Chapter 1
Introduction, Background and Rationale
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Introduction, Background and Rationale

Cell growth and proliferation is an outcome of controlled signaling, transcriptional and metabolic networks, predominantly maintained by the availability of nutrients and energy. Its control involves balancing positive regulation of anabolic processes with negative regulation of catabolic processes. Cells respond to mitogens, besides other growth factors thereby activating various signaling networks important for their survival (Dufner and Thomas, 1999; Martin and Blenis, 2002). The ability of these signalling networks to promote cellular growth and proliferation depends mainly upon the extent of phosphorylations carried out by proteins, collectively known as kinases. The protein kinases are, hence, the key regulatory enzymes which change the properties of the substrates by attaching phosphate group to Ser, Tyr or Thr residues (Pearce et al., 2010). Efforts leading to discern the molecular events that connect the phosphorylation and activation of growth factor receptors lead to the identification of ribosomal S6 kinases (RSKs), a family of protein kinases that respond to the nutrient and growth factor signals (Anjum and Blenis, 2008). RSKs were initially identified as protein kinases after studying inducible phosphorylations on ribosomal protein S6 (rpS6), and accordingly p90 ribosomal S6 kinase was identified from Xenopus oocytes (Anjum and Blenis, 2008; Erikson and Maller, 1985) which in mammals comprise a family of four enzymes termed RSK 1-4. However, later observations revealed that RSKs play a minor role in rpS6 phosphorylation (Anjum and Blenis, 2008). Meanwhile a distinct enzyme, the 70 kDa ribosomal S6 kinase (S6K), responsible for playing major role in rpS6 phosphorylation in somatic cells (Anjum and Blenis, 2008; Chung et al., 1992; Price et al., 1992; Stewart et al., 1996), was purified from mitogen stimulated Swiss mouse 3T3 cells (Jeno et al., 1988). Cloning of 70 kDa ribosomal S6 kinase gene prototype, S6K1, was soon accomplished after protein sequencing of the 70 kDa S6 kinase purified from rat liver and screening of a rat and rabbit cDNA libraries (Banerjee et al., 1990; Harmann and Kilimann, 1990; Kozma et al., 1990). Identification and cloning of human orthologs of S6K genes, RPS6KB1 and RPS6KB2 encoding S6K1 and S6K2 (Gout et al., 1998; Grove et al., 1991; Koh et al., 1999; Lee-Fruman et al., 1999; Saitoh et al., 1998; Shima et al., 1998) thus respectively lead to emergence of a second family of S6 kinases, which acts as principal kinases for rpS6 in somatic cells (Chung et al., 1992).
1.1 S6 Kinase Protein family; Domain architecture and Cellular localisation

S6Ks belong to AGC family of protein kinases. The term AGC Kinases define the subgroup of Ser/Thr protein kinases that, on the basis of sequence alignments of their catalytic kinase domain, are most related to cAMP-dependent protein kinase 1 (PKA; also known as PKAC), cGMP-dependent protein kinase (PKG; also known as CGK1α) and protein kinase C (PKC) (Hanks and Hunter, 1995). The structural resemblance in AGC kinases allows them to exhibit more or less a similar mode of regulation. These kinases exhibit bilobal structural organisation around the kinase domain which subsequently enables amino and carboxyl termini to coordinate ATP binding (Jacinto and Lorberg, 2008; Pearce et al., 2010). At the beginning of the C-terminus lobe lies an activation segment or loop (commonly known as the T-loop), phosphorylation of which brings in conformational changes important for phosphoryl transfer. Two other important phosphorylation sites, the TM (turn motif) (so-named due to its location at the cusp of a structural turn in the PKA tail) and HM (hydrophobic motif) sequentially follow the kinase domain. The phosphorylated HM site engages a hydrophobic pocket within the N-lobe. The phosphorylated TM site stabilizes phospho-HM binding to the N-lobe hydrophobic pocket. Together, these three critical phosphorylation events stabilize a catalytically competent conformation (Jacinto and Lorberg, 2008; Pearce et al., 2010).

RPS6KB1, human ortholog of S6K1 genes, encodes two isoforms, p70S6K1 and p85S6K1 (formerly known as p70S6KII and p70S6KI respectively) through alternative translational start sites (Grove et al., 1991). p70S6K1, a 502 amino acid protein and its larger isoform p85S6K1, having nuclear localisation sequence (NLS) within the 23 amino acid N-terminal extension, were earlier believed to remain localized in the cytoplasm and nucleus respectively. However, the data generated of late, contests the nuclear localization of p85S6K1 while showing its sub-cellular localization to be cytoplasmic and at the same time claims p70S6K1 localization to be both nuclear and cytoplasmic (Rosner and Hengstschläger, 2011).S6K2, encoded by RPS6KB2 gene, constitutes another member of S6 kinase family that, via alternative start sites, produces two nuclear isoforms, owing to the presence of nuclear localisation sequence at the C-terminus (Koh et al., 1999).The longer isoform, p56S6K2 comprises a 13 amino acid extension at N terminus than its shorter isoform, p54S6K2 (Gout et al., 1998).Moreover S6K2 isoforms have been characterised by the
The presence of proline rich domain at the C-terminal (Schalm and Blenis, 2002). S6K1 and S6K2 have been structurally dissected into several regulatory domains viz an acidic N-terminus that contains the TOS (TOR signalling) motif; the kinase domain that contains the Activation/T-loop; a linker region that contains the TM and HM sites; and a basic C-terminus containing an auto inhibitory pseudosubstrate domain (Fig 1.1).

Figure 1.1: S6K structure and domain organization with potential phosphorylating kinases along with their sites of action. S6K isoforms, domain structure and phosphorylation sites: S6K1 isoforms include p70-, p85- and p31-S6K1; alternative start site usage lengthens the p85- and p31-S6K1 N-termini by 23 amino acids (note that p31-S6K1 lacks most of the kinase domain). S6K2 isoforms include p54- and p56-S6K2; alternative start site usage lengthens the p56-S6K2 N-terminus by 13 amino acids. NLSs lie within the N-terminal extensions of p85-S6K1 and p56-S6K2, whereas S6K2 additionally contains an NLS within the C-terminus as well as a proline-rich domain (Pro). S6Ks contain an acidic N-terminal domain (NTD), kinase domain (KD), linker region and acidic C-terminal domain (CTD). The N-terminal domain contains the TOS motif, whereas the CTD contains the auto-inhibitory pseudosubstrate domain and RSPRR motif. mTORC1 phosphorylates the HM (hydrophobic motif) site (Thr412) in the linker region and PDK1 phosphorylates the T-loop site (Thr252) within the kinase domain. Other regulatory phosphorylation (P) sites, including the TM (turn motif) site (Ser394), are shown.
These proteins are homologous to each other within appreciable limits; their catalytic domains share about 83% identity at the amino acid level (Gout et al., 1998). However, the differences observed in the extreme N- and C-terminal regions, direct these kinases to distinct compartments or to different molecular targets. Thus, e.g. the C-terminal PDZ binding domain in S6K1 allows recruitment to the actin cytoskeleton via binding to neurabin (Burnett et al., 1998a) and S6K2 containing a proline-rich region in its C-terminus facilitates interaction with SH3 domain or WW repeat containing protein (Gout et al., 1998). Besides full length forms of S6K1 and S6K2, a novel kinase domain truncated splice variant, p31S6K with potential oncogenic properties, has also been reported (Karni et al., 2007).

1.2 S6 Kinase Regulation; a historical perspective for prevalent mode of activation

Research carried out over the past 20 years identified diverse growth factors and mitogens with the ability to activate the S6 kinases (Fenton and Gout, 2011; Meyuhas and Dreazen, 2009). Seemingly among these multiple inputs, insulin/IGF pathway, which signals via PI3K (phosphoinositide 3-Kinase) and mTORC1 appears to be the best studied activator of S6K1 (Foster and Fingar, 2010; Zoncu et al., 2011). Besides PI3K, other independent signaling pathways like Ras/MAPK (mitogen activated protein kinase) have also been implicated in S6K1 activation (Chou and Blenis, 1996; Romanelli et al., 1999). S6K1’s contribution in controlling cell growth and proliferation being paramount made the basis for studying its structure and function. The data accumulated over a period of time, hence revealed the molecular insights that govern activation of S6K1 by mitogens and identified, to a large extent, the complex interactions between its specific domains and phosphorylation sites. These observations eventually became the reason for putting forth the models that explain the activation of S6K1 by stepwise multisite phosphorylations (Alessi et al., 1998; Ferrari et al., 1992; Keshwani et al., 2011; Mukhopadhyay et al., 1992; Weng et al., 1998). These models have suggested that the phosphorylations on multiple C-terminal sites prime the event for mTORC1 mediated phosphorylation of the HM site (Thr412) in the linker domain and PDK1 mediated phosphorylation of the T-loop (Thr 252) on the activation loop. Since the phosphorylations at the HM and T-loop sites exhibit strong cooperativity, the temporal order of these two phosphorylations has not yet been convincingly deciphered. However, the data available till date has put forth two
models for S6K1 activation wherein the conventional and widely accepted model suggests mTORC1 mediated phosphorylation at Thr 412 to precede PDK1 mediated phosphorylation at Thr 252 (Alessi et al., 1998; Dennis et al., 1998; Pullen et al., 1998). An alternate model however, suggests that the phosphorylation at T loop (Thr 252) precedes HM phosphorylation (Thr 412) (Keshwani et al., 2011; Weng et al., 1998) (Fig 1.2). Though the temporal occurrence of TM (Ser394) site phosphorylation also remains largely obscure, recent study however suggests it to be a co-translational phosphorylation, carried out by a constitutive kinase (Keshwani et al., 2011). The detailed regulation of S6K1 activation as a multisite phosphorylation event is documented up next.

