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DISSERTATION

SKAR: a novel target of S6 kinase 1

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von

Kristina Karen Jülich

aus Berlin

Gutachter: 1. Prof. Dr. Markus Schülke
2. Prof. Dr. Andreas Kulozik
3. Prof. Dr. Heide Hörtnagl

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Table of contents

Zusammenfassung **5**

Abstract **6**

1. Introduction **8**

1.1 Regulatory mechanisms in a cell 8

1.1.1 Regulation of cell growth 8

1.1.2 The role of S6 kinases 9

1.2 Activation of S6 kinase 1 10

1.2.1 The PI3 kinase pathway 11

1.2.2 The mTOR pathway 13

1.3 The role and clinical potential of rapamycin 15

1.4 The structure of S6 kinases 17

1.4.1 A homologue, S6 kinase 2 18

1.5 Substrates of S6 kinases 19

2. Aims **21**

3. Materials and methods **22**

3.1. Materials 22

3.1.1 Chemicals 22

3.1.2 Buffers 22

3.1.3 Plasmids and library 25

3.1.4 Enzymes and oligonucleotides 25

3.1.5 Antibodies 26

3.1.6 Cell lines 26

3.2 Methods 26

3.2.1 Molecular biology 26

3.2.2 Bacteria 27

3.2.3 Tissue culture 28

3.2.4 Protein biochemistry 29
3.2.5 Immunofluorescence 31
3.2.6 Yeast 32

4. Results **33**
4.1 The Yeast Two-Hybrid Screen 33
4.1.1 Isolation of a novel protein that interacts with S6 kinase 1 34
4.2 Localization of SKAR 37
4.2.1 SKAR is a nuclear protein 37
4.2.2 SKAR co-localizes with ALY 38
4.3 Interaction of SKAR with S6 kinases 40
4.3.1 in vitro interaction 40
4.3.2 In vivo interaction 41
4.4 SKAR is a substrate of S6 kinase 1 45

5. Discussion **48**

6. References **54**

Appendix **63**
Abbreviations 63
Acknowledgements 65
Curriculum vitae 66
Eidesstattliche Erklärung 68

Zusammenfassung

Signaltransduktionswege, die das Zellwachstum beeinflussen, sind bei vielen Krankheiten und insbesondere in Tumorzellen fehlreguliert. Die Aufklärung der Signaltransduktion auf molekularer Ebene kann daher bei der Entwicklung neuer Therapieansätze hilfreich sein. Die Aktivität der beiden Kinasen PI3-Kinase (Phosphatidyl-Inositol-Phosphat-3) und mTOR (mammalian target of rapamycin) ist in Tumorzellen häufig verändert. Sie beeinflussen eine Vielzahl von Substraten, deren genaue Wirkungsweise oft nicht bekannt ist.

Mit einem genetischen Interaktionssystem, dem Yeast-Two-Hybrid Screen, habe ich SKAR (S6K1 ALY/Ref-like target), ein neues Substrat sowohl des PI3-Kinase- als auch des mTOR-Signalwegs identifiziert, und es in Hinsicht auf Lokalisation, Phosphorylierung und Interaktion charakterisiert. SKAR ist das erste nachgewiesen spezifische Substrat der S6 Kinase 1 (S6K1), einem Effektor oben genanner Signalwege, aber nicht der homologen S6 Kinase 2 (S6K2). SKAR bindet S6K1 *in vitro* und *in vivo*, und die Bindung wird für die Phosphorylierung benötigt. Ein solcher Mechanismus wird docking-site-Mechanismus genannt. Dies ist die erste Beschreibung einer docking-site für S6K1.

Die Phosphorylierung von SKAR durch S6K1 wird durch Wachstumsfaktoren, Insulin und Nährstoffe stimuliert und kann durch Rapamycin inhibiert werden. Für Rapamycin und seine Analoga gibt es derzeit eine Vielzahl klinischer Anwendungen. Sie werden zum Beispiel zur Immunsuppression, in der Chemotherapie und zur Beschichtung von Stents für die PTCA (perkutane transluminale coronare Angioplastie) verwendet. Zusätzlich laufen Studien bzw. Einzelfalltherapien zur Therapie zerebraler Raumforderungen bei tuberöser Sklerose. Die Identifizierung von Proteinen, deren Verhalten in der Zelle durch Rapamycin beeinflußt wird, hilft daher, die Wirkungsweise dieses Medikaments weiter aufzuklären.

SKAR ist im Zellkern in den sogenannten “speckles” lokalisiert, Strukturen, in denen sowohl Splicing als auch die weitere Verarbeitung der mRNA stattfinden. SKAR kolokalisiert sowohl mit dem Spliceosom als auch mit dem mRNA-Exportfaktor ALY.

ALY und SKAR sind etwa 50% homolog in einer Domäne, die ein RRM (RNA recognition motif) enthält.

Spätere Arbeit unserer Gruppe zeigte einen S6K1-vermittelten Effekt von SKAR auf die Zellgröße. Weitere Experimente werden in der Zukunft zeigen, welche Rolle SKAR bei der Verarbeitung der mRNA spielt und ob diese Funktion an die S6K1-vermittelte Regulation der Zellgröße und an Rapamycin geknüpft werden kann.

Schlüsselwörter: S6K1, Zellgröße, mTOR, PI3K, Rapamycin, ALY, Exon-Junction-Komplex, Splicing

Abstract

Signaling networks that promote cell growth are frequently dysregulated in human diseases, particularly in tumor cells. Identification of effectors of those pathways might therefore be useful to develop new approaches of therapy.
Pathways that are often altered in cancer include the PI3 kinase- and mTOR signaling pathways. These pathways affect a variety of substrates whose effects on a molecular level are often unknown. Yet they are modified by drugs like rapamycin, which is used for immunosuppression and chemotherapy.
By taking advantage of a genetic protein interaction system, the Yeast-Two-Hybrid Screen, I identified SKAR (S6K1 ALY/Ref-like target), a novel target of both PI3 kinase and mTOR signaling pathways, and characterized the novel protein in terms of localization, phosphorylation and interaction. SKAR is the first substrate proven to be specific for S6 kinase 1 (S6K1), a downstream effector of mTOR and PI3K signaling, but not for the highly homologous S6 kinase 2 (S6K2). Signaling from S6K1 to SKAR occurs via a docking-site mechanism. S6K1 is able to bind SKAR *in vitro* and *in vivo,* and binding to the kinase is required for phosphorylation. This is the first description of a docking-site mechanism for S6K1.
The phosphorylation of SKAR by S6K1 is stimulated by mitogens and nutrients and can be inhibited by treatment with rapamycin. Rapamycin and its analogues are used for a variety of clinical applications such as immunosuppression, chemotherapy, and coated stent implantation after PTCA (percutaneous transluminal coronary angioplasty), and

identification of downstream signaling targets will help to elucidate the mechanism of action.

SKAR is a nuclear protein and localizes in complexes that contain components of the splicing apparatus. It co-localizes with a spliceosomal marker as well as with a member of an mRNA export family, ALY. SKAR shares a 40% identity with ALY in a region containing an RRM or RNP type RNA binding motif.

Later work of our group showed that SKAR is involved in S6K1-mediated cell size control. Further research will be directed on the issue whether SKAR is involved in mRNA processing itself and whether mRNA processing can be linked to cell size control and the signaling pathways that are targeted by rapamycin.

Key words: S6K1, cell size, mTOR, PI3K, rapamycin, ALY, mRNA processing, exon-junction complex, splicing

1. Introduction

1.1 Regulatory mechanisms in a cell

The evolution of multicellular organisms composed of different cell types necessitates the strict regulation of cell size, proliferation and differentiation.
Breakdown of this coordination may lead to uncontrollable proliferation and result in tumor formation. Therefore, the understanding of how different signaling pathways interact and form networks to coordinate cellular growth and division will help to develop drugs that specifically inhibit oncogenic signaling.
The macrolide rapamycin (sirolimus, Rapamune®), originally identified as a bacterially derived fungicide, is currently being used for immunosuppression after organ transplantation and for chemotherapy (for reviews see Rubo-Viqueira et al., 2006; Webster et al., 2006). Moreover, it has been shown to reduce restenosis rates after coronary stent implantation (Serruys et al., 2006), and appears to be effective in signaling pathways in cell and mouse models and also in off-label treatment of tuberous sclerosis-associated tumors (Kwiatkowski et al., 2005) (see section 1.3 on rapamycin).
The signaling networks are still poorly understood, but the growing importance of rapamycin as a therapeutic agent shows the relevance to further elucidate its mechanism of action.

