lanes and GST-eIF3d (482-548) lanes). The interaction between eIF3d and SIN1 was confirmed by co-precipitation of Myc-SIN1 with flag-tagged eIF3d (Figure 7B). Furthermore, the C-terminal region of GST-eIF3d was able to pull down endogenous SIN1 (Figure 7C) while an interaction between flag-eIF3d and endogenous SIN1 was also observed in Hek293T cells (Figure 7D). These data suggest that eIF3d is an interacting partner of SIN1.

3.2.2 eIF3d is not a core component of mTORC2

SIN1 is an integral component of mTORC2. Thus, the interaction between eIF3d and SIN1 could indicate that eIF3d is part of this complex. This was tested in
Figure 7. Recombinant eIF3d interacts with SIN1 in Hek293T cells.

A Hek293T cells were co-transfected with constructs expressing Myc-tagged SIN1 and either GST, GST-eIF3d or GST-eIF3d (amino acids 482-548). GST-Pull down was then performed and the presence of Myc-SIN1 detected by anti-Myc antibody.

B Hek293T cells were co-transfected with constructs expressing Myc-SIN1 and either an empty vector control or a construct expressing Flag-eIF3d. Immunoprecipitation was performed using an anti-Flag antibody and the presence of Myc-SIN1 detected by anti-Myc antibody.

C Hek293T cells were transfected with constructs expressing either GST or GST-eIF3d (amino acids 482-548). GST-Pull down performed and the presence of endogenous SIN1 detected by immunoblot.

D Hek293T cells were transfected with either an empty vector control or a construct expressing flag-eIF3d. Immunoprecipitation was performed using an anti-Flag antibody and the presence of endogenous SIN1 detected by immunoblot. The lysate lanes contain one tenth of the lysate volume used for pull down and immunoprecipitation assays. The data are representative of 3 independent experiments.
Hek293T cells by immunoprecipitation of endogenous eIF3d. Endogenous eIF3d was able to co-precipitate endogenous SIN1 (Figure 8A) but no interaction with mTOR or the mTORC2 component RICTOR was observed (Figure 8A). In reciprocal experiments, endogenous SIN1 co-precipitated eIF3d (Figure 8B) and mTOR co-precipitated SIN1 and RICTOR, but not eIF3d (Figure 8C). Furthermore GST-pull down in cells expressing GST-eIF3d and either flag-RICTOR or HA-RAPTOR (a component of mTORC1) did not show interaction between eIF3d and these proteins (Figure 8D and E). Taken together, the data suggests that eIF3d binds specifically to SIN1, but is not part of mTORC2 under normal growth conditions.

3.2.3 Effect of stimuli on eIF3d-SIN1 binding

Activation of the mTORC2 complex is responsive to growth factors. As SIN1 is a critical component of mTORC2, it was tested whether cell stimulation with insulin-like growth factor 1 (IGF-1) affected the interaction between SIN1 and eIF3d. Hela cells co-expressing GST-eIF3d and Myc-SIN1 were serum starved for 6 hours and stimulated with 100 ng/mL IGF-1 for 20 minutes prior to cell lysis and GST-pull down. Immunoblotting using a phospho-specific antibody against S473 on AKT was used as readout of mTORC2 activity. The results showed no change in the amount Myc-SIN1 that interacted with eIF3d either under serum starvation conditions or upon 20 mins IGF-1 stimulation (Figure 9A). Similarly, IGF-1 stimulation for shorter times (5 and 10 minutes) after serum starvation did not show obvious changes in eIF3d and SIN1 binding (Figure 9B).

As the interaction between eIF3d and SIN1 appears to be insensitive to IGF-1, it was tested whether this association is responsive to an mTORC1 activating stimulus, as mTORC1 acts as an upstream regulator of mTORC2 (Dibble et al.,
Figure 8. eIF3d is not a core component of mTORC2.
Immunoprecipitation was performed in HeK293T cells using either an anti-eIF3d antibody (A), anti-SIN1 antibody (B) or and anti-mTOR antibody (C) and the presence of endogenous proteins was detected using the indicated antibodies. The double band observed for SIN1 corresponds to the Sin1.1 and Sin1.2 isoforms. D Hek293T cells were co-transfected with constructs expressing Flag-RICTOR and either GST or GST-eIF3d (amino acids 482-548). GST-Pull down performed and the presence of Flag-RICTOR detected by anti-Flag antibody. E Hek293T cells were co-transfected with constructs expressing HA-RAPTOR and either GST or GST-eIF3d (amino acids 482-548). GST-Pull down performed and the presence of HA-RAPTOR detected by anti-HA antibody. The lysate lanes contain one tenth of the lysate volume used for immunoprecipitation and pull down assays. The data are representative of 3 independent experiments.
Figure 9. IGF-1 and oxidative stress do not alter the interaction between recombinant eIF3d and hSIN1 in HeLa cells.
HeLa cells were co-transfected with constructs expressing Myc-SIN1 and GST-eIF3d. Cells were serum starved and stimulated with IGF-1 (100ng/mL), for 20 min (A), or shorter times (B). Relative protein levels and phosphorylation of AKT was determined as readout of mTORC2 activity by immunoblot. Cells were treated with H$_2$O$_2$ for 30 minutes at the indicated concentrations and phosphorylation of S6K and S6 detected by immunoblot (C). HeLa cells were treated with H$_2$O$_2$ (500μM) for 30 minutes and GST-Pull down was performed and the presence of Myc-SIN1 detected by anti-Myc antibody (D). The lysate lanes contain one tenth of the lysate volume used for pull down assays. The data are representative of 3 independent experiments.
Both PI3K/AKT and mTORC1/S6K pathways are activated under oxidative stress (Faghiri and Bazan, 2010). \( H_2O_2 \) induces mTORC1 activity in Hela cells, as determined by observing the phosphorylation of the mTORC1 target S6 kinase and its substrate S6 (Figure 9C). Thus, to test whether stress dependent activation of mTOR affects the interaction between eIF3d and SIN1, the effect of \( H_2O_2 \) treatment was tested. Hela cells co-expressing GST-eIF3d and Myc-SIN1 were treated with \( H_2O_2 \) 500\( \mu \)M for 30 minutes prior cell lysis. Under these conditions, GST-pull down showed no change in the interaction between eIF3d and SIN1 (Figure 9D). This suggests that the eIF3d and SIN1 interaction is independent of IGF-1 stimulation and \( H_2O_2 \) induced stress.

### 3.2.4 Cellular localisation of eIF3d

Activation of mTORC2 by association with ribosomes has been reported (Zinzalla et al., 2011). Thus it was hypothesised that the interaction between eIF3d and SIN1 may be mediating the recruitment of mTORC2 to the ribosome by the association of the eIF3 complex to the 40S ribosome (Hinnebusch, 2006).

To address the sub-cellular localisation of eIF3d, Hela cells transfected with constructs expressing GFP-eIF3d or Flag-eIF3d were assessed by fluorescence microscopy. Interestingly both GFP-eIF3d and Flag-eIF3d showed a predominantly nuclear localisation and a more diffuse cytoplasmic localisation (Figures 10A and B). The nuclear localisation of the overexpressed eIF3d was confirmed by immunoblots on cytoplasmic/nuclear cell fractions of Hela cells expressing flag-tagged eIF3d (Figure 10C). Importantly, endogenous eIF3d shows a similar distribution in HeLa cells, localising in the nucleus and diffusely in the cytoplasm (Figure 11A and B). This indicates that eIF3d is both a nuclear and cytoplasmic protein.
Figure 10. Recombinant eIF3d localises to both the cytoplasm and the nucleus. A Hela cells were transfected with constructs expressing either GFP or GFP-eIF3d. Localisation of the proteins was detected by fluorescence microscopy in cells fixed in 4% PFA. B Hela cells were transfected with either an empty vector control or a construct expressing Flag-eIF3d. Localisation of the Flag-eIF3d was detected by fluorescence microscopy using an anti-Flag antibody in cells fixed in 4% PFA. Scale bars 10 μm. Images are representative from 3 independent immunofluorescence experiments. C Hela cells were transfected with either an empty vector control or a construct expressing Flag-eIF3d and the cytoplasmic and nuclear fractions were isolated by cell fractionation. Proteins were detected by immunoblot using the indicated antibodies. β-Tubulin and Lamin B were used as cytoplasmic and nuclear markers respectively.
Figure 11. Endogenous eIF3d localises in the cytoplasm and the nucleus.

A Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Immunostaining was performed using an eIF3d antibody and localisation of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. Scale bars 10 μm. Images are representative from 3 independent immunofluorescence experiments. 

B Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). The cytoplasmic and nuclear fractions were isolated by cell fractionation. Proteins were detected by immunoblot using the indicated antibodies. β-Tubulin and Lamin B were used as cytoplasmic and nuclear markers respectively.
The presence of eIF3d in the nucleus raised the question of whether it contains a nuclear localisation sequence (NLS). Analysis of the eIF3d protein sequence showed two potential NLS located at amino acid positions 126-138 and 218-223 as predicted using the online tools NLStradamus (http://www.moseslab.csb.utoronto.ca/NLStradamus) and cNLS Mapper (http://nls-mapper.iab.keio.ac.jp), respectively. Initially, two GFP-eIF3d deletion constructs containing residues 1-173 and 174-548 were generated by mutagenesis and analysed by fluorescence microscopy in HeLa cells. GFP-eIF3d 1-173 localised exclusively in the nucleus, with apparent enrichment in nucleoli whereas GFP-eIF3d 174-548 located diffusely throughout the cell. (Figure 12A, upper panels).

Similarly, a flag-eIF3d 1-173 construct also showed nuclear localisation with apparent nucleolar enrichment suggesting that an eIF3d NLS is located in the N-terminal region of the protein. The deletion of the predicted N-terminal site at positions 126-128 significantly reduces the nuclear localisation of eIF3d (Figure 12B).

The apparent nucleolar localisation of the eIF3d 1-173 deletion mutant could suggest that eIF3d localises in the nucleus in a RNA-binding dependent manner by association with nucleolar ribosomal RNA. Thus, whether the previously reported RNA-binding site of eIF3d located between positions 86-118 (Asano et al., 1997) is also involved in its nuclear localisation was tested. Indeed, deletion of this region significantly reduces eIF3d nuclear localisation (Figure 12B). All the above indicates that the N-terminal region of eIF3d is responsible for its nuclear localisation and that both its RNA-binding site at 86-118 and its nuclear localisation site at 126-138 are critical.