**Figure 1.2:** Stepwise activation of S6K1 via multi-site phosphorylation

(A) **Conventional model.** The interaction of the C- and N-terminal domains results in auto-inhibition of S6K1. Step 1: mitogens promote C-terminal domain (C) phosphorylation on multiple sites to induce a more relaxed conformation. Step 2: the release of the auto-inhibitory C-terminal domain (CTD) enables mTORC1 access to the HM and thus phosphorylation of Thr412. Step 3: the release of the auto-inhibitory CTD and phosphorylation on Thr412 enables PDK1-mediated phosphorylation of the T-loop on Thr252, resulting in full activation of S6K1. Phospho-Thr412 serves as docking site for PDK1. Owing to insufficient data, the temporal order of TM site phosphorylation (Ser394) is not depicted.

(B) **Alternative model.** Step 1: an unknown kinase phosphorylates the inactive form of S6K on the TM site Ser394. Step 2: mitogens promote C-terminal domain (C) phosphorylation on multiple sites to induce a more relaxed conformation. Step 3: the release of the auto-inhibitory C-terminal domain enables PDK1 access to the T-loop. Step 4: PDK1-mediated phosphorylation of Thr252 promotes mTORC1-mediated phosphorylation on the HM site, Thr412. KD, kinase domain; N, N-terminal domain.
1.2.1 C-Terminal phosphorylation.

Findings initiated during early 1990s lead to the proposition that during inactive state, the basic C-terminal pseudosubstrate domain of S6K1 interacts with its acidic N-terminus. This interaction obstructs the phosphorylation at kinase domain and renders it inactive (Banerjee et al., 1990; Price et al., 1991). Stimulation by growth factors besides other mitogens result in the phosphorylation of four proline-directed sites at C-terminal auto inhibitory pseudosubstrate domain (Ser434, Ser441, Ser444 and Ser447) (Ferrari et al., 1992; Mukhopadhya et al., 1992). These phosphorylations induce a conformational change thereby releasing the interaction between the two termini and enables access to the HM and T-loop sites. Phosphorylation at these two critical sites ultimately leads to the full activation of the Kinase (Banerjee et al., 1990; Price et al., 1991). Although phosphorylations at these four C-terminal sites prime the event for complete S6K1 activation, their occurrence is not considered to be critical. Various reports have suggested that deletion of 101 amino acids from the C-terminus (CT) or mutation of these four sites to alanine residues modestly reduces S6K1 activation, whereas substitution with phospho-mimetic residues (D3E) increases the basal activity to modest levels (Ferrari et al., 1993; Foster and Fingar, 2010; Han et al., 1995; Schalm and Blenis, 2002). Several proline-directed kinases, including ERK1/2, JNK1/2 and CDK1 have been implicated in phosphorylation of these sites; it however remains unclear as to which kinase(s) play a dominant role in-vivo (Mukhopadhya et al., 1992).

1.2.2 Hydrophobic motif (HM) site Phosphorylation and Role of Amino-terminus:

Scientific observations during early 1990’s propounded the theory that mTOR controls the in-vivo activation of S6K1 (Brown et al., 1995). These observations were further augmented during 1998-1999 when mTORC1 was shown to directly phosphorylate the HM site (Thr412) of S6K1 in-vitro (Burnett et al., 1998b; Isotani et al., 1999). The findings put forward hence strengthened the notion that phosphorylation of Thr 412 at HM site remains pivotal for S6K1 activation. The indispensable nature of HM site phosphorylation is further supported by the evidence that mutation of Thr 412 to alanine (T412A) abolishes the S6K1 activity, whereas substitution to glutamate, for mimicking the phosphorylation, (T412E) enhances basal
activity even in absence of mitogens (Dennis et al., 1998; Schalm and Blenis, 2002; Weng et al., 1998). S6K1 amino terminus serves a regulatory role in promoting phosphorylation at HM site and thereby at T loop site which culminates in its complete activation. The regulation by amino terminus is documented to be two pronged. Firstly, it acts as a receptor of an activating input critical for Thr 412 and Thr 252 phosphorylation; and, secondly, it suppresses an inhibitory function mediated by C-terminus. These observations have strong scientific support as the data accumulated over a period of time shows that amino terminus truncation of S6K1 (ΔNT) abolishes the phosphorylation of rapamycin sensitive sites Thr 412, Thr 252, Ser 427 and renders it inactive (Cheatham et al., 1995; Dennis et al., 1996; Weng et al., 1995) where as additional deletion of C-terminus (ΔNT/ΔCT) restores all the rapamycin sensitive phosphorylations as well as the kinase activity of S6K1. The regulatory function of the amino terminus was mapped to a short stretch of amino acids (comprising of 5-9 amino acids and represented by a signature sequence FDIDL) present at extreme end known as TOS motif (Schalm and Blenis, 2002). Deletion of the TOS motif or mutagenic inactivation of the motif (F5A mutation within the FDIDL sequence) abolishes S6K1 kinase activity as well as Thr412 and Thr252 phosphorylation. However, deletion of C-terminus from the F5A mutant (F5A-ΔCT) partially restores kinase activity and Thr 412 phosphorylation (Schalm and Blenis, 2002). Besides S6K1 TOS motif has also been found in 4EBP1, another mTOR substrate. The TOS motif function has been related towards a launch pad required for mTORC1 to engage substrates and to mediate phosphorylation of rapamycin-sensitive sites (Nojima et al., 2003; Schalm et al., 2003). However, the data generated supports the view that mTOR interaction with TOS motif is indirect and mediated by another member of mTORC1 complex, a scaffold protein called Raptor (Nojima et al., 2003; Schalm et al., 2005). Although for efficient phosphorylation of S6K1 and 4EBP-1, binding with raptor has been unequivocally stated to be critical, the precise mechanism by which raptor mediates efficient phosphorylation in downstream targets of mTOR, S6K1 and 4EBP-1, remains debatable to a large extent. Two models have however been proposed to explain this mechanism. The first model suggests that raptor and mTOR associate in two states with varying affinities governed by the nutrient availability. During nutrient starved state Raptor binds tightly to mTOR and renders it inactive. While as the loose-binding complex, formed during nutrient sufficiency, activates mTOR and promotes efficient
phosphorylation of mTOR targets (Lee et al., 2007a). Furthermore, overexpression of raptor increases the amount of mTOR found in the tight-binding complex, thereby explaining the observation that overexpression of raptor inhibits mTOR activity. However, it is interesting to note that rapamycin is able to disrupt the raptor-mTOR interaction regardless of nutrient status (Kim et al., 2002), but it is phosphate dependent. The second model supports the existence of Raptor as a scaffolding protein for mTORC1 complex, wherein Raptor has been shown to preferentially bind unphosphorylated forms of mTOR targets and recruit the substrates to the mTOR complex for phosphorylation. Role of S6K1 amino terminus in suppressing the inhibitory function C-terminus remained unclear till identification of an RSPRR motif (a short stretch of 5 amino acids, 433-437) in C-terminus (Schalm et al., 2005). This motif has been suggested to negatively regulate S6K1 activation. Evidences, though scant, have propounded that RSPRR motif functions as a docking site for a negative regulator, such as a phosphatase, that is suppressed by mTORC1 (Schalm et al., 2005). R3A mutation of RSPRR motif within the dead ΔNT or TOS motif-mutant (F5A) backbone (NT-R3A or F5A-R3A) has been shown to rescue insulin-stimulated Thr412 phosphorylation and S6K1 activation (Magnuson et al., 2012). These findings though place TOS motif in exhibiting negative control over RSPRR motif inhibition, the exact mechanism behind this regulation still remains largely a mystery.