1.1.1 Regulation of cell growth

Whereas cell cycle regulators and cellular proliferation have been studied for a long time, the mechanisms of cell growth (defined as an increase in mass and size) and maintenance of a characteristic cell size have come under intense investigation only recently.
Studies have shown that cell size is regulated by growth factors, but also by nutrients such as amino acids (Fingar et al., 2004).
It has been suggested that cells progress through the cell cycle only when sufficient mass, size and protein biosynthesis have been reached. Inactivation of various genes encoding for cell cycle regulators in yeast results in arrest of cell cycle, but not of cell

growth. In contrast, inhibition of cell growth by nutrient depletion inhibits division and growth (Johnston et al., 1977).

Similarly, disruption of cell cycle regulators in *Drosophila melanogaster* leads to cell cycle arrest at a large cell size (Weigmann et al., 1997; Neufeld et al., 1998), suggesting that growth proceeds in the absence of proliferation and that proliferation and cell size are coordinated, but separate entities.

1.1.2 The role of S6 kinases

Stimulation of cellular proliferation is promoted by factors that are also required to regulate cell growth.

Cell size is regulated through mTOR (mammalian target of rapamycin) and PI3K (phosphatidyl-inositol-3 kinase) dependent signals. Both pathways activate S6 kinases, and overexpression of S6K1 results in a significant increase in mammalian cell size. Similarly, treatment of mammalian cells with drugs that prevent S6 kinase activation such as rapamycin results in a decrease in cell size (Fingar et al., 2002).

Cellular growth requires the production of many components of the protein synthetic apparatus. This was believed to be regulated at translational level.

Many of the mRNAs that contain a 5' terminal oligo pyrimidine sequence (5'TOP) in their untranslated region encode for ribosomal proteins and translation elongation factors (Pullen and Thomas, 1997). Mitogen- and nutrient-induced phosphorylation of the 40S ribosomal protein S6 results in recruitment of these 5'TOP mRNAs from a pool of stored messenger ribonucleoproteins (mRNPs) to the translating polysomes (Jefferies et al., 1994; Terada et al., 1994). The kinases largely responsible for S6 phosphorylation have been identified and are known as S6K1 with two isoforms of 70 and 85kD, called p70 and p85 (Banerjee et al., 1990; Kozma et al., 1990) or S6KαII & I, respectively (Grove et al., 1991), and S6K2 (Lee-Fruman et al., 1999).

However, recently this concept has been challenged when it was suggested that 5´TOP RNA translation depends neither on S6 kinases nor on S6 phosphorylation (Fingar et al., 2004; Ruvinsky et al., 2005).

In *Drosophila melanogaster,* homozygous deletion of the *dS6K* gene resulted in an extreme delay of development, female sterility, and severe growth reduction (Montagne et al., 1999) (Fig. 1).

from Montagne et al.,1999

Figure 1: Disruption of the *dS6K* gene in *Drosophila melanogaster* results in severe growth reduction, developmental delay and female sterility (left side wild type, right side *dS6K* knockout). Growth reduction is due to reduction in cell size rather than cell number. (Figure reprinted with permission from AAAS)

Consistent with the *Drosophila* phenotype, pancreatic β-cells of S6K1-deficient mice are smaller than wild type cells (Shima et al., 1998), indicating that S6 kinase 1 plays a role in the regulation of cellular growth. S6K1-deficient mice were also hypoinsulinaemic and subsequently glucose intolerant (Pende et al., 2000). Pancreatic β-cell growth, like S6K1 activity, is sensitive to insulin and growth factors, as well as to nutrients, including glucose and amino acids (Swenne et al., 1992; Hugl et al., 1998). Thus, impaired function of this signaling component in conjunction with other genetic and environmental factors may contribute to the development of specific forms of diabetes mellitus. Interestingly, these mice also exhibit increased lipolysis and are protected from diet-induced obesity (Um et al., 2004), possibly due to a failure in transduction of adipogenic stimuli such as insulin and amino acids.

1.2 Activation of S6K1

Growth factor- and nutrient-dependent signaling pathways converge upon several common effectors such as the S6 kinases 1 and 2.
S6K1 is activated by numerous stimuli like PDGF (platelet derived growth factor), IL-2, IGF (insulin-like growth factor), EGF (epidermal growth factor) and insulin (Grammer et al., 1996) through the PI3 kinase pathway.
Beside mitogenic stimuli it also requires the presence of amino acids and glucose (i.e. nutrients) whose levels are sensored by mTOR (Iiboshi et al., 1999) (Fig. 2).

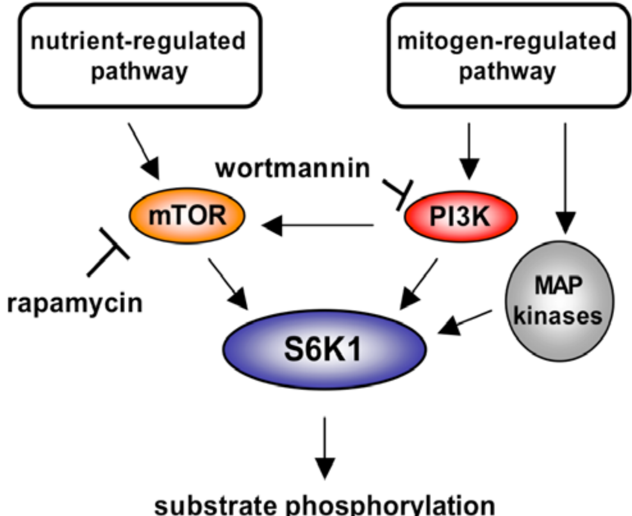


Figure 2: Signal transduction through nutrient- and mitogen regulated pathways: Activation of S6 kinase 1 occurs through phosphorylation on multiple sites. It is mediated through the nutrient-dependent mTOR pathway as well as through the mitogen-dependent PI3 kinase and MAP kinase pathways. mTOR-mediated activation is blocked by the immunosuppressant rapamycin, PI3K-mediated activation is blocked by wortmannin.

1.2.1 The PI3 kinase pathway

PI3K is involved in the regulation of cell growth, survival, motility, vesicle trafficking, transcription, protein synthesis and proliferation (Franke et al., 1997; Rameh and Cantley, 1999). Components of the PI3K/Akt signaling pathway are frequently altered in human malignancies such as ovarian, cervix, pancreas, breast and stomach tumors (Bellacosa et al., 1995; Cheng et al., 1996; Shayesteh et al., 1999; Ma et al., 2000). Binding of insulin or IGF to their receptors results in recruitment of IRS-1 (insulin receptor substrate) to the membrane and subsequently docking and activation of PI3K. Activated PI3K catalyzes the conversion of Phosphatidyl-Inositol(3,4)P_2 (PIP_2) to Phosphatidyl-Inositol(3,4,5)P_3 (PIP_3). The tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome 10) antagonizes PI3K by dephosphorylating PIP_3. The drug wortmannin also inhibits PI3K action. Activation of Akt/PKB occurs through recruitment to the membrane by binding to PIP_3 and subsequent phosphorylation. Several studies suggested Akt as an upstream regulator of S6K activity. It was demonstrated that human and *Drosophila* TSC2 (tuberous sclerosis complex 2, also known as tuberin) is efficiently phosphorylated by Akt *in vitro* which promotes activation of S6K and phosphorylation of the cell cycle regulator 4E-BP1

(Inoki et al., 2002). *In vivo*, TSC2 exists in a heterodimeric complex with TSC1 (known as hamartin). Growth factor-induced phosphorylation of the TSC2/TSC1 complex by Akt results in its dissociation and in turn in the activation of mTOR via the GTPase Rheb. This promotes the phosphorylation of S6K and 4E-BP1. Phosphorylation of S6K has recently been shown to result in a negative feedback loop by inactivation of IRS-1 (Harrington et al, 2004) (Fig. 3).