SIN1, as well as mTORC2, have been reported to be present in both the cytoplasm and the nucleus (Rosner and Hengstschläger, 2008). Thus, whether SIN1 and eIF3d co-localise in the nucleus was tested. Co-expression of GFP-eIF3d and
Figure 12. eIF3d nuclear localisation determinants. A Hela cells were transfected constructs expressing either GFP, GFP-eIF3d, GFP-eIF3d (amino acids 1-173) or GFP-eIF3d (amino acids 174-548). Localisation of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. B Hela cells were transfected with either an empty vector control or constructs expressing Flag-eIF3d, Flag-eIF3d (amino acids 1-173), a deletion mutant lacking the RNA-binding region (Flag-eIF3d (∆86-123)), and a deletion mutant lacking a predicted nuclear localisation site (Flag-eIF3d (∆126-138)). Immunostaining was performed using an anti-Flag antibody and localisation of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. Scale bars 10 μm. Images are representative from 3 independent immunofluorescence experiments.
Myc-SIN1 was performed in Hela cells and localisation of these proteins was assayed by fluorescence microscopy. As shown in Figure 13, both eIF3d and SIN1 show a disperse distribution in the cells. Both proteins seem to co-localise in the nucleus. This suggests that the interaction previously observed by immunoprecipitation is likely to be occurring in a cellular context and this might be occurring in the nucleus.

Since the mTOR pathway can be activated under stress conditions, the effect of H$_2$O$_2$ on eIF3d and mTORC2 localisation was tested. Hela cells expressing Myc-tagged SIN1 were incubated for 30 minutes with 500μM H$_2$O$_2$ prior to cell fixation and the localisation of Myc-SIN1 and endogenous eIF3d was determined by immunofluorescence. Under these conditions eIF3d and SIN1 did not show observable changes in their localisation (Figure 14A). In similar experiments, cells expressing flag-tagged RICTOR or HA-tagged RAPTOR were also tested. After 30 minutes of 500μM H$_2$O$_2$ incubation no major changes in localisation are observed. It does appear that RICTOR becomes more abundant in a perinuclear localisation (Figure 14B), and the opposite is observed in the case of RAPTOR (Figure 14C) without obvious compartmentalisation or association with eIF3d. These data support the protein interaction studies demonstrating that eIF3d is not a component of TOR complexes.

### 3.2.5 The determinants of eIF3d binding to SIN1

Previous mapping of the binding sites in both eIF3d and SIN1 showed that the critical regions for their interaction correspond to the C-terminal region of eIF3d comprising amino acids 482-528 and the central region of SIN1 between amino acids 219-340, which contains the conserved region in the middle (CRIM) domain.
Figure 13. eIF3d localises with SIN1 in the nucleus.
Hela cells were co-transfected with constructs expressing either GFP or GFP-eIF3d and either an empty vector control or a construct expressing Myc-SIN1. Immunostaining was performed using an anti-Myc antibody and localisation of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. Scale bars 10 μm. Images are representative from 3 independent immunofluorescence experiments.
Figure 14. eIF3d localisation is not affected by H₂O₂.
Hela cells were transfected with constructs either expressing Myc-SIN1 (A), Flag-RICTOR (B) or HA-RAPTOR (C). Cells were incubated with 500 µM H₂O₂ for 30 minutes before fixation. Immunostaining was performed using the indicated antibodies and localisation of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. Scale bars 10 µm. Images are representative from 3 independent immunofluorescence experiments.
(Whitmarsh, unpublished results). To further dissect the site on eIF3d required for SIN1 interaction, progressive deletions on the C-terminal region were generated (Figure 15A). GST-pulldowns from Hek293T cells co-expressing the different GST-eIF3d constructs and Myc-tagged SIN1 were analysed by immunoblot. The removal of the acidic C-terminal region located between amino acid positions 529-548 reduced the interaction between the two proteins but residual binding is still observed (Figure 15B). Further deletion analysis shows a loss of interaction between eIF3d and SIN1 following the removal of the residues between positions 517-548 of eIF3d (Figure 15C). Since the eIF3d 482-522 construct is still able to pull down SIN1, it appears that the region of eIF3d between the positions 517-522 contains a critical determinant for SIN1 interaction.

Unexpectedly, when the tag was switched from GST to Flag, progressive C-terminal deletions did not show the same binding pattern to SIN1. Removal of the C-terminal region after residue 517 did not alter the binding of SIN1 to eIF3d (Figure 16A, upper panel). Similarly, removal of the residues between the positions 510-522, containing the potential binding site according to the GST-pulldown analysis did not show major changes in the interaction between eIF3d and SIN1 either (Figure 16A, middle panel). Further deletion of the C-terminus of flag-eIF3d up to residue 509 or 481 resulted in constructs that were poorly expressed or insoluble, respectively (Figure 16B). Moreover, internal deletion of residues 481-509 did not show an alteration in SIN1 binding to eIF3d either (Figure 16A, bottom panel), making it difficult to determine where exactly the SIN1 interacting site on eIF3d is.
Figure 15. Determinants of eIF3d binding to SIN1 using GST-eIF3d deletions.

A  Sequence alignment of the C-terminal region (amino acids 482-548) of eIF3d. The numbers indicate the positions in which the protein was truncated to generate the deletion mutants.

B  Hek293T cells were co-transfected with constructs expressing Myc-SIN1 and either GST, GST-eIF3d (amino acids 482-548) or GST-eIF3d (amino acids 482-529). GST-Pull down was performed and Myc-SIN1 detected using anti-Myc antibody.

C  Hek293T cells were co-transfected with constructs expressing Myc-SIN1 and either GST, GST-eIF3d (amino acids 482-548), GST-eIF3d (amino acids 482-522), GST-eIF3d (amino acids 482-517) and GST-eIF3d (amino acids 482-509). GST-Pull down was performed and Myc-SIN1 detected using anti-Myc antibody. The lysate lanes contain one tenth of the lysate volume used for pull down. The data are representative of 3 independent experiments.
Figure 16. Determinants of Flag-eIF3d binding to SIN1. A Hek293T cells were co-transfected with a construct expressing Myc-SIN1 and either an empty vector control, a construct expressing Flag-eIF3d or constructs expressing Flag-eIF3d (Δ510-522), Flag-eIF3d (amino acids 1-517) or Flag-eIF3d (Δ479-509). Immunoprecipitation was performed using anti-Flag antibody and Myc-SIN1 detected using anti-Myc antibody. B Hela cells were transfected with constructs expressing Flag-eIF3d (amino acids 1-509) or Flag-eIF3d (amino acids 1-481). The cytoplasmic and nuclear fractions were isolated by cell fractionation. Proteins were detected by immunoblot using the indicated antibodies. The data are representative of 3 independent experiments.
3.3 Conclusions

Overall, the data is consistent with the idea of eIF3d being a novel interacting partner of SIN1. The difference observed in the level of SIN1 interacting with full length eIF3d and the C-terminal region of the protein might be indicative of a regulatory mechanism allowing a stronger interaction of eIF3d to SIN1 when the C terminal region is exposed. Whether such regulation could be regulated by stimuli known to modulate mTOR activity, such as growth factor stimulation and oxidative stress was tested, but no change in the association of eIF3d and SIN1 was observed under these conditions. It has to be considered that these experiments were performed using overexpressed proteins. Therefore, the high abundance of the proteins may render them insensitive to regulatory mechanisms. On the other hand the effect observed in the interaction of SIN1 and the C terminal region of eIF3d could be an artifact, effectively exposing or creating a stronger SIN1 interaction site, but that is inaccessible and may be irrelevant under physiological conditions.

The interaction between eIF3d and SIN1 appears to be independent of other mTORC2 components. SIN1 exists in at least five isoforms of which three appear to be part of mTORC2 complex (Frias et al., 2006). Thus, the SIN1/eIF3d interaction could be part of an mTORC2 independent complex. eIF3d might also associate with mTORC1 and mTORC2 components other than RAPTOR and RICTOR. It has to be also considered that the association of the mTOR to RAPTOR and RICTOR is sensitive to the detergent used in the preparation of cell lysates (Sarbassov et al., 2004). Similarly, there might be a weak or transient interaction of eIF3d with the mTORC2 complex that is lost in the processing of the samples.

According to the localisation assays, the association between eIF3d and SIN1 is likely to be occurring in the nucleus independently of the mTORC1 and mTORC2
complexes. Other eIF3 subunits have been previously found in the nucleus, possibly participating in ribosome biogenesis (Shi et al., 2009). This process is also regulated by S6K activity (Chauvin et al., 2014) which could be indicative of regulation by mTOR, although the exact function of nuclear mTOR is still unknown (Betz and Hall, 2013). Understanding the role of nuclear eIF3d and nuclear mTOR would reveal the possible relevance of a nuclear interaction between SIN1 and eIF3d.

The precise SIN1 interaction site in the C-terminal region of eIF3d remains to be elucidated. The discrepancy between GST-pull downs and Flag tagged eIF3d interaction approaches could be due to the influence of the tag used in each experimental approach. The difference in size and properties of the two tags may alter the ability of the protein to bind other partners or promote conformational changes within the protein altering its ability to bind SIN1. Also, the influence of the N-terminal region of the protein has to be taken into account. The SIN1 interaction site on eIF3d could be formed from distant residues within the protein. Thus different sites in the C-terminal region may participate in the interaction to SIN1 but disruption of these sites may not be sufficient to fully prevent the association of the two proteins. In addition, the interaction of eIF3d and SIN1 could be mediated by a third interacting partner, forming a complex rather than being dependent solely on the direct interaction between the two proteins.

One of the main experimental limitations in the elucidation of the SIN1 binding sites on eIF3d was the effects on the solubility of the Flag-tagged C-terminal mutants. This is likely to be occurring due to exposure of hydrophobic patches in the mutants, thus promoting aggregation of the proteins. Identification of such regions and design of mutants that do not compromise the solubility of the protein would allow the further study of the interaction between eIF3d and SIN1.
In summary, eIF3d is a SIN1 binding protein but is not a core component of mTORC2, and the interaction between eIF3d and SIN1 is likely to be occurring in the nucleus.
Chapter 4: eIF3d Regulates mTOR activity

4.1 Introduction

Having provided evidence that eIF3d interacts with the mTORC2 component SIN1, it was important to determine the functional significance of this interaction and whether eIF3d has any role in the regulation of the mTOR pathway. It has been previously described that the RAPTOR-mTOR complex and S6K interact with other members of the eIF3 complex in an insulin-stimulation dependent manner, suggesting a link between mTORC1 activity and its association with the translational pre-initiation complex (Holz et al., 2005). Furthermore, even though the mechanisms via which growth factors activate mTORC2 have been elusive, mTORC2 has been shown to be physically associated with the ribosome and ER in an insulin-stimulated manner (Zinzalla et al., 2011), spatially linking the activation of mTORC2 with the translational machinery. Furthermore, in yeast, the eIF3d orthologue Moe1 has been identified as a regulator of cytoskeleton organization (Chen et al., 1999), and is required for optimal protein translation (Bandyopadhyay et al., 2002). Recently eIF3d has also been linked to regulation of cell proliferation in mammalian cells (Li et al., 2015). These eIF3d functions, its potential location on the ribosome and its participation in anabolic processes make it an interesting candidate for study in the context of mTOR activity. Thus, the association of eIF3d with SIN1 might have a functional consequence on the activity of both mTOR complexes in mammalian cells. The aim of this chapter is to determine whether eIF3d has a role in the regulation of mTOR substrate phosphorylation using RNAi approaches.
4.2 Results

4.2.1 eIF3d regulates the phosphorylation of mTOR substrates.