1.2.3 Activation/T-loop site phosphorylation by PDK1

Co-ordinate phosphorylation of Thr252 in the Activation/T-loop and Thr412 at HM site leads to maximal activation of S6K1 (Alessi et al., 1998; Pullen et al., 1998; Weng et al., 1998). Set out to identify the potential kinases responsible for Thr 252 phosphorylation, in-vitro followed by in-vivo studies show PDK1, also corroborated as a kinase for AKT, to directly phosphorylate Thr252 at T loop site (Alessi et al., 1997, 1998; Pullen et al., 1998). PDK1 null embryonic stem cells, PDK1−/− or T252A mutation rendered S6K1 enzymatically dead. This finding supported the role of PDK1 for promoting T-loop phosphorylation (Weng et al., 1998; Williams et al., 2000). Further it is observed that PDK1 mediated phosphorylation of C-Terminus truncated variant of S6K1, S6K1-ΔCT remains significantly higher than full length S6K1 while mutation in c-terminal phosphorylation sites to alanine results in poor S6K1 activation (Alessi et al., 1998). Moreover, PDK1 poorly activates S6K1 T412A-ΔCT or T394A-ΔCT in vitro. All these findings reveal a possible role for intact unphosphorylated c-
terminus in bringing an impediment towards full activation of S6K1 by blocking the access of PDK1 to activation loop and the importance of HM and TM sites towards binging PDK1 mediated S6K1 activation (Alessi et al., 1998).

1.2.4 Turn Motif (TM) Site Phosphorylation

Phosphorylation of Ser394 (or Ser371 in P70S6K isoform) at turn motif site also remains of much significance for complete activation of S6K1, as S394A substitution of S6K1 renders it completely inactive (Moser et al., 1997). Notwithstanding the importance of turn motif site, the data accumulated on regulation and function of this phosphorylation event has progressed, albeit rather slowly. Whatever little the quantum of data may be available for regulation around TM site phosphorylation till date, it concordantly reflects that this site does not represent an autophosphorylation event (Moser et al., 1997). Some more interesting observations reveal that even the addition of T412E substitution fails to restore the activity of S394A mutant, thus supporting the notion that this site plays an important yet independent role in regulating the activity of S6K1 (Moser et al., 1997). Evidences, though scant, have also emerged which corroborate mTOR in promoting Ser394 phosphorylation in vitro (Saitoh et al., 2002). However, since data accumulated in certain other cases does not correlate phospho-Ser394 well with mTORC1 activity, intermediation of some other kinase besides mTORC1 is also speculated. Reports based on analogy to cotranslational phosphorylation of TM site (Thr450) in AKT are tempting to speculate that S6K1 TM site phosphorylation also represents an early event that occurs cotranslationally prior to T-loop and HM site phosphorylation (Oh et al., 2010). Indeed, a recent report supports such an idea, as Ser394 phosphorylation occurs simultaneously with the production of S6K1 protein from a transfected plasmid (Keshwani et al., 2011).

1.3 Rapamycin, S6K1 inhibition; a chronology of events.

In 1970s, a bacterial strain, Streptomyces hygroscopicus was isolated from a soil sample from Easter Island (“Rapa Nui” in the native language) (Vezina et al., 1975). This strain was found to produce an antifungal metabolite (Sehgal et al., 1975). Post purifications, the metabolite was characterized as a macrocyclic lactone and named “Rapamycin” after its birthplace (Fig 1.3) (Vignot et al., 2005).
Figure 1.3: Electron-density model of a molecule of the immunosuppressant drug rapamycin, also known as sirolimus: It is mainly used to prevent rejection in organ transplantation, and is used in kidney transplants. It also has anti-fungal and anti-cancer properties, and was originally derived from soil bacteria found on Easter Island. The chemical formula is C51.H79.N.O13. The atoms are represented here as color-coded blobs: carbon (green), hydrogen (white), nitrogen (blue) and oxygen (red) (Adapted from Dr. Tim Evans Science photo library).

The strong ability of rapamycin to modulate cellular proliferation besides its promising role in immune suppression for treating various cancers lead to a desperate search in decoding its mode of action and simultaneously initiated hunt for nailing down its targets. The search continued for about two decades and finally culminated in early 1990s with identification of a target protein in yeast, whose mutant conferred resistance to the growth inhibitory effects of rapamycin. The protein was subsequently named as TOR (Target of Rapamycin) (Heitman et al., 1991; Kunz et al., 1993; Petroulakis et al., 2006; Wullschleger et al., 2006). Shortly after, the mammalian counterpart of TOR (mTOR) was identified as physical target of rapamycin by three groups separately in 1994, 1995 respectively (Brown et al., 1994; Sabatini et al., 1994; Sabers et al., 1995). However effective mechanism of rapamycin action remained oblivious till intracellular cofactor, the peptidyl-prolyl cis/trans isomerase, immunophilin FK506-binding protein 12 (FKBP12) was shown to bind rapamycin as a gain-of-function component to mediate its inhibitory effect on TOR (Wullschleger et al., 2006). Thus, TOR is also referred to as FKBP12 rapamycin associated protein.
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(FRAP) (Fingar and Blenis, 2004; Hay and Sonenberg, 2004). Rapamycin-FKBP12 complex binds to the FKBP12-rapamycin binding (FRB) domain of TOR (Figure 2) (Fingar and Blenis, 2004; Gingras et al., 2001; Petroulakis et al., 2006; Schmelzle and Hall, 2000) to inhibit its intrinsic kinase activity, including autophosphorylation, thereby inhibiting access of TOR to its substrates (Wullschleger et al., 2006). This finding is however in contradiction with the earlier reports wherein it was shown that rapamycin has little effect on mTOR kinase activity (Peterson et al., 2000). In mammals, rapamycin in complex with FKBP12 acts as an allosteric inhibitor of mTOR complex1 (mTORC1). Only mTORC1 is acutely sensitive to inhibition of rapamycin. However, long term exposure to rapamycin has been shown to inhibit mTORC2 in certain cell types (Laplante and Sabatini, 2012; Li et al., 2014). It is believed that the rapamycin-FKBP12 complex prevents the association between mTOR and raptor; therefore, downstream targets that depend on raptor binding are specifically inhibited (Hara et al., 2002; Kim et al., 2002). S6K1 being a downstream effector of mTOR, shows inhibitory response to rapamycin as is evidenced by loss of its ability to phosphorylate its substrate, ribosomal protein S6. Early 1990’s witnessed a rigorous search for identifying the rapamycin-sensitive regulatory phosphorylation sites in S6K1 and during 1995 three sites, T252 in the activation loop, T412 in hydrophobic motif and S427 in the linker domain, which connects the auto inhibitory domain to the catalytic domain were identified as principal rapamycin sensitive sites (Pearson et al., 1995). These sites were shown to be dephosphorylated by rapamycin in hierarchical fashion T412 > S427 > T252 with T412 dephosphorylation most closely paralleling loss of kinase activity (Pearson et al., 1995). Besides, these sites were shown to be responsive to mitogenic stimulation as well (Han et al., 1995). Though all these three sites show rapamycin responsiveness, their conversions to either acidic or neutral amino acids reveal that T252 and T412 were critical regulatory sites where as T427 appeared to play a modulatory function (Pearson et al., 1995). Further T412 was shown to be principal site of rapamycin-induced S6K1 inactivation as T412E showed increased basal activity and was largely rapamycin resistant (Dennis et al., 1996; Pearson et al., 1995). In 1995 Weng et al reported the cooperativity among the two termini for exhibiting their regulatory effects on S6K1 activation by showing that amino-terminus truncated mutant, S6K1-ΔNT, was inactive. These findings were further supported in the subsequent year while it was shown that S6K1-ΔNT mutant lacked all three rapamycin sensitive sites
in response to mitogens (Dennis et al., 1996). However, additional deletion of carboxy terminus, generating S6K1-∆NT/∆CT, rescued its phosphorylation and activation state. Surprisingly it was observed that the mutant was rapamycin resistant (Cheatham et al., 1995) however, its responsiveness towards wortmannin inhibition did not get compromised. These observations combined with the findings that the carboxy-terminal truncated S6K1 (S6K1-∆CT) retains rapamycin sensitivity raised the speculations during later half of 1990’s that the inhibitory effect of the rapamycin–FKBP12 complex on S6K1 was either due to blockade of an upstream activator or activation of some phosphatase which mediate their influence through the two termini (Westphal et al., 1999). The presence of Thr412 phosphorylation on S6K1-∆NT/∆CT isolated from rapamycin-treated cells questioned the idea that mTORC1 represents the sole S6K1 Thr412 kinase. In ∆NT/∆CT, serum and insulin promote Thr412 phosphorylation and kinase activation in a completely rapamycin-resistant manner, suggesting that a rapamycin-insensitive kinase mediates Thr412 phosphorylation (Cheatham et al., 1995; Dennis et al., 1996). This conundrum was resolved in 2005 with the discovery that rapamycin-insensitive mTORC2 mediates non-physiological S6K1 Thr412 phosphorylation in S6K1 mutants lacking a C-terminus (Ali and Sabatini, 2005). In the dead ∆NT allele, mTORC1 (mTOR/raptor) cannot dock to S6K1 and phosphorylate Thr412, and mTORC2 (mTOR/riCTOR) cannot phosphorylate Thr412 due to steric hindrance imposed by the extended C-terminus (Ali and Sabatini, 2005). In the partially rapamycin-resistant ∆CT mutant, both rapamycin sensitive mTORC1 and rapamycin-insensitive mTORC2 cooperatively mediate Thr412 phosphorylation. In ∆NT/∆CT (and F5A-∆CT), only rapamycin insensitive mTORC2 mediates Thr412 phosphorylation.