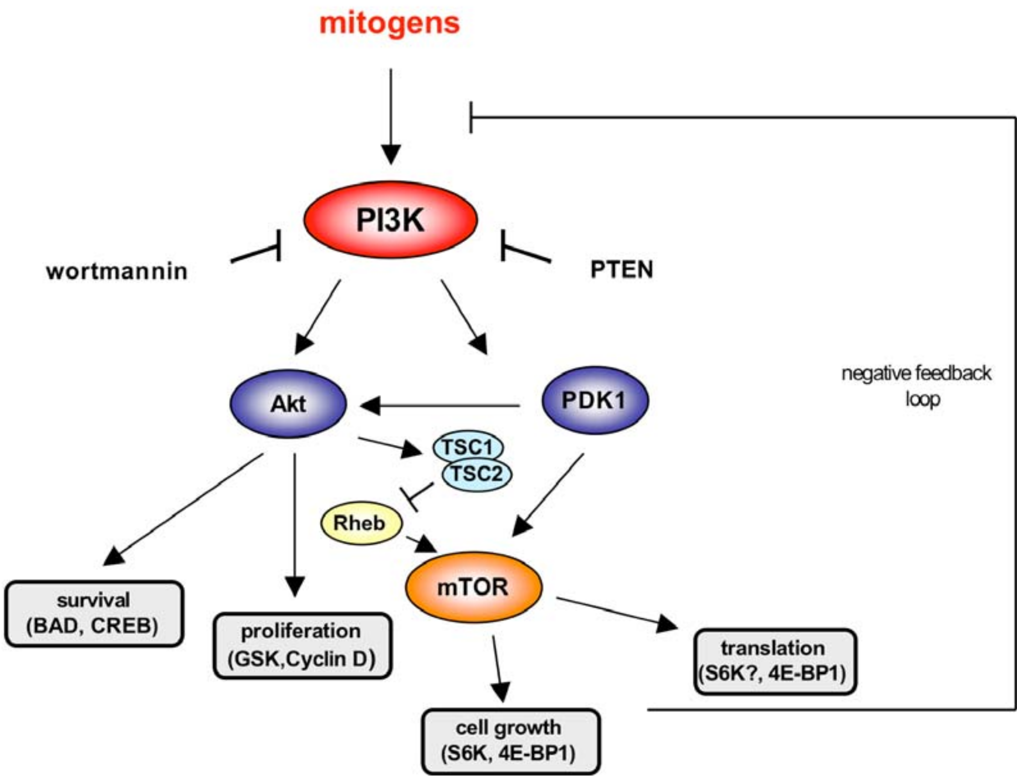


Figure 3: PI3 kinase signal transduction pathways: Activation of PI3 kinase through mitogens results in proliferation, cell survival and in enhanced protein translation and cellular growth, the latter two through mTOR signaling. The "intact" TSC1/TSC2 complex inhibits Rheb-mediated activation of mTOR. Phosphorylation of the complex by Akt results in its dissociation and hence inactivation leading to mTOR activation and ultimately phosphorylation of its substrates. Activation of S6 kinase results in a negative feedback loop by phosphorylation and inactivation of IRS-1. PI3 kinase-dependent pathways can be blocked by treatment with the drug wortmannin. PTEN phosphatase antagonizes PI3K action.

Mutation of *TSC1* or *TSC2* results in a constitutive activation of mTOR and in a disease called tuberous sclerosis. This is a neurocutaneous disorder characterized by impaired neurodevelopment with multiple hamartomas of the brain, often resulting in severe epileptic seizures. Besides the neural tissue also kidneys (angiomyolipomatosis), heart (rhabdomyomas) and skin (hypomelanotic macules, angiofibromas, shagreen patches, subungual fibromas) are affected (Fig. 4).

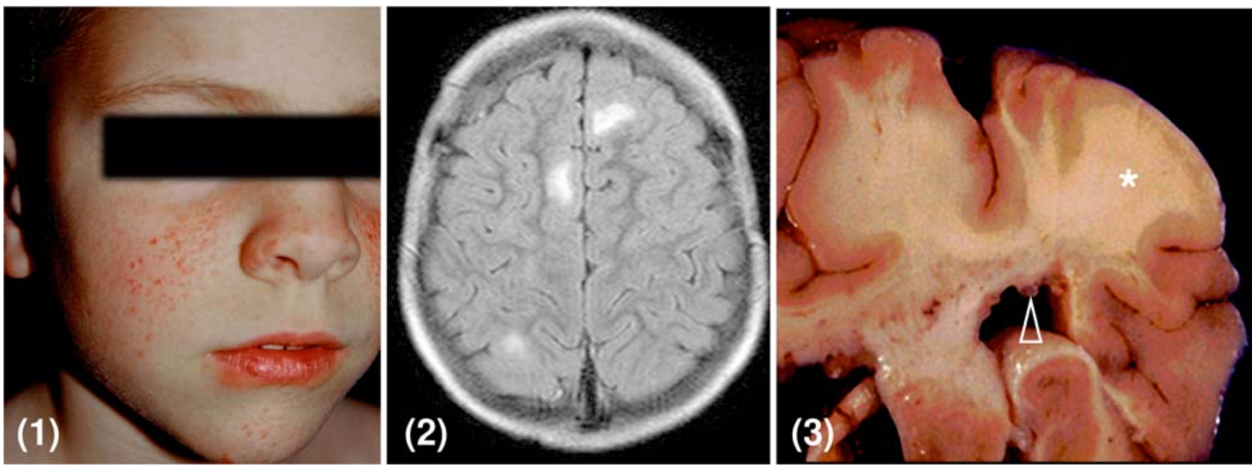


Figure 4: Clinical features of tuberous sclerosis, a disease caused by alteration of the TSC-/mTOR signaling pathway: (1) adenoma sebaceum of the skin, (2) multiple cerebral tubera (MRI scan, FLAIR), (3) subependymal nodules (arrow) and tuber (star).

1.2.2 The mTOR pathway

mTOR (mammalian target of rapamycin) exists in two heteromeric protein complexes called mTORC1 and mTORC2. mTORC1 consists of the proteins mTOR, raptor (rapamycin-sensitive adaptor protein of mTOR), PRAS40 (proline-rich protein kinase B substrate 40 kD) and mLST8 (mammalian leukocyte specific transcript 8), mTORC2 of mTOR, rictor (rapamycin-insensitive companion of mTOR), mSin1 (mammalian stress-activated protein kinase-interacting protein-1) and mLST8 (for review on mTORC signaling and composition see Sabatini, 2006). mTORC pathways are regulated through many signals including nutrients (i.e. amino acids), glucose, intracellular ATP levels, growth factors, insulin and stress. mTORC1 signaling is believed to modify the timing of cellular growth and is the target of the immunosuppressant rapamycin (Hara et al., 2002; Kim et al., 2002). mTORC2 has been shown to regulate actin polymerization

and cell spreading and was thought to be rapamycin-insensitive (Jacinto et al., 2004; Sarbassov et al., 2004), although recently some reports suggest otherwise (for review, see Corradetti et al., 2006). Here I concentrate on the mTORC1 pathway.

mTORC1 regulates cellular growth and cell cycle progression via two different effectors, 4E-BP1 and S6 kinases.

Hyperphosphorylation of the eukaryotic initiation factor binding protein (4E-BP1) through mTOR leads to the release of eIF-4E and enhanced translation of cell cycle proteins containing an N-terminal Cap-structure such as c-Myc and cyclin D1 (Sonenberg and Gingras, 1998; Gingras et al., 2001) (Fig. 5).

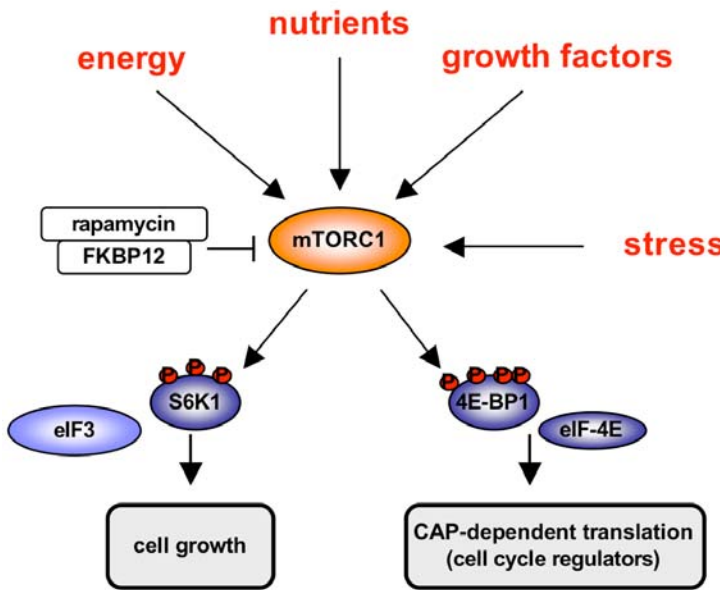


Figure 5: Activation of mTORC1 results in enhanced CAP-dependent mRNA translation (mediated by 4E-BP1) and in promotion of cellular growth (mediated by S6K1). mTORC1 activation can be blocked by the rapamycin-FKBP12-complex.