In order to study whether eIF3d has a role in the mTOR pathway, a siRNA approach was taken to knockdown eIF3d in HeLa cells. Two commercial target-specific siRNAs pools were used to knockdown eIF3d in Hela cells, eIF3ζ siRNA from Santa Cruz Biotechnology (data not shown) and eIF3S7 ON-TARGETplus duplex from Dharmacon (Figure 17). Phosphorylation of AKT, PKCα and SGK-1 detected with phopho-specific antibodies by immunoblot was used as functional readout of the activity of mTORC2. Similarly, phosphorylation of S6K, S6, 4E-BP1, PRAS40 and ULK-1 were used as functional readouts for mTORC1 activity. The use of siRNA significantly decreased the amount of eIF3d protein in Hela cells (Figure 17). Silencing eIF3d does not appear to significantly change the expression or phosphorylation of AKT at residue S473 in basal conditions. Interestingly, for both AKT and PKCα, there is a decrease in phosphorylation of their turn motif sites, T450 and T641, respectively, suggesting that eIF3d might regulate mTORC2 activation of specific targets (Figure 17A). On the other hand, eIF3d knockdown appears to increase the phosphorylation of S6K, S6 and PRAS40 suggesting a possible role of eIF3d as a negative regulator of mTORC1 (Figure 17B). However, no change in the phosphorylation of 4E-BP1 and a decrease in the phosphorylation of ULK-1 are observed. These opposing effects on mTORC1 substrate phosphorylation suggest that eIF3d is a selective regulator of mTORC1 by directing its activity toward certain substrates rather than inhibiting mTOR catalytic activity. This might indicate that eIF3d is a negative regulator of anabolic mTORC1 activities and a potential activator of autophagy through ULK-1 phosphorylation.

In order to further study the relevance of eIF3d in the mTOR pathway and investigate the effects of long-term knock-down of eIF3d, six non-overlapping
Figure 17. eIF3d regulates the phosphorylation of mTOR substrates. Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA, eIF3S7 ON-TARGETplus, Dharmacon) or with a non-targeting siRNA (control siRNA, Non-specific, Eurofins Genomics). Relative protein and phosphorylation levels of mTORC1 (A) and mTORC2 (B) targets were determined by immunoblot using the indicated antibodies. The data are representative of 3 independent experiments.
shRNA sequences targeting the coding region of eIF3d were generated and cloned into the pKLO.1 vector system and tested by transient transfection in Hela cells (Figure 18A). The shRNA that most efficiently decreased eIF3d expression was selected and its target sequence was mutated in the pCDNA-flag-eIF3d construct in order to generate an eIF3d mutant resistant to shRNA silencing. Hela cells were co-transfected with pKLO.1-eIF3d shRNA and pCDNA-shRNA-resistant flag-eIF3d plasmid. The activity of mTORC1 and mTORC2 was measured by immunoblot using phospho-specific antibodies against their substrates as previously described. As observed in Figure 18B, the increased phosphorylation of S6K, S6 and AKT at the T450 site observed after shRNA knock-down can be rescued to basal levels by the re-expression of eIF3d. This indicates that eIF3d is responsible for regulating the changes in phosphorylation observed in these mTOR targets.

4.2.2 eIF3d promotes AKT and PKCα stability

The changes observed in phosphorylation of AKT and PKCα at their turn motif site is interesting since this site is reported to be co-translationally phosphorylated and is non-responsive to stimuli (Facchinetti et al, 2008). In contrast, phosphorylation of their hydrophobic motifs is growth factor responsive and is critical for the full activation of these kinases (Jacinto and Lorberg, 2008). This raises the question of the functional consequence of specific dephosphorylation of T450 on AKT and T641 on PKCα. Turn motif site phosphorylation has been implicated in the stability of both proteins as the loss of phosphorylation of AKT and PKCα makes them dependent on Hsp90 for stability (Facchinetti et al, 2008). Thus, the stability of AKT and PKCα in cells treated with the Hsp90 inhibitor, Geldanamycin, was tested. Hela cells were transfected with eIF3d siRNA and treated with 2μM Geldanamycin for 4 and 8 hours prior cell lysis. The amount of AKT and PKCα protein was assessed by immunoblot. Under Geldanamycin treatment, no significant change is observed in
Figure 18 eIF3d regulates the phosphorylation of mTOR substrates. A Hela cells were transfected with constructs expressing either control shRNA or six distinct shRNA sequences targeting eIF3d (shRNA seq. 1 to 6). Relative protein and phosphorylation levels were determined by immunoblot using the indicated antibodies. B Hela cells were co-transfected with a construct expressing either the shRNA sequence 6 (eIF3d shRNA) or control shRNA and an empty vector control or a construct expressing a Flag-eIF3d mutant not targeted by the shRNA (shRNA resistant eIF3d). Relative protein and phosphorylation levels were determined by immunoblot using the indicated antibodies. The data are representative of 3 independent experiments. Quantification of immunoblot band intensities were normalised to the TUBULIN band intensity and presented as fold change of control shRNA co-transfected with the empty vector control. Error bars were calculated as SEM (n=3). Two-way ANOVA followed by Bonferroni corrected post hoc t test was performed among the different conditions (*p-value≤0.05, **p-values≤0.01).
control siRNA conditions but disruption of Hsp90 promotes a 50% decrease in the amount of both AKT and PKCα when eIF3d is knocked down (Figure 19).

4.2.3 eIF3d regulation of S6K phosphorylation is mTORC1 dependent

Phosphorylation of T389 on S6K has been widely used as an indicator of mTORC1 activity. Thus, in order to confirm whether the increase in phosphorylation observed after eIF3d knockdown was via mTOR, it was determined if this effect was blocked by inhibition of mTOR. Hela cells were transfected with control and eIF3d siRNA and treated with 50 and 100nM of the mTOR inhibitor Rapamycin for 1 hour in order to block mTORC1 activity. To block both mTORC1 and mTORC2 activities cells were treated with 5 and 10 mM Torin 1 for 1 hour. The increase in the phosphorylation of S6K, and consequently S6, by eIF3d knockdown is fully blocked by both Rapamycin and Torin1 treatment (Figure 20). Furthermore, blocking AKT activity by incubation of Hela cells with 2 and 4 μM GSK69069 for 1 hour also blocks the increase in S6K phosphorylation observed after eIF3d knockdown (Figure 21A). This suggests that the increase in S6K phosphorylation observed following eIF3d knockdown is a mTORC1-dependent event, possibly mediated by AKT-dependent activation of mTORC1.

To further test the dependency of the increase in S6K phosphorylation on mTORC1, the effect of inhibition of PI3K, as an upstream mTORC1 regulator was tested in Hela cells under eIF3d knockdown conditions. Additionally, as ERK participates in the activation of S6K by phosphorylation of its C-terminal domain and releasing S6K from auto-inhibition (Lehman et al, 2006), inhibition of MEK was also tested. Even though both LY294002 and Wortmannin significantly reduce S6K phosphorylation, the use of these PI3K inhibitors does not fully block the increase of phosphorylation
Figure 19. eIF3d promotes AKT and PKC stability.

A Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were treated with 2μM Geldanamycin (Hsp90 inhibitor) for 4 and 8 hours before lysis. Relative protein and phosphorylation levels were determined by immunoblot using the indicated antibodies. The data are representative of 3 independent experiments. Quantification of immunoblot band intensities for AKT (B) and PKCα (C) were normalized to the TUBULIN band intensity and presented as fold change of control siRNA transfected cells untreated (0h Geldanamycin). Error bars were calculated as SEM (n=3). Two-way ANOVA followed by Bonferroni corrected post hoc t test was performed among the different conditions (*p-value≤0.05, **p-value≤0.01)
Figure 20. eIF3d regulation of S6K phosphorylation is mTOR dependent.

Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were treated with 50 and 100nM Rapamycin (mTORC1 inhibitor) for 1 hour (A) and 5 and 10 mM Torin 1 (mTOR inhibitor) for 1 hour (B) before lysis. Relative protein and phosphorylation levels were determined by immunoblot using the indicated antibodies. The data are representative of 3 independent experiments.
Figure 21. eIF3d regulation of S6K phosphorylation is mTOR dependent. Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were treated with 2 and 4 μM of GSK69069 (AKT inhibitor) for 1 hour (A), 4.0μM LY294002 and 200nM Wortmannin for 1 hour (PI3K inhibitors) (B) and 10μM U0126 (MEK inhibitor) (C) for 1 hour before lysis. Relative protein and phosphorylation levels were determined by immunoblot with the indicated antibodies. The data are representative of 3 independent experiments.
promoted by eIF3d silencing (Figure 21 B). At the same time, inhibition of MEK by 10μM U0126 for 1 hour did not cause any significant change in S6K phosphorylation either in basal or eIF3d knockdown conditions (Figure 21C). This data further confirms that the increase in S6K phosphorylation observed by eIF3d knockdown is mediated by an increase in mTORC1 activity.

4.2.4 Disruption of mTORC2 signalling increases phosphorylation of S6K under eIF3d knockdown conditions

Interestingly, mTORC2, acting through the phosphorylation of AKT, has also been described as an upstream regulator of mTORC1 (Laplante and Sabatini, 2012). The fact that eIF3d interacts with SIN1, a critical component of mTORC2, but affects mTORC1 activity in an AKT dependent manner raises the question of whether the regulation of S6K phosphorylation by eIF3d is mediated by the crosstalk between the two mTOR complexes. To further test the influence of mTORC2 activity on eIF3d regulation of S6K phosphorylation, Hela cells transfected with eIF3d siRNA were treated with bisindolylmaleimide I, an inhibitor of another mTORC2 downstream substrate PKCα. Interestingly, inhibition of PKCα did not seem to alter S6K phosphorylation. However, in contrast to what was observed with AKT inhibition, the inhibition of PKCα under eIF3d knockdown conditions promoted a further increase in S6K phosphorylation (Figure 22A). Knocking down the mTORC2 components SIN1 or RICTOR by siRNA did not promote any significant change in S6K phosphorylation. However, similar to what was observed with PKCα inhibition, disruption of mTORC2 further increased S6K phosphorylation under eIF3d knockdown conditions (Figure 22B). This suggests that mTORC2 might be acting as an mTORC1 negative regulator, potentially through PKCα, in an eIF3d dependent manner.
Figure 22. Disruption of mTORC2 signalling increases phosphorylation of S6K under eIF3d knockdown conditions.

A Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were treated and 4 and 8 μM bisindolylmaleimide I (Bis-I, PKC inhibitor) for 1 hour before lysis. B Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA) and either a non-targeting siRNA (control siRNA), an siRNA targeting SIN1 or an siRNA targeting RICTOR. Relative protein and phosphorylation levels were determined by immunoblot using the indicated antibodies. The data are representative of 3 independent experiments. Quantification of immunoblot band intensities were normalized to the TUBULIN band intensity and presented as fold change of untreated cells transfected with control siRNA. Error bars were calculated as SEM (n=3). Significany of each sample is reported Two-way ANOVA followed by Bonferonni corrected post hoc t test was performed among the different conditions (*p-values≤0.05).
4.2.5 Effect of IGF-1 stimulation on eIF3d regulation of mTOR substrates

To further investigate the role of eIF3d in mTORC1 signalling, the effect of eIF3d knockdown on the induction of mTORC1 activity by IGF-1 stimulation was analysed. Hela cells were transfected with eIF3d siRNA and serum starved for 6 hours and stimulated with 100ng/mL IGF-1 for 20 minutes prior to cell lysis. Serum starvation decreased S6 phosphorylation compared to cells grown in serum while IGF-1 stimulation increased S6 phosphorylation as would be expected (Figure 23A). Under eIF3d knockdown conditions, there was an increase in S6 phosphorylation as previously observed. Serum starvation partially blocked this increase while IGF-1 caused an increase. However, the increase observed in S6 phosphorylation after IGF-1 stimulation was comparable to that observed under control siRNA conditions, indicating that eIF3d knockdown might not further contribute to S6 phosphorylation by IGF-1 mediated activation.

To further study the role of eIF3d in growth factor dependent S6K activation, the temporal dynamics of IGF-1 induction of mTORC1 activity was tested following eIF3d knockdown. After IGF-1 treatment of both control and eIF3d siRNA transfected cells, S6K phosphorylation increased progressively over time (Figure 23B). Interestingly at early time points (1 and 5 minutes after IGF-1 stimulation) there was an increase in AKT phosphorylation at S473 in control cells. This increase was also observed under eIF3d knockdown conditions but it did not reach the same amplitude as in the control cells, suggesting an impairment of IGF-1 dependent AKT phosphorylation. Rapamycin treatment blocked S6K phosphorylation. It also delayed AKT phosphorylation but this was still impaired in eIF3d knockdown cells. In both untreated and Rapamycin treated cells, AKT phosphorylation levels appeared to be comparable between control and eIF3d knockdown conditions after
Figure 23. IGF-1 stimulation does not further increase S6K phosphorylation under knockdown conditions. Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were serum starved for 6 hours and stimulated with IGF-1, 100 ng/mL for 20 min before lysis (A). Alternatively cells were starved for 6 hours and stimulated with IGF-1 for the indicated times before lysis (B). Relative protein and phosphorylation levels were determined by immunoblot using the indicated antibodies. The data are representative of 3 independent experiments. Quantification of immunoblot band intensities were normalized to the Actin band intensity and presented as fold change of control siRNA transfected cells untreated. Error bars were calculated as SEM (n=3). Two-way ANOVA followed by Bonferonni corrected post hoc t test was performed among the different conditions. Significany of each sample is reported (*p-value<0.05).
about 20 minutes of IGF-1 stimulation and, as expected, mTOR inhibition by Torin 1 fully blocked both, S6K and AKT phosphorylation. This suggests that the effect of eIF3d knockdown on S473 AKT phosphorylation is transitory and occurs at early times after IGF-1 induced mTOR activation. This is consistent with the fact that differences in the phosphorylation of AKT at residue S473 were not observed in unstimulated cells.

4.2.6 Effect of oxidative stress on eIF3d regulation of mTOR substrates

mTORC1 is also activated by different stress signals. To determine the role of eIF3d in regulating mTORC1 activity in response to oxidative stress, a combination of eIF3d knockdown and treatment with either H₂O₂ or sodium arsenite was tested. Hela cells were transfected with eIF3d siRNA and incubated with either 500μM sodium arsenite or 500μM H₂O₂ for 30 minutes prior to cell lysis.

As previously described (Thedieck et al., 2013), arsenite induced S6K phosphorylation (Figure 24B). Interestingly, under eIF3d knockdown conditions, both arsenite and H₂O₂ further increased S6K phosphorylation (Figure 24A and B). This suggests that activation of mTORC1 by oxidative stress does not completely release the complex from eIF3d dependent inhibition and eIF3d might be participating in preventing hyperactivation of mTORC1 in response to stress.
Figure 24. Activation of mTORC1 by oxidative stress further increases S6K phosphorylation under eIF3d knockdown conditions.
Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were treated with either 500 μM H$_2$O$_2$ for 30 minutes (A) or 500 μM sodium arsenite for 30 minutes (B) before lysis. Relative protein and phosphorylation levels were determined by immunoblot with the indicated antibodies. The double band observed in S6K and P-S6K immunoblots corresponds to the p70 S6K and p85 S6K isoforms. The data are representative of 3 independent experiments. Quantification of immunoblot band intensities were normalized to the TUBULIN band intensity and presented as fold change of control siRNA transfected cells untreated. Error bars were calculated as SEM (n=3). Two-way ANOVA followed by Bonferroni corrected post hoc t test was performed among the different conditions. Significance of each sample is reported (*p-value ≤ 0.05).
4.3 Conclusions

Altogether, the data in this chapter indicates that eIF3d inhibits both, S6K and S6 phosphorylation, as observed by increase in phosphorylation of both kinases under eIF3d knockdown conditions. This might be occurring as a consequence of modulation of mTORC1 activity. Inhibition of AKT prevents the increase of S6K phosphorylation in a similar fashion as the inhibition of mTOR with Rapamycin and Torin 1 (Figure 20 and Figure 21 A). This suggests that eIF3d might inhibit the activation of the mTORC1 thought an AKT-dependent mechanism. As the opposite effect is observed by disruption of SIN1 and RICTOR (Figure 22), this AKT-dependent activation seems to be independent of mTORC2. Since IGF-1 seems to be unable to significantly increase the S6 phosphorylation levels under eIF3d knockdown conditions (Figure 23A) eIF3d could be modulating the growth factor activation of mTORC1 through AKT activity in an event independent of mTORC2. A recent report showed that in response to insulin, AKT phosphorylation by PDK1 is sufficient to promote its phosphorylation of SIN1 prior to mTORC2 activation (Yang et al., 2015). In a similar way, AKT could be mediating mTORC1 activation by phosphorylating PRAS40 or another mTORC1 regulator in an mTORC2-independent manner.

eIF3d knockdown decreases the phosphorylation of both AKT and PKCα at their turn motifs. These residues participate in regulating the stability of these proteins and they become dependent on Hsp90 to prevent degradation when their turn motifs are not phosphorylated (Figure 19) (Facchinetti et al., 2008; Ikenoue et al., 2008). This could result in decreased function of AKT and PKCα under stress conditions. At the same time, the further induction of S6K phosphorylation under eIF3d knockdown conditions by mTORC2 disruption could be mediated by PKCα. Thus, decreased PKCα activity after eIF3d disruption may account for the increased mTORC1 activity and thus, mTORC2 dependent activation of PKCα may account
for a novel point of communication between mTORC1 and mTORC2. The observation that eIF3d acts as an mTORC2 activator may be related to its direct binding to SIN1. Association of SIN1 to eIF3d might facilitate the localisation of SIN1 and mTORC2 to the ribosome, promoting the phosphorylation of AKT and PKCα at their turn motifs. In addition, eIF3d might be acting as a scaffold for the recruitment of these mTORC2 substrates. Through the regulation of mTORC2, eIF3d may indirectly affect mTORC1 activity as a consequence of the crosstalk between the two complexes. Alternatively, eIF3d binding to SIN1 might restrict the interaction of SIN1 to common mTORC1 and mTORC2 components. Changes in the availability of these components might impact the integrity and function of mTORC1.

It is interesting to note that eIF3d may be regulating the temporal dynamics of AKT phosphorylation at S473 after IGF-1 stimulation (Figure 23B) possibly affecting the initial steps in IGF-1 mediated AKT phosphorylation. The effect of eIF3d disruption on AKT and S6K phosphorylation may be an uncoupled event, since the changes in phosphorylation dynamics for both targets differ. There is a delay of the peak of AKT phosphorylation after IGF-1 stimulation (Figure 23B), while there is a steady increase in phosphorylation on S6K. Additionally, rapamycin treatment, as expected, completely blocks S6K phosphorylation whereas it only delays AKT activation but does not prevent it.

It seems that oxidative stress further increases the activation of S6K under eIF3d knockdown conditions. Oxidative stress participates in both activation and inhibition of mTORC1 (Thedieck et al., 2013). In the case of both arsenite and hydrogen peroxide, inhibition by eIF3d might be preventing the hyperactivation of the mTORC1 complex as a result of persistent exposure to stress. This could be happening by a different mechanism compared to growth factor induced S6K phosphorylation.
In summary, eIF3d is an mTOR regulator. eIF3d acts as an mTORC2 activator, promoting the phosphorylation of AKT and PKCα at their turn motifs and AKT hydrophobic motif early phosphorylation after growth factor stimulation. eIF3d is an mTORC1 inhibitor towards S6K through inhibition of the AKT-mediated mTORC1 activation and through mTORC2-mediated mTORC1 inhibition.
Chapter 5: eIF3d regulates cell Growth and Proliferation

5.1 Introduction

The mTOR pathway is an attractive target to study due to its role in cell growth, proliferation and survival, all of which are highly associated with the pathogenesis and progression of cancer and metabolic diseases. mTORC1 positively regulates cell growth and proliferation by promoting anabolic processes, such as protein synthesis and organelle biogenesis, and by negatively regulating catabolic processes such as autophagy (Wullschleger et al., 2006). At the same time, mTORC2 has been linked to cell survival, metabolism and proliferation due to the high dependency of these processes on the activation of AKT through the phosphorylation of various effectors (Manning and Cantley, 2007). Thus, since the effects of eIF3d observed on the activity of mTORC1 and mTORC2 suggest a potential participation of this factor in mTOR-related processes, a key issue was to determine whether eIF3d has a role in mTOR related phenotypes such as cell growth and proliferation. eIF3d, as part of the eIF3 complex, has been linked to protein synthesis and thus to cellular growth (Hinnebusch, 2006). Furthermore, eIF3d has been implicated in cytoskeletal organisation and the regulation of cell growth and morphology in Schizosaccharomyces pombe (Chen et al., 1999).