1.4 Other modes of S6K1 regulation

Although phosphorylation represents the best understood mechanism underlying S6K1 regulation, roles for other PTMs (post-translational modifications) have been proposed, including phosphatase-mediated dephosphorylation, acetylation and ubiquitination

1.4.1 Dephosphorylation

Rapamycin action on phosphorylation state of S6K1 suggested a possible role for phosphatase to regulate this event. Indeed, S6Ks have been suspected to represent
targets of PP2A (protein phosphatase 2A)-like phosphatases. In S. cerevisiae, TOR regulation of several substrates occurs via suppression of PP2A-like phosphatases (Düvel and Broach, 2004). In mammals, PP2A reportedly co-immunoprecipitates with S6K1 (Westphal et al., 1999); moreover, an independent study showed that PP2A binds wild-type but not ΔNT/ΔCT S6K1 (Peterson et al., 1999). It is however important to note that since the late 1990s there has been little follow-up regarding the role of PP2A-like phosphatases in S6K1 regulation. Recent work in Drosophila melanogaster demonstrates that genetic ablation of the PP2A regulatory subunit B’ (PP2A-B’) leads to dS6K (DrosophilaS6K) deregulation and a variety of metabolic defects (Hahn et al., 2010). High levels of phosphorylated S6K (on Thr412) were also detected in human cells upon knock down of PPP2R5C, the human PP2A-B’ orthologue (Hahn et al., 2010). Whether mTOR suppresses a PP2A like phosphatase to modulate S6K1 Thr412 phosphorylation in mammals as in yeast still remains largely obscure.

1.4.2 Acetylation and ubiquitination

Though poorly understood in comparison to protein phosphorylation, acetylation and ubiquitination are two such post translational modifications (PTMs) that have been implicated in S6K regulation. Two acetyltransferase enzymes, p300/CBP (cAMP-response-element-binding protein binding protein) and PCAF (p300/CBP-associated factor), reportedly interact with and acetylate S6K1 and S6K2 both in vitro and in vivo (Fenton et al., 2010a). Acetylation of S6K1 occurs at the extreme C-terminus (Lys516) in response to mitogens, and remains largely independent of phosphorylation (Fenton et al., 2010b). Although the function of acetylation remains unclear, this PTM has been speculated to stabilize S6Ks, as treatment of cells with the HDAC (histone deacetylase) inhibitor trichostatin A increases S6K2 acetylation and protein abundance (Fenton et al., 2010a). Polyubiquitination of proteins induces their degradation by the 26S proteasome. Both S6K1 and S6K2 appear to experience this PTM in response to mitogen stimulation (Gwalter et al., 2009; Wang et al., 2008). Certain findings report ubiquitin ligase, ROC1 to interact with and ubiquitinate S6K1 (Panasyuk et al., 2008). As RNAi (RNA interference) against ROC1 increases steady-state levels of S6K1, these results suggest that polyubiquitination may destabilize S6K proteins and thus may function as a mechanism for signal attenuation. Indeed, it has been reported recently that Akt phosphorylation on its HM site (Ser473) induces Akt
polyubiquitination and subsequent degradation as a means to attenuate Akt signalling (Wu et al., 2011). Although regulation of S6K1 through ubiquitination still remains in its infancy; it however has opened a new window towards understanding detailed mechanistic inputs of proteasome mediated S6K regulation and function. A detailed representation of upstream regulators and downstream effectors of S6K1 is described in fig 1.4

![Fig 1.4 Key upstream regulators and downstream effectors of S6K signalling: Major components of the signalling pathways regulating S6K activation are shown. Besides S6K downstream substrates are also shown along with their relation with other pathways.](image)

1.5 mTOR (Mammalian Target of Rapamycin); Principal Upstream Regulator of S6K1

Target of Rapamycin (TOR) one amongst various other upstream kinases of S6K1 is considered to be responsible for carrying out the critical rapamycin sensitive phosphorylation at T412 in the hydrophobic motif (HM motif). The structural complexity of TOR allows it to respond to various environmental cues vis a vis energy status, amino-acid sufficiency, growth factor stimulation and mediate its influence on downstream effectors thereby regulating cell growth and proliferation. TOR is hence often regarded as master cell growth regulator.
1.5.1 mTOR; Structure and function

TOR is approximately 290 kDa protein belonging to the phosphoinositide kinase-related kinase (PIKK) family. Despite its significant homology to phosphoinositide 3-kinases (PI3Ks) and PI4Ks, TOR is exclusively considered as Ser/Thr protein kinase that exhibits no lipid kinase activity. It is highly conserved in all eukaryotes and is ubiquitously and constitutively expressed. Apart from yeasts, where two paralogous genes have been identified, TOR is encoded by a single gene in most of the organisms. TOR comprises of a multi domain structure (Fig1.5) with amino terminus containing up to 20 tandem HEAT repeats (a protein–protein interaction domain whose name is derived from Huntington, elongation factor 3, PP2A and TOR). This is followed by a FAT (FRAP, ATM, TRRAP) domain. The carboxy-terminal portion of TOR contains the kinase domain and the FKBP–rapamycin binding (FRB) domain. The C terminus contains a FATC domain that is paired with the upstream FAT domain in all PIKKs to modulate kinase activity in an unknown manner.

Figure 1.5 Structural Organisation of mTOR: Schematic representation of structural organisation of mTOR at domain level.
TOR associates with various proteins to generate two structurally and functionally distinct complexes (Fig 1.6). In mammals, mTOR (also known as mechanistic target of rapamycin) associates with mammalian lethal with SEC13 protein 8 (mLST8), proline-rich AKT substrate of 40 kDa (PRAS40; also known as AKT1S1) and regulatory-associated protein of mTOR (RAPTOR) to form the rapamycin-sensitive mTOR complex 1 (mTORC1). mTORC1 remains highly sensitive to inhibition by rapamycin in the nanomolar range. mTORC2 consists of mTOR in association with mLST8, proline-rich protein 5 (PRR5; also known as PROTOR1), mitogen-activated protein kinase-associated protein 1 (MAPKAP1; also known as mSIN1) and rapamycin-insensitive companion of mTOR (RICTOR). It is generally insensitive to inhibition by rapamycin, although in certain cell types, mTORC2 may show sensitivity after prolonged rapamycin treatment (Sarbassov et al., 2006). The functions of mTORC1/2 components have been depicted in table 1.1

![Figure 1.6 mTOR complex1 and mTOR complex2](image)

**Figure 1.6 mTOR complex1 and mTOR complex2**: Schematic representation of various components of mTORC1 and mTORC2. Growth factors and metabolic conditions like energy levels, nutrient sufficiency influence mTORC1. 4EBP1 and S6K1 are the major effectors of mTORC1. Growth factors activate mTORC2 to activate AKT, the major downstream effector. Cell growth, proliferation are the major functions of mTORC1 while mTORC2 controls cytoskeleton organization along with survival and cell proliferation.
mTORC1 responds to growth factor, nutrient stimulation besides sensing cellular energy status (ATP/AMP ratio) to execute various downstream functions. Growth factors such as insulin stimulate mTORC1 through the PI3K–phosphoinositide-dependent kinase 1 (PDK1)-AKT pathway (FIG. 1a). The data accumulated over the years places tuberous sclerosis complex (TSC), a heterodimer that comprises TSC1 and TSC2 subunits, as an intermediator between PI3K/Akt and mTOR (Gao et al., 2002; Inoki et al., 2002; Tee et al., 2002). Growth factor activation of AKT prompts it to phosphorylate TSC2 (also known as tuberin) at multiple sites (Ser939, Ser981, Ser1130, Ser1132 and Thr1462) to inhibit TSC1 (also known as hamartin) – TSC2 complex (Lee et al., 2007b). This complex acts as a GTPase-activating protein (GAP) for the small GTPase RAS homologue enriched in brain (RHEB) (Dibble and Manning, 2013; Inoki et al., 2002). During inhibition of the TSC complex, GTP-loaded RHEB binds the mTOR catalytic domain to activate mTORC1 through largely unknown mechanism (Long et al., 2005). The nutrient (amino acids) signal to mTORC1 is transduced through the RAS-related GTP-binding protein (RAG) family of small GTPases (Kim et al., 2008; Sancak et al., 2008). Activated