Phosphorylation of S6K1 results in dissociation from the translation preinitiation complex eIF3 (Holz et al., 2005) and phosphorylation of its targets, namely the ribosomal protein S6. The model that S6K1-mediated S6 phosphorylation controls cellular growth through enhanced translation of 5'TOP mRNAs has come under discussion recently. However, knock-in mice incapable of S6 phosphorylation due to substitution of serine residues to alanine display hypoinsulinaemia and impaired glucose tolerance, and MEFs (mouse embryonic fibroblasts) derived from these cells

are smaller compared to wild type MEFs (Ruvinsky et al., 2005), a phenotype resembling the one from S6K1-deficient mice. These results suggest a role for control of cellular growth through S6 phosphorylation, but the exact mechanism remains unclear.
The mechanism of mTOR regulation of S6K1 activity has been discussed controversially for a long time: However, it seems that mTORC1 is able to phosphorylate S6K1 directly after associating with the eIF3/S6K1 complex, a step that is a prerequisite for S6 kinase activation.
S6K activation is further regulated by a variety of other events that are not yet fully understood.

1.3 Role and clinical potential of rapamycin

Rapamycin was originally identified as a fungicide isolated from the soil bacterium *Streptomyces hygroscopicus* on Easter Island (Rapa Nui).
It belongs to a group of immunosuppressive agents (together with Cyclosporin A and FK506) that bind to cytoplasmic receptors resulting in a rapamycin/FKBP12 complex.
The rapamycin/FKBP12 complex blocks mTORC1, and possibly also mTORC2 signaling (for review, see Corradetti et al., 2006) and inhibits downstream signaling to the mTORC1 substrates S6K and 4E-BP1.
Inhibition of S6K activation prevents S6 phosphorylation (Jefferies et al., 1997), inhibition of 4E-BP1 phosphorylation reduces cap-dependent mRNA translation (Gingras et al., 1998).
Rapamycin and its analogues CCI-779 (temsirolimus), RAD001 (everolimus) and AP23573 are currently in use or in trials for a wide range of clinical applications (Table 1). They inhibit T cell activation and proliferation and have been used as potent immunosuppressives in renal, liver and cardiac transplantation. A number of pharmaceutical companies are investigating the effect of rapamycin derivatives in treating autoimmune disorders including rheumatoid arthritis and psoriasis (Young and Nickerson-Nutter, 2005).
Furthermore, rapamycin has a pro-apoptotic effect in several types of tumor cells (Chen et al., 2005; Peponi et al., 2005; Teachey et al., 2005).

Function	Mechanism	Application	Literature
Immunosuppression	inhibition of B- and T-cell proliferation	in use: prevention of GvHD after organ transplantation in trials: autoimmune disorders such as psoriasis and rheumatoid arthritis	Young et al., 2005 (review)
Antitumor activity	inhibition of proliferation, pro-apoptotic effects	in trials: renal cell carcinoma, lung cancer, breast cancer, glioblastoma multiforme, sarcomas, malignant melanoma and hematological malignancies	Smolewski et al., 2005 (review)
Prevention of restenosis after PTCA	inhibition of vascular smooth muscle cell growth	in use: coated PTCA stents	Moses et al., 2003 Poon et al., 1996
Tuberous sclerosis	inhibition of the TSC-signaling pathway	cell and mouse models of tuberous sclerosis-related tumors, off-label treatment of astrocytomas in trial: renal angiomyolipomas	El-Hashemite et al., 2004 Franz et al., 2006 Kenerson et al., 2005 Lee et al., 2005
Diabetes	prevention of diabetic nephropathy	reduction of early renal structural changes in rats	Yang et al., 2007

Table 1: Summary of mechanisms, functions and clinical and experimental applications of rapamycin.

The mTOR pathway also plays a key role in the regulation of vascular smooth muscle cell (VSMC) growth. Studies demonstrated rapamycin-induced inhibition of VSMC proliferation via blockage of cell cycle progression at the G_1/S transition (Marx et al., 1995). As a substantial number of patients experience restenosis after implantation of a coronary artery stent, the implanted material was coated with rapamycin to prevent this.

Thus it has been shown in humans that the rate of restenosis 270 days after PTCA was reduced from 21% to 8.6% (Moses et al., 2003). Interestingly, rapamycin also appears to be effective in cell and mouse models of tuberous sclerosis-associated tumors (El-Hashemite et al., 2004; Kenerson et al., 2005; Lee et al., 2005). In addition, off-label treatment of individual patients showed a regression of tuberous sclerosis-related astrocytomas. It is also tested in clinical trials against renal angiomyolipomas (Franz et al., 2006). One study showed a reduction of early structural changes in diabetic nephropathy in rats (Yang et al., 2007).

However, it is often unclear which components of the mTOR signaling pathways account for the therapeutic effects. Thus, the wide range of clinical applications stresses the importance of investigating these pathways in more detail.

1.4 The structure of S6 kinases

S6K1 is a Ser/Thr kinase that is activated upon phosphorylation through different signaling pathways.

Its substrate recognition depends on a block of arginines N-terminal of the phosphorylation site (R-X-R-X-X-S/T). The kinase consists of four domains, an N-terminal acidic domain, a catalytic domain, a linker domain and the C-terminus with the basic pseudosubstrate domain (pseudosubstrate due to the resemblance to the phosphorylation motif in the 40S ribosomal protein 6). S6K1 has two isoforms, the predominantly cytosolic p70 or αII, and the nuclear p85 or αI (Coffer and Woodgett, 1994; Reinhard et al., 1994). These isoforms are identical except for a 23 amino acid extension at the N-terminus of p85 (Fig. 6).

This 23 amino acid extension contains sequence motifs similar to those required for nuclear targeting, which includes an NLS (nuclear localization signal). Immunolocalization assays of the endogenous and microinjected p85 isoform show a predominantly nuclear localization (Reinhard et al., 1994). So far specific targets of the p85 isoform are unknown.

In growth factor-depleted cells, S6 kinase maintains basal activity. The initial step of S6K1 activation is mitogen-induced phosphorylation of the C-terminal phosphorylation sites (S404, S411, S416, T421 and S424).

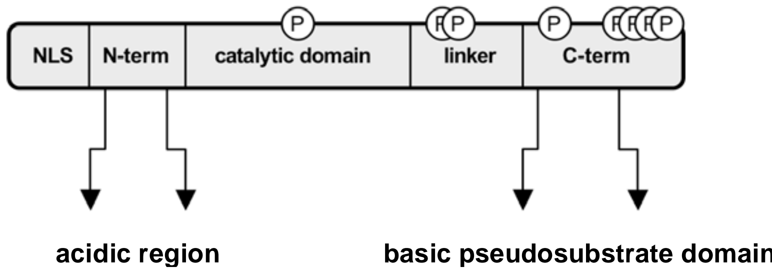


Figure 6: Structure of S6 kinase 1: S6K1 isoforms consist of the N-terminus with an acidic domain, the catalytic domain, the linker domain and the C-terminus with the basic pseudosubstrate domain. One model suggests an interaction between the acidic domain with the basic pseudosubstrate domain to cover the catalytic center when the kinase is inactive. The numerous phosphorylation sites (Thr229 in the catalytic center, S371 and T389 in the linker domain, and S404, S411, S416, Thr421 and S424 in the C-terminus) are marked with circled marks. The nuclear isoform p85 has an additional nuclear localization signal (NLS) at the N-terminus but is otherwise identical to the cytosolic isoform p70.

The MAP kinases ERK and p38 are thought to mediate this step (Weng et al., 1998). Phosphorylation of the C-terminus is a prerequisite for all subsequent events and for full kinase activity.

Mutation of the four C-terminal phosphorylation sites to acidic residues mimics phosphorylation and results in a higher basally active kinase (Cheatham et al., 1995).

Subsequently, phosphorylation of T389 through mTOR occurs which facilitates association and phosphorylation of T229 in the catalytic center through PDK-1.

Full activation is dependent on both PI3K and mTOR pathways, as evidently, inhibitors of PI3K (wortmannin) and mTOR (rapamycin) block kinase activation.

However, the precise mechanism and pathway for S6 kinase activation is still not fully understood (for review, see Martin and Blenis, 2002).

1.4.1 A homologue, S6K2

Apparently, disruption of the *S6K1* gene in mice leads to a marked reduction in animal size due to a reduction in cell size but not cell number. However, S6 phosphorylation levels in these mice were not reduced in response to mitogen stimulation (Shima et al., 1998; Lee-Fruman et al., 1999). This led to the discovery of a 54kD homologue called S6K2. S6K2 shares more than 70% overall homology with S6K1, with the greatest

sequence homology in the kinase and adjacent linker domain (Shima et al., 1998; Koh et al., 1999; Gout et al., 1998; Lee-Fruman et al., 1999).
S6K2 has been shown to phosphorylate S6 *in vitro* and is regulated by the Phosphoinositide 3 (PI3)-Kinase and mTOR pathways *in vivo* (Lee-Fruman et al., 1999). It is similarly sensitive to inhibition by rapamycin and wortmannin. However, S6K2 deficient mice do not display a reduced body mass compared to wild type animals (Pende et al., 2004). Also, S6K2 is nuclear whereas one of the S6K1 isoforms is cytosolic (Koh et al., 1999).
This indicates a difference in function of the two homologues and the presence of other S6K2 substrates that have not yet been identified.