Interestingly, S. pombe lacking eIF3d have more microtubule bundles compared with wild type cells, showing abnormalities in the spindle (Chen et al., 1999). Deletion of both eIF3d and eIF3E orthologs, in combination with defects in Ras1 have been described to lead to chromosome missegregation due to the defect in proper spindle formation (Yen and Chang, 2000), further suggesting a link between eIF3d and cell proliferation in S. pombe.

The aim of this chapter is to determine whether eIF3d has a role in cell growth and proliferation, as these are major processes regulated by mTOR activity. This is
addressed by RNAi-mediated eIF3d knockdown in combination with flow cytometry and immunofluorescence assays.

5.2 Results

5.2.1 eIF3d regulates cell size.

The increased mTORC1 activity observed under eIF3d knockdown conditions results in an increase in S6K phosphorylation and consequently S6 phosphorylation. This raised the question of whether this will have an impact on cell growth, as it has been reported that S6K controls cell size in Drosophila (Stocker et al., 2003). Thus, it was initially tested whether eIF3d knockdown had an impact on cell size. Hela cells were transfected with control and eIF3d siRNA and cell size was measured by forward scattering with flow cytometry and by directly measuring cell diameter using an automated cell counter. Consistent with the increase in S6 phosphorylation, knockdown of eIF3d caused an increase of 10% in cell size and in cell diameter in HeLa cells (Figure 25).

5.2.2 eIF3d regulates cell proliferation

The observations that knockdown of eIF3d makes AKT unstable if Hsp90 is inhibited (Figure 19) and impairs IGF-1 stimulated AKT phosphorylation at S473 (Figure 23 B) suggests that phenotypes associated with changes in mTORC2 and AKT activities could be also affected. To test the effects of eIF3d disruption on cell proliferation in Hela cells, crystal violet assays were performed using both siRNA and shRNA directed against eIF3d. Crystal Violet accumulation was used as an estimate of cell proliferation, as the dye accumulation in the cell nucleus of fixed cells, correlates with the nuclear DNA content and thus with cell number. There was
Figure 25. eIF3d negatively regulates cell size.
Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cell size was analysed by measurement of the forward-scattered light (FSC). The graph shows the mean measured FSC value from three independent experiments. Error bars were calculated as standard error of the mean (SEM) (A). Direct cell diameter was measured using an automated cell counter with cells stained with trypan blue. (B). The graph shows the mean measured values from three independent experiments. Error bars were calculated as standard error of the mean (SEM)
Figure 26. eIF3d regulates cell proliferation.
A Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were stained with crystal violet to assess cell proliferation.
B Hela cells were co-transfected with a construct expressing an anti-eIF3d shRNA or a control shRNA sequence control shRNA and either an empty vector control or a Flag-eIF3d mutant not targeted by the shRNA (shRNA resistant eIF3d). Cells were stained with crystal violet to assess cell proliferation. The absorbance of methanol-solubilized crystal violet was measured at 590nm. The graph shows the mean measured values from three independent experiments. Error bars were calculated as standard error of the mean (SEM) (*p-value < 0.05).
reduced proliferation in siRNA transfected cells from the third day following transfection with around 30% reduction in cell number compared with the non-specific siRNA control (Figure 26A). This reduction in proliferation was confirmed by shRNA directed against eIF3d and was fully rescued by re-introduction of an eIF3d mutant where the mRNA is not targeted by the shRNA used for eIF3d silencing (Figure 26B). Taken together, these results indicate that eIF3d participates in the regulation of cell proliferation.

5.2.3 eIF3d knockdown impairs cell cycle progression

In order to investigate possible causes for the proliferation defect observed under eIF3d knockdown, the cell cycle distribution of Hela cells was analysed by propidium iodide staining of DNA using flow cytometry. Hela cells were transfected with eIF3d siRNA and collected at the exponential growing phase. Propidium iodide staining showed a higher population of cells at G2/M phase in eIF3d knockdown cells compared with cells transfected with a non-specific siRNA (Figure 27A). To further understand the nature of this difference, analysis of cell cycle progression of synchronised cells was performed. Hela cells were transfected with eIF3d siRNA and synchronised by double thymidine blocking using 2mM thymidine for 12 hour block/release cycles. The results showed an overall delay in progression from G0/G1 to S phase and S to G2/M phase following release from thymidine block (Figure 27B). Interestingly, after 18 hours of release, there is an apparent G2/M phase arrest, consistent with the increased population undergoing G2/M in non-synchronised cells. This suggests that eIF3d knockdown might be impairing the proliferation rate by promoting cell cycle arrest in Hela cells.
Figure 27. eIF3d regulates cell cycle progression.

A Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cell cycle distribution was measured by flow cytometry using propidium iodide (PI) staining. B Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were synchronized by double thymidine blocking using 2mM thymidine in 12 hour block/release cycles and harvested at the indicated time points after release. Cell cycle distribution was measured by flow cytometry using propidium iodide (PI) staining. (n=3) *p < 0.05 in comparison of G2/M phases in control versus eIF3d siRNA. Data shown was analysed with the ModFit DNA analysis program.
5.2.4 elf3d knockdown induces mitotic spindle multipolarity

In yeast, elf3d has been described as a microtubule associated protein participating in the assembly and stability of microtubules (Chen et al., 1999). To test whether human elf3d showed phenotypes associated with defects in microtubule organization, immunofluorescence of β-tubulin was performed in HeLa cells under control and elf3d siRNA knockdown conditions. Whereas no obvious change was observed in resting cells, cells undergoing mitosis showed a clear increase in spindle multipolarity (Figure 28A). The incidence of multipolar spindles rose from 20% to 60% of total mitotic cells under elf3d knockdown conditions in a process that might be related to mTOR activity, as inhibition of mTOR by torin1 and rapamycin partially rescue this phenotype (Figure 28B and C). This suggests that the cell proliferation defect due to G2/M arrest in elf3d knockdown cells may originate due to defects in spindle formation.
Figure 28. eIF3d regulates spindle multipolarity.

A Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Immunostaining was performed using the indicated antibodies and localization of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. Images are representative from 3 independent immunofluorescence experiments. B Quantification of spindle number observed in A expressed as percentage of total mitotic cells in sample. C Hela cells were transfected with a siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were incubated with either DMSO, Torin1 or Rapamycin for 24 hours before fixation. Quantification of spindle number expressed as percentage of total mitotic cells observed. Scale bars 10 μm. (n=3) *P < 0.05 in comparison of spindle multipolarity in control versus eIF3d siRNA.
5.3 Conclusions

eIF3d knockdown increases cell size (Figure 25A), which is consistent with an increase in S6 and S6K activity (Montagne et al., 1999; Fingar et al., 2002). This is comparable to the increase in cell size observed by disruption of the mTORC1 inhibitors TSC2, DEPTOR and TBC1D7 in mammalian cells (Peterson et al., 2009; Dibble et al., 2012). This was an indication that the effects observed on the phosphorylation of mTOR targets by eIF3d are physiologically relevant. Together with cell size, another important mTOR-activity readout is cell proliferation. eIF3d appears to be important for cell proliferation and cell cycle progression in G2/M (Figures 26 and 27). The defect observed in mitotic spindle assembly may explain the cell cycle arrest observed but the exact molecular mechanism of the spindle defect remains to be elucidated. Assembly of the spindle apparatus and other regulatory components such as the activation of polo-like kinases as well as positioning of CENTRIN and AURORA-A will need to be assessed in order to determine the role of eIF3d in spindle formation. It is possible that eIF3d is functioning in the nucleus as a scaffold in order to facilitate the positioning of critical regulatory components to the spindle apparatus, thus contributing to its correct assembly. It has to also be considered that, in addition to the increase in S6 and S6K activity, the increase in cell size observed under eIF3d knockdown might be a consequence of the delay in cell cycle progression. The prolonged transition from G1 to S phase observed, may be leading to larger cells due to ongoing macromolecule synthesis resulting in increased cellular content and overall cell growth rather than specifically triggering a cell size related pathway.

In summary, eIF3d is a negative regulator of cell size but promotes cell proliferation and is necessary for proper mitotic spindle bipolarity in Hela cells.
Chapter 6: eIF3d and regulation of stress granule formation

6.1 Introduction

It has been reported that upon oxidative stress, ASTRIN inhibits mTORC1 by recruiting the mTORC1 component RAPTOR to stress granules, thereby disassociating it from mTOR and preventing mTORC1-hyperactivation (Thedieck et al, 2013). Interestingly, knockdown of other eIF3 subunits causes a failure to induce stress granules in RDG3 cells (Ohn et al 2008), which might suggest that eIF3d knockdown might induce mTORC1 activity by impaireing its inhibition by stress granules. On the other hand, the mTORC2 downstream target PKCα has also been described as a component of stress granules by its association with RNA-binding proteins such as G3BP2. Furthermore, stress granule assembly is suppressed by PKCα downregulation (Kobayashi et al, 2012), suggesting a link between mTOR regulation and stress granule formation. The aim of this chapter is to determine whether eIF3d localises in stress granules by immunofluorescence microscopy and whether eIF3d has any role in stress granule formation by knockdown of eIF3d by RNAi.

6.2 Results

6.2.1 eIF3d localises in stress granules in response to arsenite

To test whether eIF3d localises to stress granules upon stress, HeLa cells were incubated with 500 μM arsenite for 30 minutes and granule formation was assayed by immunofluorescence. Upon arsenite stress, eIF3d nuclear localisation decreases and it forms granular cytoplasmic structures (Figure 29A) that co-
Figure 29. eIF3d localises in stress granules in response to arsenite stress.

A Hela cells were treated with 500 μM sodium arsenite for 30 minutes before fixation. Immunostaining was performed using an anti-eIF3d antibody and localisation assessed by fluorescence microscopy in cells fixed in 4% PFA. B Hela cells were treated with 500 μM sodium arsenite for 30 minutes before fixation. Immunostaining was performed using anti-eIF3d antibody and anti-G3BP1 antibody as a stress granule marker. Localisation of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. Scale bars 10 μm. Images are representative from 3 independent immunofluorescence experiments.
localise with the stress granule marker G3BP1, whereas no co-localisation was observed in non-stressed cells (Figure 29B). This suggests that eIF3d is a component of stress granules.