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**Table 1.1 mTOR complex components and their function**

<table>
<thead>
<tr>
<th>Components</th>
<th>Associated Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>mTOR</td>
<td>Catalytic subunit of mTORC1 and mTORC2</td>
</tr>
<tr>
<td>Raptor</td>
<td>Essential component of mTORC1, recruits mTOR substrates to mTORC1 and promotes the activity of mTORC1 to 4EBp1 and P70S6K</td>
</tr>
<tr>
<td>Rictor</td>
<td>Promotes the assembly and activity of mTORC2, stabilizes mSIN1</td>
</tr>
<tr>
<td>PRAS40</td>
<td>An mTORC1 binding partner and negatively regulates the activity of mTORC1 by binding to mTORC1</td>
</tr>
<tr>
<td>msIN1</td>
<td>A necessary component of mTORC2, promotes the assembly and the activity of mTORC2 to phosphorylate Akt at serine 473</td>
</tr>
<tr>
<td>mSLT8</td>
<td>A necessary component for the stability of Rictor-mTOR interaction and activity of mTORC2</td>
</tr>
<tr>
<td>Deptor</td>
<td>Negatively regulates the activity of both mTORC1 and mTORC2</td>
</tr>
<tr>
<td>Protor-1</td>
<td>A rictor binding subunit in mTORC2</td>
</tr>
</tbody>
</table>
RAG in the form of heterodimer mediates the translocation of mTORC1 from the cytoplasm to lysosomal surface, where mTORC1 encounters and is subsequently activated by RHEB. A high cellular energy state (having increased ATP/AMP ratio) activates mTORC1 by preventing the activation of AMP-activated protein kinase (AMPK). In the absence of energy (high AMP/ATP ratio) AMPK carries out activating phosphorylation of TSC2 at Ser1387 (a site distinct from that phosphorylated by AKT). Besides it also phosphorylates RAPTOR at Ser792. Together these phosphorylations lead to mTORC1 inhibition.

The earliest identified and best characterized substrates of mTORC1 are S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1). mTORC1 activates S6K1 and inhibits 4EBP1 to promote translation and cell growth (Nojima et al., 2003). S6K1, a major ribosomal protein S6 (rpS6) kinase in mammalian cells, is pointed as a key player in the control of cell growth and proliferation (Avruch et al., 2001; Montagne et al., 1999; Radimerski et al., 2002). S6K1 after being activated by mTORC1 is thought to regulate protein synthesis through phosphorylation of the 40S ribosomal subunit protein S6, which has been suggested to increase the translational efficiency of a class of mRNA transcripts with a 5’-terminal oligopyrimidine (5’-TOP) (Jefferies et al., 1994; Terada et al., 1994). However, it has of late been found that neither S6K1 activity nor rpS6 phosphorylation is required for the translational regulation of TOP mRNAs (Pende et al., 2004; Stolovich et al., 2002). New data indicate that mTOR and S6K1 control on and off the eukaryotic initiation factor 3 (eIF3) translation initiation complex in a growth factor- and rapamycin-sensitive manner (Holz et al., 2005). S6K1 associates with the eIF3 complex when inactive, but dissociates from the eIF3 complex upon stimulation by insulin or amino acids. Activated S6K1 then phosphorylates its translational targets, including the 40S ribosomal protein S6 and eIF4B, promoting translation initiation.

Unlike mTORC1, the data available for regulation of rapamycin insensitive variant mTORC2 has remained relatively scant till date. Although mTORC2 responsiveness to its only stimulant i.e. growth factors largely depends on mediation through PI3K- dependent mTORC2-ribosome association (Zinzalla et al., 2011), the signalling steps beyond phosphatidyl inositol- 3, 4, 5triphosphate (PtdIns (3, 4, 5) P3) remain largely unknown and distinct from those upstream of mTORC1 (Cybulski and Hall,
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2009; Sparks and Guertin, 2010). Active mTORC2 has been shown to phosphorylate the hydrophobic motif in a subset of AGC family kinases, among which AKT is best described substrate of mTORC2. As a consequence, Rictor⁻/⁻, mSin1⁻/⁻ and mLST8/GβL⁻/⁻ mouse embryonic fibroblasts show significant reduction in AKT phosphorylation at Ser473 position (Guertin et al., 2006; Jacinto et al., 2006). In addition to mTORC2 other kinases have also been reported to mediate AKT HM (Hydrophobic motif) site phosphorylation on Ser473 like DNA-PK (DNA dependent protein kinase), and the IKK-related kinases TBK1 and IKKe (Feng et al., 2004; Joung et al., 2011; Ou et al., 2011; Xie et al., 2011). This goes to show that many upstream kinases co-operate to regulate AKT. Besides AKT, mTORC2 has also been implicated in phosphorylation of SGK1 (serum and glucocorticoid-induced protein kinase 1) and PKCα on their hydrophobic motif sites (Ser473, and Ser 657, respectively) (García-Martínez and Alessi, 2008; Sarbassov et al., 2006). Despite its relatively less known regulatory aspects, the functions performed by mTORC2 have accumulated over the years. mTORC2 has been shown to play a significant role in diverse cellular processes like cell survival, metabolism, proliferation and cytoskeleton organization. Also, mTORC2 may modulate actin cytoskeleton in yeast and mammals (Jacinto et al., 2004; Sarbassov et al., 2004). Of late, mTORC2 has also been implicated in carrying out S6K1 phosphorylation as it has been reported that carboxy terminus truncated S6K1 is phosphorylated by mTORC2 as well (Ali and Sabatini, 2005). These findings, though in their infancies have nevertheless opened a new dimension towards understanding role of mTORC2 in regulating AGC kinases and/or their counterparts. Seemingly so mTORC2 still remains an important area for future research .Unlike mTORC1, TSC1/2 has been shown to positively affect mTORC2 (Huang, J. et al., 2008). The physiological importance of mTOR is undoubtedly demonstrated by the fact that the knockout of mTOR in mice is primordially embryonic lethal, and the dysregulation of the mTOR pathway is associated with increased transformation and oncogenesis.
1.6 Some other S6K1 Kinases

1.6.1 hVps 34

The mammalian homologue (hVps34) of Vps34p, a class III PI 3 kinase is claimed to be a nutrient regulated lipid kinase (Byfield et al., 2005). This Kinase in association with another protein kinase Vps15 is involved in multiple vesicular trafficking pathways (Herman and Emr, 1990; Stack et al., 1993) besides being implicated in autophagy in yeasts as well as mammalian cells. Beclin1, a hVps34-associated protein has also been shown to be required for autophagy in mammalian cells (Liang et al., 1998; Yue et al., 2003). Although these data suggest that hVps34 is involved in nutrient regulated pathways and a study by Byfield et al. has hinted at the possibility that hVps34 may itself be regulated by the cellular nutritional state and hVps34 is required for insulin stimulation of S6K1. Instead hVps34 is not regulated by insulin, nor does it affect insulin-stimulated phosphorylation of Akt or TSC2. However amino acid, glucose levels and AMPK remain involved in the regulation of hVps34 (Byfield et al., 2005). These data hence suggest a novel role for hVps34 in nutrient sensing, and in the integration of signalling from amino acids and glucose to mTOR and S6K1. Of late, hVps34 has been shown to regulate S6K1 activation such that over expression of hVps34 or the associated hVps15 kinase activates S6K1. hVps34 is not part of the insulin input to S6K1, as it is not stimulated by insulin, and inhibition of hVps34 has no effect on phosphorylation of Akt or TSC2 in insulin-stimulated cells. However, hVps34 is inhibited by amino acid or glucose starvation, suggesting that it lies on the nutrient-regulated pathway to S6K1. Consistent with this, hVps34 is also inhibited by activation of the AMP-activated kinase, which inhibits mTOR/S6K1 in glucose starved cells. hVps34 appears to lie upstream of mTOR, as small interfering RNA knock-down of hVps34 inhibits the phosphorylation of another mTOR substrate, eIF4E-binding protein-1 (4EBP1) suggesting that hVps34 is a nutrient-regulated lipid kinase that integrates amino acid and glucose inputs to mTOR and S6K1 (Byfield et al., 2005).