1.5 Substrates of S6 kinases

When inactive, S6K1 is associated with the translation preinitiation complex eIF3 (Holz et al., 2005). In response to nutrients, energy, hormones and mitogenic agents, S6K1 is activated, dissociates from the complex and phosphorylates the ribosomal protein S6 and the translation factor eIF4B (Stewart et al., 1994; Raught et al., 2004). Until recently the ribosomal protein S6 was the best-characterized substrate of both S6K1 and S6K2. S6 phosphorylation seems to be involved in cell size control, but the exact mechanism is yet unknown. Phosphorylated eIF4B contributes to translation by associating with the translation preinitiation complex.
S6K1 activation also leads to a decrease in IRS-1 levels, moreover, S6K1 has been shown to phosphorylate IRS-1 directly on several residues. IRS-1 phosphorylation results in downregulation of insulin-mediated signaling (Haruta et al, 2000, Harrington et al., 2004, Shah et al., 2004). Taken together, these data suggest that chronic S6K1 activation eventually leads to insulin resistance. Consistent with this, S6K1-deficient mice are hypersensitive to insulin possibly due to the lack of the negative feedback mechanism. In contrast to data suggesting otherwise, S6K1 has also been proposed to phosphorylate mTOR directly, possibly as a positive feedback mechanism (Holz et al., 2005).
Within the last years other S6 kinase targets have been proposed, such as elongation factor 2 kinase (eEF2k), BAD, CREM and CBP80, a subunit of the cap binding complex. Phosphorylation of these proteins by S6K resulted in inhibition of eEF2 phosphorylation,

inhibition of the pro-apoptotic function of BAD, an increase in CREM transactivation, and enhanced pre-mRNA splicing (Wang et al., 2001; Harada et al., 2001; de Groot et al., 1994; Wilson et al., 2000) (Fig. 7).

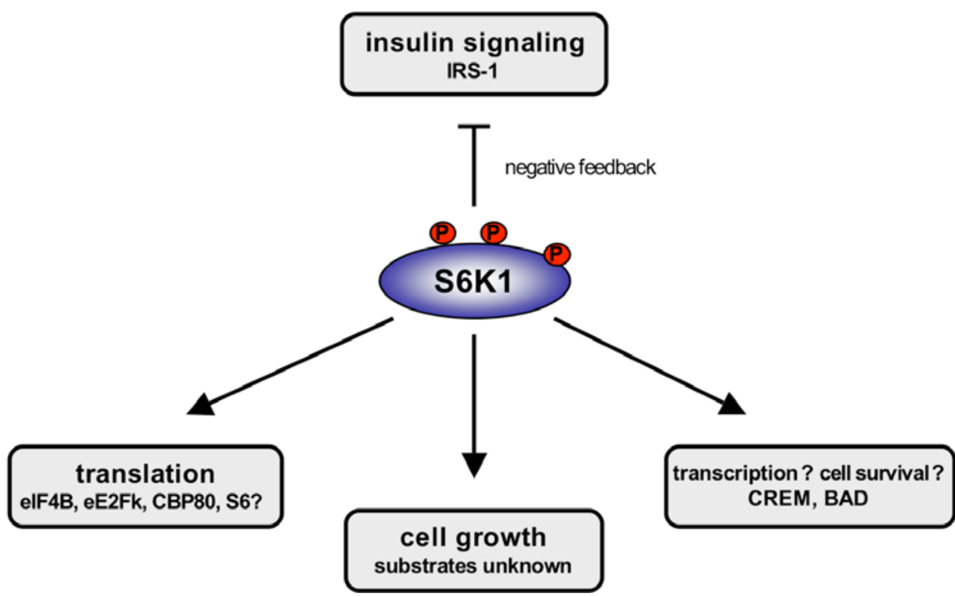


Figure 7: Activation of S6 kinase 1 results in translation and cell growth and possibly also plays a role in transcription and cell survival. Known substrates include the ribosomal protein S6 and the translation initiation factor eIF4B. Other substrates (Cremτ, CBP80, BAD, eEF2k) have been proposed in recent years by various authors, however, none of the substrates has been shown to be specific for S6K1. The mechanism of cell growth regulation through S6K1 is unclear.

However, none of the substrates was specific for either S6 kinase 1 or 2 and most of them had only been studied *in vitro*. A downstream target involved in cell growth control other than possibly S6 had not been identified at that time.

2. Aims

Ongoing clinical work shows that inhibition of the mTOR signaling complex has promising clinical potential in a variety of diseases such as prevention of GvHD (Graft versus host disease after organ transplantation), various tumors, restenosis after PTCA and possibly also tuberous sclerosis. My aim was to further elucidate the downstream signaling components that are influenced by treatment with rapamycin.

I thus decided to employ a genetic method, the Yeast Two-Hybrid Screen, to identify new components of the mTOR signaling pathway by searching for new specific S6 kinase 1 interactors or substrates.

S6K1 has been shown to regulate cellular growth, since overexpression of S6K1 in mammalian cells led to an increase in cell size, an effect that could be blocked by rapamycin.

In addition, S6K1 deficient mice and Drosophila are smaller than wild type animals. However, the reduced cell size of S6K1 deficient mice could not be attributed to a reduction of translation, since S6 phosphorylation levels and 5'TOP mRNA regulation were normal, possibly due to the S6K1 homologue S6K2. None of the known substrates of S6K1 had been shown to have an influence on cell size.

S6K1 exists in two isoforms, the predominantly cytosolic p70 and the nuclear p85. A potential functional difference between the two isoforms had not been described yet.

Thus the aims of my work were the following: (i) to identify a new S6K interactor using a Yeast Two-Hybrid Screen, (ii) to characterize this protein in terms of interaction with S6K and phosphorylation, (iii) to identify its potential specificity for S6K1, and (iv) to evaluate potential differences of the two S6K1 isoforms with regard to this interactor.

3. Materials and methods

3.1 Materials

3.1.1 Chemicals

3-Amino-1,2,4-Triazole (3AT)	Sigma
ATP	Boehringer Mannheim
Bovine serum albumin (BSA)	GibcoBRL
Chemiluminescent	NEN
DAPI	Hoechst
DMEM	GibcoBRL
Fetal bovine serum (FBS)	GibcoBRL
Insulin	Sigma
IPTG	Sigma
Leupeptin	Sigma
Lipofectamine	GibcoBRL
Normal goat serum (NGS)	GibcoBRL
PDGF	GibcoBRL
PMSF	Sigma
Protein A/G sepharose	Pharmacia Biotech
Prestained protein molecular weight standards	Pharmacia Biotech
Rapamycin	S.N. Seghal (Wyeth-Ayerst)
Trypsin	GibcoBRL
Wortmannin	Sigma

3.1.2 Buffers

Molecular biology		
DNA sample buffer (6 x)	0.25% 40% (w/v) in H2O	bromophenol blue / xylene cyanol sucrose
TAE 10x	48.8 g 10.9 g 2.92 g H_2O	Tris-base Glacial acetic acid EDTA added to a final volume of 1l

Bacteria		
LB medium	10 g	bacto-tryptone
	5 g	bacto-yeast extract
	10 g	NaCl
	H_2O	added to a final volume of 1l
	pH 7.0	
TfbI	30 mM	K_2CH_3COO
	100mM	KCl
	10mM	$CaCl_2$
	50 mM	$MnCl_2$
	15%	glycerol
	adjust pH to 5.8 with 0.2 M acetic acid	
	sterilize by filtration	
TfbII	10 mM	MOPS
	75 mM	$CaCl_2$
	10 mM	KCl
	15%	glycerol
	adjust pH to 6.5 with KOH	
	sterilize by filtration	
GST–fusion protein prep		
Elution Buffer	50mM	Tris pH 8.0
	20mM	Glutathione
	100mM	NaCl
	1mM	DTT
	pH 8.0	
Tissue culture		
2x HEPES buffered saline (2xHBS)	50 mM	HEPES
	280 mM	NaCl
	1.5 mM	Na_2HPO_4
	12 mM	dextrose
	10 mM	KCl
	pH 6.95 dissolve in 90 ml ddH_2O, adjust pH to 7.5 with 1 N NaOH and add ddH_2O to 100 ml	
Phosphate buffered saline (PBS)	8 g	NaCl
	0.2 g	KCl
	1.44 g	Na_2HPO_4
	0.24 g	KH_2PO_4
	add H_2O and adjust pH to 7.4 with HCl	
Lysis buffer	10 mM	K_3PO_4
	1 mM	EDTA
	10 mM	$MgCl_2$
	50 mM	β-glycerophosphate
	5 mM	EGTA
	0.5%	Nonidet P-40 [NP-40]
	0.1%	Brij 35
	0.1%	sodium deoxycholate
	1 mM	sodium orthovanadate
	40 mg/ml	phenylmethylsulfonyl fluoride
	10 µg/ml	leupeptin
	5 µg/ml	pepstatin
	pH 7.28	
TE	10 mM	TrisHCl, pH 8
	1 mM	EDTA, pH 8
Protein biochemistry		
SDS-page and immunoblots		
TBST	100 mM	Tris pH 7.5
	9%	NaCl
	1%	Triton X-100