It was next tested whether eIF3d co-localises with mTOR related components upon arsenite stress. No co-localisation of SIN1 or RICTOR was observed with G3BP1 in either stressed or unstressed cells (Figure 30), indicating that mTORC2 components do not localise in stress granules. Similarly, co-localisation of eIF3d with SIN1, RICTOR and RAPTOR under arsenite stress conditions was tested. Hela cells were transfected with plasmids expressing either Myc-SIN1, Flag-RICTOR or HA-RAPTOR, incubated with 500 μM arsenite for 30 minutes and localisation was assayed by immunofluorescence microscopy. The granular structures formed by eIF3d do not co-localise with overexpressed SIN1 or RICTOR (Figure 31A and B), but, at small degree, some co-localisation with the mTORC1 component RAPTOR can be observed under these conditions (Figure 31C). This suggests that upon arsenite stress, eIF3d and RAPTOR can localise in stress granules.

6.2.2 eIF3d knockdown does not block stress granule formation

Stress granules are cytoplasmic messenger ribonucleoprotein (mRNPs) complexes that are formed when translation initiation is impaired in response to stress (Buchan and Parker, 2009). eIF3 components are linked to stress granule formation as part of the translation preinitiation complex (Ohn et al. 2008). To test the effect of silencing eIF3d on stress granule formation, Hela cells were transfected with eIF3d siRNA and treated with 500 μM arsenite for 30 minutes prior to cell fixation. Stress granule formation was assayed by immunofluorescence of G3BP1. As observed in Figure 32, Hela cells lacking eIF3d still form stress granules. Interestingly, these stress granules formed under knockdown conditions appear to be smaller and more
Figure 30. SIN1 and RICTOR do not localise in stress granules in response to arsenite stress.

Hela cells were treated with 500 μM sodium arsenite for 30 minutes before fixation. Immunostaining was performed using either anti-SIN1 (A) or anti-RICTOR (B) antibodies. Anti-G3BP1 antibody was used as a stress granule marker. Localisation of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. Scale bars 10 μm. Images are representative from 3 independent immunofluorescence experiments.
Figure 3. eIF3d localises with RAPTOR in stress granules in response to arsenite stress. Hela cells were transfected with constructs either expressing Myc-SIN1 (A), Flag-RICTOR (B) or HA- RAPTOR (C). Cells were treated with 500 μM sodium arsenite for 30 minutes before fixation. Immunostaining was performed using the indicated antibodies and localisation of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. Scale bars 10 μm. Images are representative from 3 independent immunofluorescence experiments.
Figure 32. eIF3d does not block stress granule formation. Hela cells were transfected with siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were treated with 500 μM sodium arsenite for 30 minutes before fixation. Immunostaining was performed using the indicated antibodies and localisation of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. Scale bars 10 μm. Images are representative from 3 independent immunofluorescence experiments.
abundant compared to control. As mTORC1 inhibition by stress granule formation is ASTRIN dependent (Thedieck et al., 2013), localization of ASTRIN after elf3d disruption was tested. elf3d knockdown does not seem to affect the localisation of ASTRIN to stress granules (Figure 33).

6.2.3 elf3d regulates the dynamics of stress granule formation

To further study the effects of elf3d disruption on stress granules, it was tested whether it has a role in the dynamics of stress granule formation. Hela cells were transfected with control or elf3d siRNA and treated with a time course of 500μM sodium arsenite prior to cell fixation. The formation of stress granules was assayed by immunofluorescence microscopy of G3BP1. Interestingly, whereas elf3d silencing does not inhibit stress granule formation, its disruption promotes a delay in their maturation, as assayed by stress granule size. At 15 minutes of arsenite incubation, around 80% of control cells show formation of stress granules whereas the formation of stress granules in the elf3d siRNA cells is around 20%. Furthermore, at 20 minutes after arsenite incubation, even though both control and elf3d siRNA transfected cells show stress granule formation in the majority of the cells, elf3d knockdown cells present predominantly immature granules appearing to be smaller and more abundant than those observed in control cells (Figure 34).

To test whether this impairment of stress granule formation is related to the changes observed in mTORC1 activity, arsenite treatment was combined with 100 nM rapamycin treatment. The results show that the effect of elf3d knockdown on stress granule formation appears to be mTORC1 independent (Figure 35).
Figure 33. eIF3d does not affect ASTRIN localisation in stress granules. Hela cells were transfected with siRNA targeting eIF3d (eIF3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were treated with 500 μM sodium arsenite for 30 minutes before fixation. Immunostaining was performed using the indicated antibodies and localisation of the proteins was assessed by fluorescence microscopy in cells fixed in 4% PFA. Scale bars 10 μm. Images are representative from 3 independent immunofluorescence experiments.
Figure 34. elf3d Regulates the dynamics of stress granule formation. Hela cells were transfected with siRNA targeting elf3d (elf3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were treated with 500 μM sodium arsenite for the indicated times before fixation in 4% PFA. Immunostaining was performed using an anti-G3BP-1 antibody and localisation was assessed by fluorescence microscopy. Images are representative from 3 independent immunofluorescence experiments. Quantification of number of cells showing stress granule formation is expressed as percentage of total cells ± SEM. **Mature SG**: Cell showing predominantly G3BP-1 granules > 2.0μm of diameter. **Immature SG**: Cell showing predominantly G3BP-1 granules < 2.0μm of diameter. **No SG**: Cells showing no G3BP-1 Granules. (n=3) *P<0.05 in comparison of G3BP1 positive granules in control versus elf3d siRNA.
Figure 35. elf3d Regulation of the dynamics of stress granule formation is mTORC1 independent. Hela cells were transfected siRNA targeting elf3d (elf3d siRNA) or with a non-targeting siRNA (control siRNA). Cells were incubated with 100 nM Rapamycin and treated with 500 μM sodium arsenite for the indicated times before fixation. Immunostaining was performed using an anti G3BP-1 antibody and localisation was assessed by fluorescence microscopy. Quantification of number of cells showing stress granule formation is expressed as percentage of total cells ± SEM. Mature SG: Cell showing predominantly G3BP-1 granules > 2.0μm of diameter Immature SG: Cell showing predominantly G3BP-1 granules < 2.0μm of diameter. No SG: Cells showing no G3BP-1 Granules. (n=3) *P<0.05 in comparison of G3BP1 positive granules in control versus elf3d siRNA.
6.2.4 The RNA-binding domain of eIF3d is required for its localisation to stress granules

Stress granules are composed of mRNA molecules associated with preinitiation factors and other RNA-binding proteins. (Kedersha et al., 2013) Therefore it was tested whether deletion of the RNA-binding domain of eIF3d affected its localisation to stress granules. Hela cells were transfected with a construct expressing flag-eIF3d Δ86-118 and treated with 500μM sodium arsenite for 30 minutes prior cell fixation. Formation of stress granules was assayed by fluorescence microscopy. After arsenite stress, cells expressing Δ86-118 eIF3d showed a decrease of around 60% in eIF3d-positive stress granules.
Figure 36. The RNA-binding domain of eIF3d is required for its localisation to stress granules.  

A  Hela cells were transfected with either a pCDNA vector expressing a Flag-tagged eIF3d (Flag-eIF3d) or a pCDNA vector expressing a Flag-tagged eIF3d mutant lacking the RNA binding domain (Flag- eIF3d Δ86-123). 48 hours later cells were treated with 500 μM sodium arsenite for the indicated times prior being fixed with 4% paraformaldehyde. Immunostaining was performed using an anti-Flag antibody and localisation was assessed by fluorescence microscopy in cells fixed in 4% PFA. Scale bars 10 μm. Images are representative from 2 independent immunofluorescence experiments.  

B  Quantification of number of cells showing stress granule formation is expressed as percentage of total transfected cells ± SEM (n=2). **Mature SG:** Cell showing predominantly G3BP-1 granules > 2.0μm of diameter. **Immature SG:** Cell showing predominantly G3BP-1 granules < 2.0μm of diameter. **No SG:** Cells showing no G3BP-1 Granules.
6.3 Conclusions

Taken together, the data suggests that eIF3d, potentially via its association with RNA, participates in stress granule formation in Hela cells. It seems that the localisation of eIF3d in stress granules is independent of the mTORC2 components RICTOR and SIN1 (Figure 31). The role of eIF3d in stress granules may depend on its association with the eIF3 complex, as other subunits have been reported to localise to stress granules. Since mTORC1 activity is inhibited by stress granule sequestration of RAPTOR mediated by ASTRIN, eIF3d could be participating in such inhibition by its association to stress granules. Additionally if the association of eIF3d and SIN1 is relevant for mTORC2 activity, the incorporation of eIF3d into stress granules may play an indirect role in the regulation of the mTORC2 complex by disrupting the eIF3d-SIN1 association. Additionally, whether ASTRIN or RICTOR are interacting with eIF3d under stress conditions remains to be elucidated.

Disruption of eIF3d prevents efficient stress granule formation (Figure 34). Although it has to be taken into account that these effects are assessed by a knockdown approach, whether complete or chronic disruption of eIF3d fully impairs stress granule formation has to be elucidated. At the same time, it is proposed that eIF3d knockdown affects the maturation of stress granules, but this was evaluated only by granule size. Changes in stress granule maturation such as eIF2α phosphorylation levels have to be evaluated over time as large G3BP-induced stress granules show strong phosphorylation of eIF2α (Reineke et al., 2012). A slight decreased in eIF2α phosphorylation is observed under eIF3d knockdown (Figure 24B) but the relationship of this observation to the dynamics of stress granule formation has to be evaluated. In summary, eIF3d localises in stress granules and participates in the dynamics of stress granule formation in Hela cells.
Chapter 7: Discussion

The mTOR pathway is known to participate in the response to environmental cues regulating cell growth and proliferation. As a central regulator of cell metabolism, it has gained attention due to its role in human disease (Laplante and Sabatini, 2012). Thus, great efforts are being made to understand and modulate this pathway as it represents an important pharmacological target. The discovery that mTORC2 is a key regulator of the activity of the AGC kinases AKT, PKC and SGK1 that are involved in cellular processes such as proliferation and cell survival has stimulated the study of the mechanisms involved in its activation and regulation (Oh and Jacinto, 2011). SIN1 is a critical regulator of mTORC2 activity and a role as a scaffold has been proposed for the specific recruitment of mTOR substrates (Cameron et al, 2011; Jacinto et al, 2006). eIF3d, a non-core component of the eIF3 complex has been identified as a novel SIN1 interacting protein (Whitmarsh, unpublished results), but the biological meaning for this interaction and its relevance on mTORC2 biology are still unknown. eIF3d possesses no recognisable conserved functional domains except for its RNA binding domain, and has only recently been studied in mammalian models making it difficult to predict its functional role in mammalian cells (Bandyopadhyay et al, 2002; Yen and Chang, 2000; Li et al., 2015).