1.6.2 DRAK 2 (Death associated protein kinase related 2)

Death-associated protein-kinase-related 2, (DRAK2) is a member of DAP (death-associated protein) family of serine/threonine kinases. DAP kinases are known to induce apoptosis when over-expressed in cell lines (Honey, 2005). DRAK2 shares
50% identity in the kinase domain with other members of the family (Deiss et al., 1995). Unlike some other members of this family, DRAK2 does not have a calmodulin regulatory domain in the C-terminal and resides in both nuclei and cytosol (Matsumoto et al., 2001; Sanjo et al., 1998). DRAK2 has been found to interact with calcineurin homologous protein (CHP) and be inhibited by it in a calcium dependent manner (Kuwahara et al., 2003). For a long time, DRAK2 was believed to be expressed specifically by lymphocytes but in situ hybridization analysis has revealed that DRAK2 expression is ubiquitous at the midgestation stage in embryos, followed by more focal expression in various organs in the perinatal period and adulthood, notably in the thymus, spleen, lymph nodes, cerebellum, suprachiasmatic nuclei, pituitary, olfactory lobes, adrenal medulla, stomach, skin, and testes (Mao et al., 2006). A recent study by Mao et al suggested role of this serine/threonine kinase in regulating S6 kinase activity, it was seen that DRAK 2 phosphorylated S6K in an in-vitro kinase assay and DRAK 2 expression positively regulated phosphorylation at the hydrophobic motif (T412) such that its over expression in NIT-1 cells leads to increased T412 phosphorylation and its down regulation using DRAK 2 specific siRNA produced the opposite effect. It is possible to link p70S6 kinase activation with islet apoptosis and that inhibition of p70S6 kinase phosphorylation by rapamycin contributes to the reduction of islet apoptosis after transplantation. This also suggests that inflammatory cytokines activate both the DRAK2/P70S6 kinase and mTORC1/P70S6 kinase pathways and that inhibiting one of them is only partially effective in reducing cell apoptosis. Indeed, when DRAK2 up-regulation stimulated by cytokines was prevented by siRNA, islet apoptosis was decreased but was not totally prevented. Similarly, rapamycin only partially protected islet apoptosis from the cytokines. Dual inhibition of mTORC1 (with rapamycin) and DRAK2 (with DRAK2 inhibitors that are to be developed) might achieve better results in islet protection in terms of cytokine-induced cell apoptosis. Thus it seems probable that mTOR/S6K pathway runs parallel to DRAK2/ S6K pathway and molecular cross talk between these two pathways is circumstantial and prevalent under conditions involving cell apoptosis and other survival mechanisms.

1.6.3 Glycogen Synthase Kinase 3 Beta or GSK3-β

GSK 3 is a Ser/Thr kinase that was initially isolated and purified from rat skeletal muscle cells. It was designated as Glycogen Synthase Kinase as it phosphorylates the
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last enzyme of glycolysis, Glycogen Synthase to inactivate it (Embi et al., 1980; Woodgett, 1990). It has two isoforms GSK3α and GSK 3β with similar structure but different substrate specificities. GSK 3 which was originally thought to be important in metabolism is now known to phosphorylate greater than 40 proteins and more than 12 transcriptional factors (Sutherland, 2011). GSK3A and GSK3B encode 51 and 47 kDa proteins respectively (Patel and Woodgett, 2008). Along with possessing a glycine rich extension at the amino terminus GSK3α also differs from GSK3β in having only 36% identity in the carboxy terminal (Doble and Woodgett, 2003). Both the isoforms prefer primed substrates and remain active in non-stimulated cells (McCubrey et al., 2014). They act on substrates that have been phosphorylated by other kinases like MAPK, ERK or AMPK.

GSK3β is inactivated by phosphorylation at S9 as it induces a pseudosubstrate conformation in the substrate docking motif of GSK3β (Patel and Woodgett, 2008; Sutherland, 2011). In addition, phosphorylation of GSK3β at S9 causes inactivation by proteosomal degradation and consequent pathological conditions including cancer. GSK3β is phosphorylated by various kinases like protein kinase A (PKA), Protein Kinase B (AKT), p90 ribosomal S6 kinase (p90Rsk), p70 ribosomal S6 kinase (Doble and Woodgett, 2003; Patel and Woodgett, 2008). Insulin signaling and other mitogenic/growth factors result in activation of AKT and subsequent phosphorylation and inhibition of GSK3β. S9 phosphorylation causes a pseudosubstrate to form which folds and binds the positively-charged pocket. As a result, phosphorylation of substrates at the catalytic groove is blocked. This is a competitive mechanism of inhibition as the elevated level of substrate out-competes the pseudosubstrate and get phosphorylated (Frame et al., 2001). The requirement of priming of the substrate at N+4 position depends on presence of Arginine at position 96. The R96A mutation changes the GSK-3beta pocket of positive charge to prevent the phosphorylation of the primed substrates. The R96A mutation prevents the S9 phosphorylated GSK-3beta pseudosubstrate from folding back and results in the phosphorylation of unprimed substrates even when GSK-3beta is phosphorylated at S9 (Zhang et al., 2009).

Wnt pathway, responsible for cellular growth and organismal development phosphorylates GSK3β to inactivate it. However, when it is not inhibited by Wnt pathway, GSK3β acts on TSC1/TSC2 to influence mTOR pathway. GSK3β can phosphorylate TSC2 on two serine residues, 1341 and 1337, with AMPK
phosphorylating serine residue 1345. AMPK primes the TSC2 at 1345 to facilitate GSK3β to act on target serine residues. These phosphorylations activate TSC1/TSC2 to result in mTORC1 inactivation (Majid et al., 2012). On the other hand GSK3β activates S6K1 by phosphorylating turn motif at Ser394 and inhibiting PP2A. This goes to show the co-operation between mTOR and GSK3β in regulation of P70S6K1 (Shin et al., 2011). mTORC1 has been shown to be responsible for deactivating GSK3β via P85S6K1 wherein inhibition of S6K1 abrogated the phosphorylation of GSK3β (Embi et al., 1980; Wang et al., 2011; Woodgett, 1990). GSK3β has a history of being a paradoxical player in oncogenesis acting as a tumor suppressor in some cases and promoting tumor in others. These complex patterns in oncogenesis along with its baffling interplay with mTOR and its substrate kinases P85S6K1 and P70S6K1 has promoted the rationale of co-targeting mTOR and GSK3β for treating cancer (Wang et al., 2011). In fact, mTOR kinase inhibitors used in cancer therapy are antagonized by GSK3β inhibitors while constitutively active GSK3β sensitizes cancer cells response to mTOR inhibition (Koo et al., 2014). It raises two possibilities; mTOR and GSK3β act in concert to promote oncogenesis and possibly S6K1 and/or mTOR and GSK3β act independent of each other on cancer cells and S6K1.