Blocking buffer	1x PBS containing:	
	2%	BSA
	0.2%	Tween 20
	0.05%	sodium azide
Stripping buffer	100 mM	Tris pH 8
	100 mM	ß-mercaptoethanol
	2%	SDS
Protein sample buffer (4x)	500 mM	Tris, pH 6.8
	8%	SDS
	20%	ß-Mercaptoethanol
	33% (v/v)	glycerol
	2.5 ng	Bromophenol blue
Coomassie Brilliant Blue (10x)	0.05% (w/v)	coomassie blue
	50% (v/v)	methanol
	10% (v/v)	acetic acid
	in ddH_2O	
Ponceau Stain	1%	acetic acid
	0.5%	Ponceau stain
Transfer buffer	192 mM	glycine
	25 mM	Tris base
Kinase assay		
Buffer A	10 mM	Tris
	100 mM	NaCl
	1%	Nonidet P-40 [NP-40]
	1 mM	EDTA
	2 mM	Dithiothreitol
	1 mM	sodium orthovanadate
	40 mg/ml	phenylmethylsulfonyl fluoride
	10 µg/ml	leupeptin
	5 µg/ml	pepstatin
	pH 7.2	
	10 mM	Tris
	1 M	NaCl
	0.1%	Nonidet P-40 [NP-40]
	1 mM	EDTA
	2 mM	dithiothreitol
	1 mM	sodium orthovanadate
	40 mg/ml	phenylmethylsulfonyl fluoride
	10 µg/ml	leupeptin
	5 µg/ml	pepstatin
1.5x Kinase buffer	30 mM	HEPES pH 7.2
	15 mM	$MgCl_2$
	150 µg/ml	BSA
Kinase assay reaction buffer	3 µg/ml	protein kinase A inhibitor
	10 µM	ATP
	2 µg	GST-S6 or as indicated
ST	50 mM	Tris-HCl
	5 mM	Tris base
	150 mM	NaCl
	pH 7.28	
STE	ST buffer with 1mM EDTA	
Yeast		
YPD-Plates (1l)	10 g	yeast extract
	20 g	Bacto-Peptone
	17.5 g	Bacto-agar
	950 ml	H_2O
	2%	sterile glucose (add after autoclaving)
YPD-Media	same as plates but without the agar	
Amino acid concentration	40 µg/ml	Adenine

	20 μg/ml 60 μg/ml 40 μg/ml 20 μg/ml	Histidine Leucine Tryptophan Uracil
Elution buffer	50mM 20mM 100mM 1mM pH 8.0	Tris pH 8.0 Glutathione NaCl DTT
STES	0.5M 0.2M 0.01M 1%	NaCl Tris-HCl pH 7.6 EDTA SDS
One step transformation buffer	0.2N 40% 100mM	LiOAc PEG 3350 DTT
PEG/LTE	1x 0.1M 40%	TE LiAc PEG 3350

3.1.3 Plasmids and library

HA-S6K1 wild type (WT), a C-terminal deletion mutant (ΔCT) and an N- and C-terminal deletion mutant (ΔNT/ΔCT) were generated as previously described (Cheatham *et al.,* 1995) and subcloned into the mammalian expression vector pRK7 under the control of the CMV promoter. The (ΔCT) clone has residues Glu401 and Lys402 in the wild type sequence changed to Asp and an amber stop codon, respectively. This results in the expression of a truncated protein lacking the 101 C-terminal residues. The ΔNT/ΔCT-HA clone lacks both the first 30 residues and the C-terminal 101 residues. All S6K2 constructs were cloned into the pcDNA3 mammalian expression vector under the control of the CMV promoter. SKAR was cloned as pCDNA3-Flag-SKAR for coimmunoprecipitation assays. The Two-Hybrid library was a gift from Stan Hollenberg, the Two-Hybrid plasmids and the yeast strain from Philipp James.

3.1.4 Enzymes and oligonucleotides

Restriction enzymes and the T4 DNA ligase were purchased from New England Biolabs and the cloned Pfu polymerase was purchased from Stratagene. The oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

3.1.5 Antibodies

mouse α-HA polyclonal	kindly provided by M. Chou
rabbit α-SKAR polyclonal	raised against the SKAR sequence MKKESELPRRVNSASS (Invitrogen)
rabbit α-GST	Amersham Biosciences
human α-ALY	kindly provided by R. Reed
α-mouse HRP	Boehringer Mannheim
α-rabbit HRP	Life Science
FITC-conjugated α-mouse	Jackson Immunoresearch
Rhodamine-conjugated α-rabbit	Jackson Immunoresearch
FITC-conjugated α-human	Jackson Immunoresearch

3.1.6 Cell lines

Human embryonic kidney cells (HEK293E) were cultured in DME medium containing 10% (v/v) heat inactivated fetal bovine serum, 20 U/ml penicillin and 20 µg/ml streptomycin. NIH3T3 fibroblasts cells were cultured in DME medium containing 10% (v/v) calf serum, 20 U/ml penicillin and 20 µg/ml streptomycin. Cells were kept at 37°C with 5% CO_2. HeLa cells were cultured in DME medium containing 10% (v/v) heat inactivated fetal bovine serum, 20 U/ml penicillin and 20 µg/ml streptomycin.

3.2 Methods

3.2.1 Molecular biology

DNA preparation Plasmids were prepared with the help of Qiagen (Santa Clarita, CA) Mini and Maxi kits according to the supplied instructions, and checked by DNA electrophoresis.

DNA electrophoresis For analytical or preparative gels, agarose was dissolved in TAE buffer (microwave) to a final concentration of 1-2 % (w/v). Ethidium bromide with the

final concentration of 1 µg/µl was added to the solution. The DNA samples were mixed with 6x DNA loading buffer and run together with a molecular weight marker at 60 mA in TAE buffer. The DNA bands were visualized with UV light.

DNA concentration The absorbance of the solubilized double strand DNA was measured at 260 nm. The DNA concentration was measured using the formula: A_{260} x 50 = concentration in µg/ml.

Digestion of DNA Restriction enzyme cutting of DNA for analytical and preparative purpose was performed in 10 µl sterile ddH_2O using 1 µg DNA, 1x restriction enzyme buffer and 0.5 µl restriction enzyme. The digest was checked by DNA electrophoresis.

DNA gel extraction DNA was gel purified using the QiaEx gel purification kit from Qiagen and following the protocol.

Ligation 25 ng or 50 ng insert was mixed with the cut plasmid in a mol-ratio 1:1 or 4:1, 1 unit T4 ligase and 1x T4 ligase reaction buffer and incubated at 16°C over night or for 3-4 hours at room temperature.

Sequencing of DNA was done by Harvard sequencing facility.

3.2.2 Bacteria

Generation of competent bacteria E.coli DH1 cells were streaked out from a frozen stock onto LB-plates with ampicillin and incubated at 37°C over night. Then a single colony was inoculated in 5 ml LB medium containing ampicillin and grown for 3 h at 37°C. The suspension was subcultured into 100 ml prewarmed LB medium with ampicillin and grown until an OD 550 of 0.48. The culture was chilled on ice for 5 min and centrifuged for 5 min at 4°C at 2500 rpm. The pellet was resuspended in 40 ml TfbI (ice-cold) and left on ice for 5 minutes. After an additional centrifugation at 2500 rpm for 5 min at 4°C the cells were resuspended in 4 ml TfbII (ice-cold) and incubated for 15 min. Cells were aliquoted, frozen in aliquots on dry ice and stored at –80°C.