The findings presented in this thesis indicate that eIF3d is a novel interacting partner of SIN1 that does not form an integral part of the mTORC2 complex. Despite not being a core component of either mTORC1 or mTORC2, reduced eIF3d expression decreases the phosphorylation levels of AKT and PKCα turn motifs (Figure 19), decreases the phosphorylation level at the AKT hydrophobic motif after IGF-1 stimulation (Figure 23B), and increases mTOR dependent S6K phosphorylation (Figure 20).
7.1 eIF3d localisation and role as an mTOR regulator

The activities of both mTOR complexes depend on their specific cellular localisation. mTORC1 activation is highly dependent on its association to the lysosome (Sancak et al., 2010), and active mTORC2 is recruited to the ribosome in response to growth factor stimulation (Zinzalla et al., 2011). Since both the phosphorylation of AKT and PKCα at their turn motifs occurs co-translationally at the ribosome and the interaction of S6K with eIF3 participates in its phosphorylation via mTOR, the selectivity of mTOR substrate phosphorylation might be due to a role of eIF3d in the recruitment of mTORC2 to ribosomes, contributing to the regulation of compartment specific mTORC1 and mTORC2 pools. The data presented in this thesis demonstrates eIF3d nuclear localisation and co-localisation with SIN1 in this compartment (Figure 13). Interestingly, mTORC2 components have been previously reported to shuttle between the cytoplasm and the nucleus, as well as upstream mTOR regulators such as IRS1, PI3K and PDK1 (Rosner and Hengstschläger, 2008). In addition, phosphorylated AKT is found in this compartment (Meier et al., 1997) but the nuclear function of mTORC2 is still unknown. Defining the specific localisation in which the eIF3d-SIN1 interaction occurs, may explain the selectivity observed in the changes in phosphorylation of mTOR substrates. As eIF3d does not appear to be part of mTORC2, the eIF3d-SIN1 interaction could be part of an mTORC2-independent complex that acts indirectly on mTORC2 activity by the recruitment of SIN1.

The mTOR pathway is responsive to different stimuli and since the mTORC1 recruitment to eIF3 is promoted by IGF-1 (Peterson and Sabatini, 2005), it is possible that eIF3d may regulate mTOR activity and localisation by interacting with SIN1 in response to growth factors or other stimuli. However, no change is observed in the interaction between SIN1 and eIF3d under IGF-1 stimulation or oxidative stress (Figure 9). Thus, this interaction could be either constitutive or it
might be regulated by other mTOR activators not assessed in this study. Alternatively, the eIF3d-SIN1 interaction could be regulated by stimuli not affecting mTOR. Even though eIF3d does not appear to directly interact with mTORC1, it is possible that its association with SIN1 indirectly affects mTORC1 activity. As SIN1 is essential for mTORC2 assembly, if the eIF3d-SIN1 interaction promotes mTORC2 stability, reduced eIF3d expression might cause a disruption of mTORC2 integrity releasing its components from SIN1 interaction. The resulting increase in the availability of shared components between the mTOR complexes might positively impact mTORC1 formation and activity, resulting in an increase in mTORC1 substrate phosphorylation, as S6K. However, the functional consequence of the SIN1-eIF3d interaction and the mechanism by which eIF3d modulates mTOR activity are yet to be elucidated.

7.2. eIF3d as an mTORC1 regulator.

The enhanced increase in S6K phosphorylation by eIF3d knockdown in mTORC2 disrupted cells (Figure 22) indicates that eIF3d could be mediating a negative feedback regulatory loop between the two mTOR complexes in which mTORC2 acts as an mTORC1 inhibitor. This is consistent with previous reports showing enhancement in S6K phosphorylation after insulin stimulation in SIN1 knockdown and knockout cells (Jacinto et al., 2006). Furthermore, under eIF3d knockdown conditions, serum starvation does not fully block S6K phosphorylation and IGF-1 stimulation does not further increase the S6K phosphorylation levels (Figure 23A). One possibility is that mTORC1 could be activated by growth factor stimulation by releasing the eIF3d-mediated inhibition. Thus, eIF3d knockdown may be mimicking mTORC1 growth factor stimulation, resulting in enhanced S6K phosphorylation. Since S6K phosphorylation in response to oxidative stress stimuli such as arsenite and hydrogen peroxide is further increased following eIF3d knockdown, the participation of eIF3d in IGF-1-induced activation and oxidative stress-induced
activation of mTORC1 is likely to be occurring at different levels in the pathway. The observation that S6K activation appears to be dependent on AKT but is negatively regulated by PKCα (Figure 22), indicates that eIF3d could be inhibiting the growth factor activation of AKT independently of mTORC2. This could result in the activation of mTORC1 through PRAS40 phosphorylation and by inhibition of mTORC2 by preventing SIN1 phosphorylation (Yang et al., 2015). However, whether the participation of eIF3d in regulating mTORC1 activity is direct, or is exerted by modulation of mTORC2 activity remains to be elucidated. As no direct interaction to RAPTOR or RICTOR is observed, it is also possible that the interaction with SIN1 is mediated by association to other mTOR complexes components. eIF3d might be associating to PRAS40, DEPTOR or PROTOR and regulating mTOR activity indirectly through modulating the inhibitory effects of these proteins.

Alternatively, although phosphorylation through mTORC1 is the best characterised mechanism for the regulation of S6K1, effects on dephosphorylation may explain the increase in S6K phosphorylation under eIF3d knockdown conditions. mTORC1 substrates are dephosphorylated by protein phosphatase 2A (PP2A) (Peterson et al., 1999 and Magnuson et al., 2011). It is possible that eIF3d may be acting as a PP2A activator, promoting S6K1 dephosphorylation. Thus, phosphatase-mediated dephosphorylation of S6K could be inhibited as a consequence of eIF3d disruption. This could explain the effects on S6K1 phosphorylation without the need for a direct interaction of eIF3d to the mTORC1 complex.

Together with S6K, 4E-BP1 is the other most studied target of mTORC1. The data presented shows that mTOR dependent phosphorylation of 4E-BP1 is not affected by eIF3d knockdown. However, it has been reported that rapamycin does not inhibit the phosphorylation of these substrates equally. Whereas rapamycin completely inhibits S6K1 phosphorylation, it only partially inhibits phosphorylation of 4E-BP1.
(Choo et al., 2008). Thus, eIF3d might be participating in the rapamycin sensitive activation of mTORC1 resulting only on the modulation of S6K activity.

In addition to providing evidence that eIF3d associates with SIN1 and regulates mTOR substrate phosphorylation, it was important to understand the cellular function of eIF3d in mammalian cells. The increase in cell size observed in Hela cells is consistent with mTORC1 activation and increased S6K activity, as this kinase has been associated with regulation of cell size (Montagne et al., 1999; Fingar et al., 2002). Additionally, the observation that eIF3d knockdown decreases cell proliferation could be explained by the differential effect of rapamycin on S6K and 4E-BP1 phosphorylation, as it appears that mTORC1 regulates cell proliferation but this occurs through 4E-BP1 rather than S6K phosphorylation (Dowling et al., 2010). The effects observed on cell proliferation might result from either a defect as a result of mTORC2 negative regulation, or an mTOR independent mechanism.

7.3 eIF3d as regulator of cell proliferation

The decrease in proliferation rate due to a cell cycle arrest in G2/M after eIF3d knockdown has been recently reported in other cancer cell lines (Gao et al., 2015; Lin et al., 2015; Li et al., 2015; Ren et al., 2015; Yu et al., 2014) but the mechanisms involved are still not clear.

The proliferation defect observed under eIF3d knockdown may be due to the increase in mitotic spindle multipolarity (Figure 28). Mitotic spindle bipolarity is essential for proper chromosomes segregation during cell division and defects in correct assembly of mitotic spindles are associated with cell cycle arrest (Maiato and Logarinho, 2014). Furthermore, the percentage of cells that appear to undergo cell cycle arrest under eIF3d knockdown conditions is comparable to that for the number of mitotic cells showing spindle formation defects (Figures 27 and 28).
suggesting that these phenotypes observed might be linked. Further study is needed to address whether spindle multipolarity is the underlying cause of the proliferation defect observed.

The mitotic spindle multipolarity phenotype observed resembles what it is observed when the mitotic-spindle-associated protein ASTRIN is knocked down in Hela cells (Gruber et al, 2002). ASTRIN has been recently described as an mTORC1 inhibitor, thus it is possible that a functional link between elf3d and ASTRIN might explain both its inhibitory effects towards mTORC1 and the effects of elf3d in cell proliferation. elf3d knockdown, unexpectedly, showed an increase in ASTRIN protein levels (Figure 24B). One possibility is that the lack of elf3d promotes mislocalisation of ASTRIN thus altering its functions and resulting in the spindle formation defect observed. Consistent with this, alterations in KINASTRIN expression have been previously implicated in aberrant spindle formation due to ASTRIN mislocalisation (Dunsch et al., 2011). Another possibility is that elf3d is participating together with ASTRIN in localising other regulatory proteins to spindle microtubules. For example, ASTRIN regulates spindle formation by mediating the correct localisation of AURORA-A kinase in mitotic spindles (Du et al., 2008), thus it cannot be ruled out that elf3d could be having a direct role in the localisation and activity of AURORA-A, Polo like kinases (Lee et al., 2014), or another microtubule associate protein in mitotic cells.

7.4 elf3d in stress granules

ASTRIN has recently been implicated in mTORC1 inhibition under stress conditions by sequestration of RAPTOR to stress granules (Thedieck et al, 2013). Furthermore, since the elf3 complex localises in stress granules (Kerdesha et al., 2002), it seemed likely that elf3d is also localised in stress granules and its
knockdown might alter their formation, as described for other eIF3 subunits (Ohn et al 2008). Alternatively, elf3d knockdown might impair ASTRIN localisation, thus blocking the ASTRIN-mediated mTORC1 inhibition. The results obtained show that elf3d disruption does not impair ASTRIN localisation in stress granules but impairs stress granule formation dynamics in an mTORC1 independent manner (Figure 35). Prevention of the efficient formation of stress granules might be sufficient to reduce ASTRIN association to RAPTOR, thus restricting its inhibitory role towards mTORC1. The further increase in S6K phosphorylation observed under mTORC2 and PKCα disruption in elf3d knockdown cells may also be due to a defect in stress granule formation, as PKCα down regulation delays stress granule formation (Kobayashi et al., 2012). This might also result in impaired RAPTOR sequestration and mTORC1 inhibition under stress.