1.6.4 AMP-activated Protein Kinase

AMPK originally identified as a protein that responds to carbon starvation in green plants (Thelander et al., 2004), has been largely considered to be responsive to diverse metabolic stresses such as nutrient starvation, hypoxia, and exercise and heat shock that disturb cellular energy homeostasis. AMPK is expressed ubiquitously in mammalian cells. The AMP-activated protein kinase was originally identified as regulatory kinase that phosphorylates hydroxymethylglutaryl-CoA reductase, key enzyme in cholesterol biosynthesis (Hardie and Carling, 1997). Many other metabolic enzymes that act as substrates of AMPK include hormone sensitive lipase, glycogen synthase and creatine kinase (Hardie and Carling, 1997). 5’-AMP levels when elevated in cells act as allosteric activator of AMPK. This binding facilitates phosphorylation by LKB1 (or CaMMKbeta in certain cell types) and inhibition of dephosphorylation and inactivation by phosphatase 2C (Davies et al., 1995). Upon ATP consumption, ADP is produced which gets converted to AMP. Accumulation of 5’AMP leads to binding and activation of AMPK.
AMPK is believed to inhibit P70S6K1 through mTOR inactivation (Baumann et al., 2007; Bolster et al., 2002). The mechanism of inactivation of mTOR is proposed through TSC1/2 (Inoki et al., 2003). Upon energy depletion or low ATP levels, AMPK is activated which phosphorylates TSC2, leading to increased GAP activity of TSC2 towards Rheb and thus, mTORC1 inactivation. Many activators of AMPK like AICAR (Kimura et al., 2003), metformin (Dowling et al., 2007) and troglitazone (d’Abramo et al., 2006) have been reported to inhibit P70S6K1 activity and phosphorylation as well. Many observations have raised the need to elaborate the effect of AMPK that operates independent of mTOR. AICAR has been shown to be unable to mediate its effect on P70S6K1 in HEK293 cells (Kimura et al., 2003) and to further phosphorylate the rapamycin-resistant mutant of P70S6K1. Effect of metformin on mTOR has been shown to be mediated through REDD1 and not by activation of AMPK (Sahra et al., 2011). Likewise, the inhibitory effect of troglitazone has been explained by inhibition of PDK1 (d’Abramo et al., 2006). Other studies support the disparateness of AMPK activation and P70S6K1 activity. The insulin sensitizing effect of AMPK has been shown to be independent of inhibition of mTOR/P70S6K1 axis (Ginion et al., 2011). PF-4708671, a specific inhibitor of P70S6K activates AMPK and inhibits rps6 (substrate of P70S6K) phosphorylation even in P70S6K1 null cells (Vainer et al., 2014). Further, AMPK and insulin/insulin-like growth factor pathways that are expected to oppose each other, work in the same direction in many cases. For instance, both insulin and AMPK activation cause increase in glucose uptake by translocation of GLUT4 to the plasma membrane. Moreover, Mechanical stretch causes concomitant increase in T412 phosphorylation of P70S6K1 and Thr172 of AMPK. Stimulation of mTOR pathway in this case does not decrease activation of AMPK. However, the presence of compound C (inhibitor of AMPK) and AICAR (activator of AMPK) showed routine effect of increasing and decreasing the phosphorylation of P70S6K1, respectively (Nakai et al., 2015). These studies suggest that the pharmacological modulators like compound C, AICAR and PF-4708671 may influence P70S6K1 through some effector that is independent of AMPK s effect on mTOR or even mTOR. In fact, lowering endogenous levels of ATP in cells by using glycolysis inhibitor DG (2 deoxyglucose) dephosphorylates P70S6K1 without affecting the phosphorylation levels of rapamycin resistant mutant of P70S6K1 (Dennis et al., 2001).
1.7 Functions Associated with S6K1

S6K1 activation occurs as a response to various coordinated signaling pathways including mTOR, PI3-kinase and MAPK, through ordered phosphorylation events directed at multiple sites that is ultimately requires for cell growth and proliferation (Schmelzle and Hall, 2000). Hence S6K1 is considered to be a multifaceted effector that regulates cell growth and proliferation by phosphorylating multiple ways. A few of the S6K1 associated functions are briefly described below:

1.7.1 Cell growth and Translational control

S6K1 has been known for its ability to phosphorylate S6 protein of 40S ribosomal subunit at five C-terminal serine residues in a sequential manner S236 > S235 > S240 > S244 > S247 to regulate cell growth. An important event that was initially attributed to S6 phosphorylation is selective translation of mRNAs characterized by 5' - oligo Pyrimidine tract (5'-TOP). Later studies however, confirmed this to be an independent event (Ruvinsky et al., 2005). The study of knock-in mice in which these five phosphorylation sites in rpS6 were replaced by alanine residues (rpS6) has provided much-needed insight into the physiological role of rpS6 phosphorylation in vivo (Ruvinsky et al., 2005, 2009). Although rpS6-/-mice are viable and fertile, they display a remarkable phenotypic overlap with S6K1-/- mice and strikingly, a cell growth defect is evident in both cases. In both rpS6P-/-and S6K1-/- mice, hypoinsulinaemia decreased cross-sectional area of the myotubes results in a reduction in size of the pancreatic cells leads to muscle weakness and cells isolated from both genotypes display defective cell growth (Pende et al., 2000; Ruvinsky et al., 2009). The cell growth phenotype common to these mouse models is particularly interesting given that S6K1-/- mice display minimal defects in rpS6 phosphorylation, while S6K2-/- mice grow to normal size despite a significant reduction in rpS6 phosphorylation (Pende et al., 2004). Selective recruitment of S6K1 and S6K2 into distinct protein complexes and sub cellular compartments suggests differential phosphorylation rpS6 or phosphorylation of rpS6 may be required at a specific developmental stage, at which S6K1 but not S6K2 is active in the affected cell lineages. The link between S6K1, rpS6 phosphorylation and cell growth is clearly an area of great interest for further study. In addition to re-establishing a role for rpS6 phosphorylation in the control of cell growth, analysis of the rpS6P-/- mice also
confirmed that translation of a specific subset of mRNAs containing an oligo pyrimidine tract at the 5' terminus (5TOP mRNAs), a process long thought to be under control of rpS6 phosphorylation occurs entirely independently of this event (Ruvinsky et al., 2005). The fact that 5/S6K1-/-/S6K2-TOP mRNA translation is unaffected in mice, which display minimal rpS6 phosphorylation, further demonstrates the lack of association between these events in vivo (Pende et al., 2004).

1.7.2 Cell cycle progression

Various studies implicate S6K1 in regulating G1 to S progression (Lane et al., 1993), however; a direct role in the process has not been established yet. A study has reported that phosphorylation of S6K1 aids in cell cycle progression thorough phosphorylation of oestrogen receptor leading to activation of its target genes resulting in proliferation (Yamnik et al., 2009). In this study, the proliferation of cell lines in which S6K1 was highly expressed due to amplification of 17q23 was sensitive to rapamycin, while those with lower S6K1 levels were relatively resistant, thus the contribution of S6K1 to proliferation may be apparent only at supra physiological expression levels. This could be advantageous in the context of targeting S6K in cancer, where the aim is to minimize effects on normal cells. S6K1 has been also shown to phosphorylate CREB (cAMP response element binding protein) and transcription factor UBF-1, which in turn activates RNA polymerase 1 driven transcription of ribosomal RNAs aiding in ribosomal biogenesis. Serum stimulation driven mTORC1 mediated activation of hNRPs especially hNRP-F by S6K2 aids in cell proliferation. Significantly however, cardiac hypertrophy, a response dependent upon ribosome biogenesis, is unaffected in S6K -/- mice, thus there is no absolute requirement for S6K function at either the transcriptional or translational levels of this process (McMullen et al., 2004). Cellular translation control remains pivotal in executing the regulated cell proliferation. S6K1 remains implicated in maintain this control as well. S6K1 has been involved in regulation of cap dependent translation by activating eIF4B at serine 422 under mTOR influence in response to nutrient supply (Raught et al., 2004). It also controls translational initiation by phosphorylating PDCD4 which is a negative regulator of eIF4A, marking it for degradation by ubiquitin ligase β TRCP (Dorrello et al., 2006). Besides it phosphorylates and inactivates elongation factor 4- kinase to regulate cell growth (Wang et al., 2001b).
1.7.3 Cellular metastasis and S6 Kinase

S6 kinase has been implicated to regulate cellular metastasis in glioma cells wherein mTOR/S6K axis has been shown to be constitutively activated and using silencing RNAs against S6K partially rescues the transformed phenotype (Nakamura et al., 2008) in addition to its reported constitutive activation in oesophageal squamous cell carcinomas (ESCC) (Hou et al., 2007).

1.7.4 Cell survival signalling

Pro-apoptotic molecule BAD, an Akt substrate has been shown to be phosphorylated by S6K1 at Ser136 causing its 14-3-3 dependent sequestration in cytoplasm and inactivating its function (Harada et al., 2001). S6K has been shown to phosphorylate P53 ubiquitin ligase MDM2 causing cell survival (Lai et al., 2010). S6K1 modulates several actions of Mdm2, like 163/183 phosphorylation, nuclear cytoplasmic shuttling, and Mdm2-mediated ubiquitination of its substrates likely through interaction between these two proteins. S6K regulates the stability of p53 - the best studied Mdm2 substrate, cell death response under genotoxic stress in normal cells. Thus, the mTOR-S6K pathway has a function in DNA damage response by transmitting pro-apoptotic signals and may also regulate tumorigenesis. Several initial studies have indicated that p38 MAPK can directly act on Cdc25 and p53; it was however later revealed that p38 MAPK can also regulate p53 stability through the mTOR-S6K Mdm2 pathway, thus highlighting the importance of p38 MAPK further in DNA damage response. Enriched environment will lead to S6K1 T412 phosphorylation and activation, enhanced S6K1-Mdm2 complex formation, and Mdm2 cytoplasmic retention, allowing maximal p53 induction upon genotoxic stress, which might be needed to counteract the strong mitogenic signals and enhanced protein synthesis mediated by the mTOR-S6K pathway. On the other hand, poor environment will downplay mTOR-S6K signalling, leading to reduced p53 induction upon genotoxic stress. This might be sufficient to cause cell-cycle arrest in the presence of weak mitogenic signals. Therefore, S6K1-Mdm2 interaction may provide the link between cells status (nutrients, energy, and growth factors) and their response to DNA damage. This study establishes that S6K1 is not only a kinase for Mdm2 S163 phosphorylation but also a physical interacting partner under genotoxic stress. S6K1 can phosphorylate Mdm2 on S163 in vivo and in vitro and is required for S163
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phosphorylation in response to DNA damage through activation of mTOR-S6K1 axis mediated through p38a MAPK. Activation of S6K1 leads to a high titre complex formation with Mdm2 which in turn inhibits Mdm2-mediated p53 ubiquitination and promotes p53 induction. Deactivation of mTOR-S6K1 signalling leads to Mdm2 nuclear translocation, which is facilitated by S163 phosphorylation, a reduction in p53 induction, and an alteration in p53-dependent cell death. These findings thus establish mTOR-S6K1 as a novel regulator of p53 in DNA damage response. S6K1–Mdm2 interaction presents a route for cells to incorporate the metabolic/energy cues into DNA damage response and links the aging-controlling Mdm2− p53 and mTOR-S6K pathways.