Heat shock transformation 40 µl of competent DH1 E.coli cells was thawed and added to the DNA. The mixture was incubated for 30 minutes on ice and placed for 60 seconds in a 42°C water bath. 800 µl LB was added and the suspension was incubated at 37°C for 1 h. The cells were pelleted for 15 seconds and resuspended in 100 µl LB. The suspension was streaked out on LB plates with ampicillin and incubated over night at 37°C.

GST fusion protein preparation A clone of transformed bacteria was grown in 50 ml of LB media containing ampicillin (100 μg/ml) over night at 37°C. It was then diluted into 1 l of LB media (+ ampicillin) and grown for 3 h at 37°C. IPTG was added to a final concentration of 0.1 mM to induce the protein production. The culture was incubated for 4 h at 37°C. The cells were pelleted at 4500 rpm for 15 min and resuspended in 25 ml of PBS. The cells were pelleted again and resuspended in 40 ml of PBS containing 10 mM EDTA. Protease inhibitors were added immediately before lysis, which was performed with a microfluidizer. Triton X-100 was added to a final concentration of 1%. The lysate was spun down. 750 μl of Glutathione Sepharose HB (per 10 ml lysate) was spun down, washed with PBS and added to the lysate. The mixture was incubated with rotation for 1 h at 4°C. The beads were spun down and washed twice with mammalian cell lysis buffer (including protease inhibitors), twice with Buffer B (+protease inhibitors), once with PBS (+protease inhibitors) and left in 500 μl PBS. Protein was eluted with 1 ml Elution Buffer by rotation and pelleting. This was repeated four times. The supernatant was dialyzed over night at 4°C in 50% glycerol, 20 mM Hepes pH 7.2, 10 mM $MgCl_2$.

3.2.3 Tissue culture

Media HEK293E cells were cultured in DME medium containing 10% (v/v) heat inactivated fetal bovine serum, 20 U/ml penicillin and 20 µg/ml streptomycin. NIH3T3 cells were cultured in DME medium containing 10% (v/v) calf serum, 20 U/ml penicillin and 20 µg/ml streptomycin. Cells were kept at 37°C with 5% CO_2.

Freezing cells Cells were washed 2x in PBS.1 ml Trypsin was added and aspirated after a few seconds. After incubation of the cells for 2 min at 37°C the cells were resuspended in 3 ml medium with 10% serum. The cell suspension was spun down at 5000 g for 3 min. The pellet was resuspended in 2 ml growth medium with 10% DMSO. The cell suspension was transfered to cryovials and kept first for 1 h at 4°C, then transferred for 30 min to -20°C and kept over night at -80°C and stored in liquid nitrogen.

Thawing cells The frozen cells were thawed quickly at 37°C and then diluted into 5 ml growth media. The suspension was spun down at 5000 g for 3 min, the media was aspirated. The pellet was resuspended in 3 ml media and transferred to a 60 mm plate.

Calcium phosphate transfection Cells were seeded at a 30% density per 60 mm-dish about 3.5 h prior to transfection. Plasmids were transfected at a total of 6 µg DNA. Cells were incubated with the calcium phosphate-DNA mixture for 20 h, washed with PBS supplemented with 0.8 mM $CaCl_2$ and 1 mM $MgCl_2$, and then recovered by incubation for 24 h in DME media containing either 10% fetal bovine serum or no serum at all for starvation. *Calcium phosphate-DNA mixture:* The DNA was added to 218 µl water and 31.25 µl of 2 M calcium chloride, the mix was incubated for 5 min at room temperature. 250 µl 2X HBS was added dropwise to the mixture while being vortexed. The mixture was then added dropwise to the cells. Cells were incubated with the precipitate for 20 h at 37°C.

Lipofectamine transfection Cells were transfected with a total of 2 µg of DNA. 1.7x 10^5 NIH3T3 cells were seeded on 35 mm plates 16 h prior transfection. Cells were incubated with the DNA-precipitate for 5 h, washed with PBS supplemented with 0.8 mM $CaCl_2$ and 1 mM $MgCl_2$, and then recovered by incubation for 16 h in DME medium containing 10% fetal bovine serum. Cells were starved for 24 h in DEM medium containing 25 mM HEPES. *Transfection mix*: 6 µl lipofectamine reagent was diluted in 100 µl serum-free medium (OPTI-MEM I). The DNA solution was transferred in a sterile tube and diluted into 200 µl serum-free medium (OPTI-MEM I). The lipofectamine cocktail was combined with the DNA solution, gently mixed and incubated at room temperature for 30 min. 5 min prior transfection the cells were washed with 2 ml serum free medium (OPTI-MEM I).

Cell stimulation and lysis Following a 24 h-starvation period in serum-free media cells were stimulated with fetal bovine serum (final concentration 10%) or 100 nM insulin for 30 min. 20 ng/ml of rapamycin was added to cells 30 min prior to stimulation when indicated. Cells were placed on ice, the medium was aspirated, and cells were washed once with ice-cold PBS supplemented with 0.8 mM $CaCl_2$ and 1 mM $MgCl_2$ and lysed in 300 µl lysis buffer (still on ice). Lysates were cleared of debris by centrifugation at 15,000 g for 10 min at 4°C, 250 µl of supernatant was used for experiments.

3.2.4 Protein biochemistry

Immunoblots Whole-cell lysate (10% of total cell extract) was boiled in 1x protein sample buffer and resolved by polyacrylamide gel electrophoresis (SDS-PAGE).

Proteins were transferred electrophoretically to a nitro-cellulose membrane in transfer buffer (50 V, 1.5 h at 4°C). The membrane was blocked with TBST containing 5% dry milk for 30 min, then incubated with a primary antibody (as indicated in the figure legends) for 1 to 3 h in TBST with 1% milk and then with the secondary HRP-conjugated antibody. Membranes were washed four times with TBST (each incubation 5 min) at room temperature after the incubation with the primary and secondary antibody. All immunoblots were visualized by enhanced chemiluminescence. ECL reagents 1 and 2 were mixed in a ratio 1:1 and the blot was incubated with the solution for 1 min.

Stripping of the immunoblot The membrane was washed three times over 30 min at 50°C in stripping buffer and afterwards in TBST.

Coimmunoprecipitations 250 μl cell lysate (from a 60 mm dish) was incubated with 5 μl anti-HA antibody for 1 h at 4°C. Then 60 μl of Protein A/Protein G sepharose beads (ratio 1:1) were added followed by another incubation for 30 min at 4°C. The mixture was spun down, the supernatant was removed and the beads were washed with lysis buffer containing protease inhibitors. The procedure was repeated three times. The beads were then resuspended in 2x Laemmeli buffer, vortexed and spun down again. Half of the buffer was used for SDS-PAGE and immunoblotting. The blot was incubated with either an anti-SKAR or an anti-GST antibody for 1 h as a primary antibody, washed four times in TBST, incubated with HRP-conjugated Protein A for 30 min and washed four times again. Detection was performed according to the immunoblot protocol.

GST pull-downs GST-fusion proteins were prepared according to the protocol. The protein was not eluted from the beads. The beads were quantified on a gel using BSA as a standard. Transfected or untransfected cells were lysed according to the protocol. 1 μg GST-fusion protein was incubated with 250 μl of cell lysate for 1.5 h at 4°C. The beads were washed three times with cell lysis buffer. The pellet was then resuspended with 25 μl 2x Laemmeli buffer and half of it was used for SDS-PAGE.

***In vitro* kinase assay** Cell lysate supernatant was incubated with an anti HA-antibody for 1 h, followed by another hour of incubation with 50 μl protein A/G sepharose. Immunoprecipitates were washed with 1 ml each of buffers A, B and ST. The pellet was resuspended in 20 μl of 1.5x kinase buffer and 10 μl of reaction buffer. Kinase assays were carried out using 5 μg of a GST-fusion protein of the last 32 amino acids of 40S ribosomal protein S6 or the indicated substrates at 30°C for 10 min. Boiling with 2x

protein loading buffer stopped the reaction. The samples were separated by SDS-PAGE, Coomassie-stained, dried, and autoradiograms were obtained. The level of radioactive isotope incorporation was quantified using a phosphoimager. Protein levels were measured via ECL-signal quantification at the BioRad station.