It is important to note that since the eIF3 complex is a key translation initiation factor, the effects observed by reduced elf3d expression may be a consequence of a decrease in protein translation. elf3d has been reported to be non-essential for global translational initiation in S. pombe (Bandyopadhyay et al, 2002), but it was suggested that elf3d may be necessary for either the optimal rate of protein translation or for translation of a specific subset of mRNAs. Thus, the effects observed in mTORC activity, cell growth and proliferation could be a consequence of a decrease in the translation of key mRNAs relevant for these processes. Consistent with this idea, elf3e has been shown to support breast cancer progression principally through its role as a component of the elf3 complex by the regulation of translation of specific mRNAs involved in cancer growth, invasion and apoptosis, rather than through a role of elf3e directly modulating the signaling pathways affected (Grzmil et al 2010). Thus, deregulation of the synthesis of critical proteins may be one mechanism by which elf3d regulates cell growth and
proliferation. However, the specific role of eIF3d in translation initiation in mammalian cells remains elusive.

7.5 Concluding Remarks.

eIF3d has been identified as a novel SIN1 interacting protein and their interaction is likely to occur in the nucleus independently of mTORC2. eIF3d participates in the regulation of the mTOR pathway, both as an mTORC2 activator and as an inhibitor of mTORC1 activity towards S6K. This eIF3d dependent inhibition of mTORC1 appears to be dependent on AKT activity, possibly by a growth factor dependent mechanism. Alternatively, under stress conditions, eIF3d-mediated mTORC1 inhibition may occur through modulation of stress granule formation directly, or by regulation of PKCα. Finally eIF3d is a regulator of cell proliferation, participating in proper mitotic spindle formation independently of mTOR. A proposed model for the role of eIF3d in mTOR activity is presented in Figure 37.

The understanding of the role of eIF3d in the regulation of the mTOR pathway and cell proliferation might give us better understanding of pathogenesis of diseases in which these are deregulated. Two different high throughput transcript expression profiling studies performed in prostate and ovarian cancer have shown correlation between low eIF3d levels and advanced tumour progression (Figure 38) (Varambally et al., 2005; Bowen et al., 2009). Changes in eIF3d functions might result in tumorigenic hyper-activation of mTORC1. However, while low eIF3d has been associated with a decrease in cell proliferation, the promotion of spindle multipolarity might account for an increase in chromosome instability thus promoting malignant transformation (Chan, 2011). Further studies in cancer models are needed to address the potential role of eIF3d in this disease.
In response to growth factors, PDK1 phosphorylates AKT at T308 (1), leading to SIN1 phosphorylation by AKT. In a similar way, AKT could be mediating the growth factor-mediated activation of mTORC1, and consequently S6K, independent of mTORC2 in a process involving PRAS40 phosphorylation (2). This leads to phosphorylation of S6 possibly increased cell size. The AKT-mediated mTORC1 activation is inhibited by eIF3d, and is released in response to growth factors by an undefined mechanism (3). Additionally, eIF3d might inhibit S6K phosphorylation by activation of PP2A or other S6K phosphatases, directly regulating S6K rather than modulating mTORC1 kinase activity (4). eIF3d is localised in the nucleus where it might associate to ASTRIN and other regulators of mitotic spindle assembly in order to ensure correct spindle bipolarity during mitosis (5). eIF3d interaction with SIN1 may facilitate SIN1 recruitment to mTORC2, thus promoting its activity resulting in increased stability and activity of both AKT and PKCα (6). By an increase in mTORC2 assembly and stability, eIF3d might as well be inhibiting mTORC1 by recruiting shared components form mTORC1 (7). Under stress conditions, eIF3d mediates the efficient formation of G3BP-1 positive stress granules, possibly by recruitment of components of the eIF3 complex and activated PKCα (8). In stress granules, Astrin-mediated sequestration of RAPTOR further contributes to mTORC1 inhibition (9). Additionally, as part of the eIF3 complex, eIF3d might be regulating the translation of key mRNAs involved in mTOR activity, cell size regulation, and proliferation.
Figure 38. eIF3d is downregulated in prostate and ovarian cancer. eIF3d GEO Profile Charts (http://www.ncbi.nlm.nih.gov/geo). The charts show the expression level of the eIF3d gene across all different samples. A: GEO Profile comparing eIF3d expression in benign, primary and metastatic in prostate cancer patient samples by Expression profiling by array using Affymetrix Human Genome U133 Plus 2.0 Array (Varambally et al., 2005). B: GEO Profile comparing eIF3d expression in normal ovarian epithelia, and ovarian adenocarcinoma patient samples by Expression profiling by array using Affymetrix Human Genome U133 Plus 2.0 Array (Bowen et al., 2009).
7.6 Future Work

7.6.1 Considerations on the knock-down approach

In this thesis, RNA interference was used to study the effects of eIF3d disruption. In the interpretation of the results obtained, it has to be considered that under siRNA and shRNA mediated knockdown, the expression of the target protein is not fully inhibited. Additionally, the risk of having phenotypes as a result of off-targets effects rather than a loss of function of eIF3d exists. Thus the development of a knockout system to address the effects of eIF3d disruption on the mTOR pathway and the phenotypes observed would be much more informative in the understanding of the role of eIF3d in mTOR signalling and its cellular functions.

7.6.2 Considerations on eIF3d-SIN1 interaction

7.6.2.1 eIF3d-SIN1 interaction site

eIF3d interacts with SIN1 through it C terminal region (amino acids 482-548) but the exact region in which this interaction is occurring is still unknown. Deletions at the C-terminal region of the protein decrease its solubility. One possibility is that these deletions promote the exposure of hydrophobic surfaces causing the protein to aggregate. This issue could be overcome by the prediction of the structure and solubility of eIF3d by computational approaches. Mutation of residues or specific regions that are predicted to not compromise the solubility of the protein will allow the further study of the interaction. Identification of the critical region for SIN1 in eIF3d and generation of a mutant unable to bind SIN1 is a key step to understand the biological function of the association of these proteins.
7.6.2.2 elf3d Localisation

elf3d-SIN1 interaction is likely to be occurring in the nucleus, but the relevance of this localisation to the role of elf3d as an mTOR regulator was not elucidated. The mutants unable to localise in the nucleus that were generated will be a useful tool to assess the role of nuclear elf3d. Additionally, cellular fractionation coupled with interaction studies such as immunoprecipitation assays would be informative of whether there is preferential association of elf3d and SIN1 in this compartment.

7.6.3 Consideration on elf3d as an mTOR regulator

elf3d does not appear to be part of the mTOR complexes based on the lack of interaction with RICTOR or RAPTOR. However, as the evidence shows that elf3d participates in mTOR regulation, it has also to be assessed whether elf3d can associate to other components mTORC2 and mTORC1 in order to regulate their activities. Immunoprecipitation and localisation studies to assess the interaction of elf3d and mLST8, DEPTOR, PRAS40 and PROTOR are proposed to fully assess the association of elf3d to mTOR. Additionally, elf3d might be regulating mTOR activity indirectly by recruiting SIN1, thus, the association of SIN1, RICTOR and RAPTOR to mTOR should be tested under elf3d knockdown conditions, to evaluate whether elf3d affects complex assembly.

The data presented also suggests that PKCα participates in mTORC1 inhibition, but a direct link between PKCα activity and mTORC1 regulation has not been previously reported. It would be interesting to assess whether
any of the mTORC1 components are PKCα substrates, or whether PKCα modulates mTORC1 indirectly through MAPK regulation (Mauro et al., 2002). Also, whether eIF3d knock-down is sufficient to produce a decrease on PKCα activity resulting in activation of mTORC1 has to be tested. Kinases assays using PKCα and AKT immunoprecipitates would indicate whether the decrease in phosphorylation observed on their turn motifs under eIF3d knockdown conditions results in a decrease in their activities.

7.6.4 Consideration on eIF3d functions

7.6.4.1 Role of eIF3d in protein translation

The results presented indicate that eIF3d knockdown increases cell size in Hela cells. Increase in cell size is associated with enhanced biosynthesis (Lloyd, 2013). The knockdown of Moe1, the eIF3d yeast ortholog, is not essential for global translation initiation (Bandyopadhyay et al, 2002), however, the role of eIF3d as part of the eIF3 complex in translation initiation in mammalian cells is unknown. Under eIF3d disruption conditions, polysome profiling studies in combination with 35S methionine labelling will be used to determine the proportion of ribosomes actively translating and total protein synthesis rate respectively, in order to address the participation of eIF3d in protein synthesis using Hela cells. In addition, combination with S6K inhibition would indicate whether this occurs by eIF3d forming part of the translation machinery, or indirectly through S6K phosphorylation by hyperactivation of mTORC1.
7.6.4.2 elf3d participation in cell proliferation

The data presented indicated that elf3d participates in cell cycle progression. However, whether the G2/M cell cycle arrest observed under elf3d knockdown occurs solely due to the defect in mitotic spindle formation observed is not clear. Changes in Cyclin B, CDK1 and phosphorylated Histone-3 need to be monitored in order to further assess G2/M progression. Additionally, the nature of the spindle defects observed still needs to be elucidated. Temporal dynamics of ASTRIN’s positioning during mitosis evaluated in synchronised cells by either assessing different time points or though live cell imaging in elf3d knockdown conditions should illustrate whether this process is affected under elf3d disruption. Additionally, evaluation of other critical regulators of spindle assembly such as expression, phosphorylation and localisation of Polo-Like Kinases, CENTRIN and AURORA-A to assess correct spindle assembly during mitosis has to be performed in order to determine the role of elf3d in spindle formation.

7.6.4.3 elf3d participation in stress granule formation.

The data presented indicates that elf3d participates in the regulation of stress granule formation dynamics. However, it has to be elucidated whether elf3d directly associates to stress granule components such as G3BP1 and whether ASTRIN and RICTOR are interacting with elf3d under stress conditions. Interaction assays including immunoprecipitation under arsenite stress conditions are proposed. It has to be assessed whether the changes observed in stress granule formation dynamics actually have an impact on
RAPTOR sequestration by ASTRIN, and whether the delay in granule formation observed is responsible for mTORC1 activation.

The association of eIF3d with stress granules seems to be dependent on its ability to bind RNA. To elucidate whether association of eIF3d to stress granules is critical for mTOR regulation, recovery experiments using the eIF3d mutants unable to bind RNA and unable to form stress granules would be informative.

7.6.4.4 eIF3d in tumorigenesis.

As mentioned in sections 7.5, there might be a correlation between eIF3d downregulation and tumour progression. It would be interesting to evaluate whether there is an association between S6K hyperphosphorylation in different cancer cell lines and low eIF3d expression levels. Additionally, if such an association exists, whether overexpression of eIF3d is able to revert the mTOR hyperactivation observed and the tumour associated phenotypes such as chromosome instability, and increased cell proliferation and migration.
Chapter 8: References


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