1.7.5 Cell migration

Cytoskeleton assembly and rearrangement dictate cellular movements across a substratum through polymerization and depolymerisation of actin filaments leading to extension of the cytoskeletal and translocation of the cell through the forward motion of the cell at the leading edge and detachment at the trailing edge (Ip et al., 2011). These cellular structures are formed in response to actin polymerization and the formation of actin stress fibres, the basic component of the cytoskeleton necessary for focal contacts (adhesion), migration and maintaining cellular shape. Complex signalling pathways regulate formation of these cytoskeletal structures involving receptor activation mediated by signalling proteins and second messengers such as PI-3,4P2, phospholipase C, PKC, Ca2+, PI 3-kinase, Rho and Rac GTPases and MAPK (Wells, 1999). Association of p70S6K with small GTPases like Rac1 and cdc42 which are known to regulate membrane ruffling, migration and actin polymerization has shown to mediate some of these effects (Chou et al., 2003). Dominant-negative Rac1 over expression in several cell types prevented growth factor-induced p70S6K activity, suggesting that Rac1 activates p70S6K. In addition, activation of p70S6K by expression of an activated allele of Rac1 is inhibited by rapamycin and the PI 3-kinase inhibitor wortmannin. The results of both experiments support a role for p70S6K in regulation of the cytoskeleton, except that rapamycin had no effect on membrane ruffling or stress fibre formation. Studies showing Rac1- mediated activation of p70S6K is unrelated to cytoskeleton reorganization have not been published. In contrast, several studies have shown a role for p70S6K in cytoskeleton regulation and
cell migration. p70S6K has been shown to co-localize with actin stress fibres, suggesting that p70S6K activation plays a role in actin polymerization. Another observation, that thrombin stimulation causes a shape change effect that is characterized by elongation and organization of stress fibres and this effect is inhibited by treatment with rapamycin (Crouch, 1997). Nitric oxide donors have been found to increase growth factor-stimulated p70S6K activity and this potentiation of p70S6K activity is associated with a prolonged shape change effect and enhancement of tails, both morphological features that may be enhanced in rapidly migrating cells (Berven et al., 1999). Based on several morphological and biochemical results, it has been proposed that p70S6K is involved in regulating the migration of 3T3 fibroblasts and thus present a potentially novel function of p70S6K. Clearly, p70S6K is important for regulation in translation. Thus, growth factor induced translocation of p70S6K to the actin cytoskeleton and leading edge of the cell followed by localized synthesis of key protein regulators of filopodia or lamella extension is one possible mechanism. In support of this idea, it has been shown that p70S6K may be targeted to nerve endings via its interaction with Neurabin, an F-actin binding protein that is highly expressed in nerve tissue (Burnett et al., 1998a). In this study, p70S6K and Neurabin were shown to co-localize in brain sections by in situ hybridization and were both enriched in the synaptosomal fraction in rat brain. Although localized protein translation in nerve terminals has not been established, it is possible that p70S6K functions to increase synthesis of proteins required for the assembly of actin cytoskeletal structures that are involved in neurite outgrowth or growth cone formation. Thus, it is possible that the function of p70S6K at the synapse may be analogous to its role in migration. Recently, it has been shown that mTOR interacts with gephyrin, a protein that is necessary for the clustering of glycine receptors at the postsynaptic terminals in spinal cord neurons. In these studies, expression of mTOR mutants that were unable to bind gephyrin failed to activate the downstream targets of mTOR, p70S6K and 4E-BP1. Furthermore, while mTOR expressed in HeLa cells appears uniformly distributed throughout the cytoplasm, coexpression of mTOR with gephyrin causes aggregation of mTOR at polarized regions of the cell, suggesting that gephyrin may influence mTOR (and consequently p70S6K) signalling through its role in clustering receptors or other signalling molecules that contain a gephyrin-binding domain (Sabatini et al., 1999).
Chapter 1

1.8 Rationale of the study

S6K1 lies downstream of growth signalling pathways like mTOR & PI3 Kinase and is activated by a series of ordered phosphorylation events in response to growth factor stimulation (Dennis et al., 1998; Mahalingam and Templeton, 1996; Mukhopadhyay et al., 1992). Its activation is reported to begin by a calcium dependent priming event to release the interaction between amino terminal and carboxy terminal domains (Hannan et al., 2003), which facilitates phosphorylation of several proline directed serine residues in the pseudosubstrate domain possibly by MEK/Akt/CDK1 (Conus et al., 1998; Wang et al., 2001). The regulation of S6K1 has been proposed to be phosphorylation dependent and accordingly three phosphorylations at turn motif, hydrophobic motif (HM) and Activation/T loop are considered to be critical. Since the turn motif phosphorylation is considered to be constitutive, the other two phosphorylations share the burden of S6K1 regulation. The phosphorylation at hydrophobic motif has been attributed to the kinase activity of mTOR (Isotani et al., 1999); which in a form of a rapamycin sensitive complex, mTORC1, is believed to be recruited by a scaffold protein, Raptor on the amino terminus of S6K1 (Chung et al., 1992; Pearson et al., 1995). In spite of the predicted model of rapamycin action, how rapamycin actually targets and carries over inhibition to S6K1 is not clearly understood (Saitoh et al., 2002), despite the discovery of TOR signalling motifs in S6K1 and its association with Raptor (regulatory associated protein of mTOR). It has been reported that these amino (FDIDL) and carboxy-terminal (RSPRR) signalling motifs mediate interaction with mTOR through raptor to phosphorylate S6K1 at Threonine 412 and carry rapamycin mediated inhibition to S6K1 (Schalm and Blenis, 2002; Schalm et al., 2005). HM phosphorylation is ensued by the phosphorylation at the activation loop (AL), supposedly carried out by PDK1 (Alessi et al., 1998; Pullen et al., 1998; Weng et al., 1995) These sequential phosphorylations have hence been considered to be indispensible for the complete activation of S6K1 as these phosphorylation exhibit a dramatic turnover upon activation and inhibition of the enzyme by rapamycin. The implication of mTORC1 to phosphorylate HM however, lacks concrete evidence and is merely based on the ability of mTORC1 to bind rapamycin. Further mTORC1 docking on amino terminus TOS motif, mediated by Raptor has also invited reservations. This is supported by our earlier findings that S6K1 mutants, lacking the ability to bind mTOR kinase still exhibit HM.
phosphorylation (Beigh et al., 2012a; Beigh et al., 2012b) Furthermore, studies of late show the presence of HM phosphorylation in S6K1 variant truncated of its both termini (S6K1∆NT/∆CT) isolated from rapamycin treated cells. This has been attributed to the rapamycin insensitive kinase mTORC2 that supposedly mediates the phosphorylation in a non-physiological manner (Ali and Sabatini, 2005). Several independent studies suggest involvement of several other kinases known to phosphorylate this site like NEK, hVsp34, and DRAK2 etc (Belham et al., 2001; Byfield et al., 2005; Mao et al., 2009). Amongst these kinases, DRAK2 has been figured as a direct kinase for HM site of S6K1. These entire observations question the idea that mTORC1 may represent the sole S6K1 T412 kinase. The ambiguity about the role of mTORC1 and mTORC2 in propagation of signals downstream to influence protein translation defines the rationale of this study to identify phosphorylation sites in S6K1 that respond to mTORC1 or mTORC2 selectively.

In this study, we have attempted to dissect the mechanistic regulation of S6K1 with inputs from both mTORC1 and mTORC2 complexes and present a model which explains, in detail, its activation as well as inhibition. Also we sought to investigate the regulatory inputs of DRAK2 on S6K1 activation and relate it with mTOR mediated activation, if any.