3.2.5 Immunofluorescence

NIH3T3 or HeLa cells were grown to 60% confluency and then starved in serum free medium for 24 h. Cells were stimulated with FBS and treated with rapamycin if indicated. After washing the cells twice with PBS+ (PBS supplemented with 0.8 mM $CaCl_2$ and 1 mM $MgCl_2$) cells were fixed for 10 min in PBS containing 3.7% paraformaldehyde at room temperature. Cell were rinsed twice with PBS+ and permeabilized for 5 min in PBS+ containing 0.2% TritonX 100. Non specific sites were blocked with 10% normal goat serum (NGS) in PBS for 30 min at room temperature. The primary antibody was diluted in the blocking solution and then incubated on the cover slips for 45 min. After 5 times washing over 10 min with PBS+ containing 0.1% NGS, cells were incubated with the secondary antibody conjugated to FITC or Rhodamine at a dilution of 1:500 and 0.4 µg/ml DAPI in PBS+ with 0.1% NGS. Cells were than rinsed 5 times over 10 min with PBS+ containing 0.1% NGS. The cover slips were washed in ddH_2O and mounted with 10 µl Mowiol Dubco-mounting solution on glass slides.

3.2.6 Yeast

Two-Hybrid screen The bait plasmid was constructed by cloning S6K1 and mutants into pGBDU-C3 containing the GAL4-DNA binding domain and transformed into the PJ69-4A yeast strain (one step transformation). Colonies were selected on selective media lacking uracil. A mouse embryonic library from days 9.5 and 10.5 in the vector pVP16 was transformed (large scale transformation), interactors were selected on plates lacking uracil, leucine and histidine but containing 2 mM 3-aminotriazole. Positives were streaked out again for purification. Colonies were replica-plated for confirmation. From the positive clones the library plasmid was isolated: a 5 ml culture lacking leucine (but containing uracil) was grown over night, a small aliquot was

transferred into fresh media (-leucine, +uracil). This procedure was repeated three or four times. The culture was then spun down, lysed, the plasmid was retransformed into bacteria, isolated and sequenced.

One-step transformation 0.5 ml of a saturated yeast culture was spun down and resuspended in 100 µl transformation buffer by vortexing. 80 µg single stranded denatured carrier DNA (salmon sperm DNA) and 3 µg plasmid DNA were added, the mixture was incubated for 30 min at 45°C. 1 ml TE was added, cells were vortexed, pelleted, resuspended in 100 µl YPD and plated onto selective media.

Large-scale yeast transformation (for library) 400 ml yeast culture was set up in selective media over night. The culture was then diluted 1:10 in 4 l of YPD and allowed to grow up to an OD 600 between 0.3 and 1 (2-3 h). Cells were pelleted at 3000 g for ten minutes, washed with water, resuspended in 80 ml water and pelleted again. The pellet was resuspended in 80 ml TE/LiAc and pelleted. The pellet was resuspended again in 4 ml TE/LiAc. 30 µl library DNA, 4 mg denatured carrier DNA (salmon sperm) and 24 ml PEG/LTE were added and incubated at 30 µC for 30 min. The mixture was then heat shocked at 42°C for 15 min, spun down, resuspended in 20 ml YPD and plated on 150 mm selective plates (200 µl per plate).

Yeast lysis for plasmid recovery Cells from a 5 ml overnight culture were collected. 100 µl STES and acid washed 0.4 mm glass beads were added. The mixture was vortexed for 5 min. 100 µl phenol/chloroform (1:1) was added and vortexed for 5 min. 100 µl STES was added, the beads were spun down and the aqueous phase was recovered. The DNA was precipitated with ethanol, the precipitate was dissolved in 30 µl TE. 2 µl were used to transfer the plasmid into bacteria.

4. Results

4.1 The Yeast-Two-Hybrid Screen

The Yeast-Two-Hybrid System, which takes advantage of the GAL4 protein of *Saccharomyces cerevisiae*, is used to detect protein-protein interactions (Fields et al., 1989). GAL4 is a transcriptional activator that consists of two separable and functionally essential domains: an N-terminal domain which binds to specific DNA sequences (DBD) and a C-terminal domain containing acidic regions, which is necessary to activate transcription (DAD).

The protein of interest functions as a bait fused to the DNA binding domain (DBD) of GAL4. Pieces of a randomly digested cDNA library encoding the so-called prey-protein are fused to the DNA activation domain (DAD). Both plasmids are transformed into a yeast strain that contains a reporter gene. Interaction of the two fusion proteins results in the "co-localization" of DNA binding and DNA activation domain and the subsequent transcription of the reporter gene (Fig. 8).

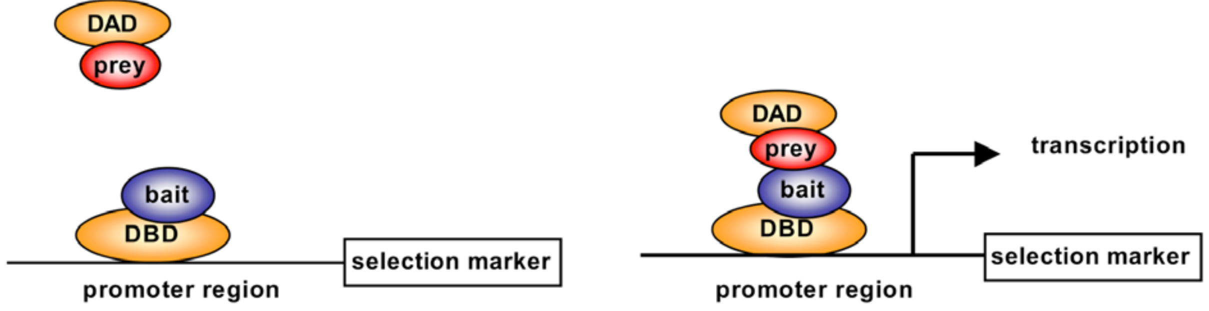


Figure 8: Yeast-Two-Hybrid System. The DNA of the protein of interest (S6K1, “bait”) is fused to the DNA binding domain (DBD) of the GAL4 transcription factor. Pieces of a DNA library (“prey”) are fused to the DNA activation domain (DAD) of the transcription factor. When interaction between the bait and the prey occurs, DNA binding domain and DNA activation domain come together which results in transcription of the selection marker or the reporter gene.

4.1.1 Isolation of a novel protein that interacts with S6K1

As bait I used full length *S6K1*, as prey a cDNA library made from day E9.5 and day E10.5 mouse embryos. 2×10^7 transformants were screened; among other genes of known function I obtained three clones from the same gene. Database research revealed them to be partial clones of a mouse gene with a human ortholog of unknown function on chromosome 22, PDIP46, which has been reported to interact by two-hybrid assay with DNA polymerase δ (Liu et al., 2003; no further functional data reported). The BLAST analysis revealed an RNA recognition motif (RRM) of clone #49 (one of the three clones encoding the same gene) in a region of about 70 amino acids (Fig. 9a). I decided to focus on this putative RNA binding protein since little was known about an effect of growth factor-stimulated kinases on mRNA processing. For my experiments I used the Yeast-Two-Hybrid clone #49 which will be referred to as #49.
In the region that comprised the RRM domain, the novel protein shared 40-50% identity with several RNA binding proteins from the REF (RNA Export Factor) family, such as ALY/Ref1-I (42% identity, 57% similarity, Fig. 9b), Ref1-II, and Ref2-I in mice, the Dip1/Dip2-ALY-like family of proteins in *Arabidopsis*, the RNPS1 and S1 RNA binding proteins in humans, and *Drosophila* RNA binding proteins such as the ALY-homologue BCDNA. There are also six repetitions of a DARX $^{K}/_{L}$ $^{I}/_{L}$ motif in the N-terminus with so far unidentified functional relevance. We hence decided to call the novel protein SKAR for <u>S</u>6<u>K</u>1 <u>A</u>LY/<u>R</u>ef-like target.
Interestingly, BLAST search revealed homologues in rat and mouse with 91% identity and a putative homologue in *Drosophila* with about 50% homology in the RRM motif, but not in lower eukaryotes.

MADISLDELIRKRGAAAKGRLNARPGVGGVRSRVGIQQGLLSQSTRTATFQQRFDARQKIGLSDARLKLG
VKDAREKLLQKDARFRIKGKVQDAREMLNSRKQQTTVPQKPRQVADAREKISLKRSSPAAFINPPIGTVT
PALKLTKTIQ *VPQQKAMAPLHPHPAGMRINVVNNHQAK*QNLYDLDEDDDGIASVPTKQMKFAASGGFLH
HMAGLSSSKLSMSALPLTKVVQNDAYTAPALPSSIRTKALTNMSRTLVNKEEPPKELPAAEPVLSPLEGT
KMTVNNLHPRVTEEDIVELFCVCGALKRARLVHPGVAEVVFVKKDDAITAYKKYNNRCLGQPMKCNLHM
NGNVITSDQPILLRLSDSPSMKKESELPRRVNSASSSNPPAEVDPDTILKALFKSSGASVTTQPTEFKIKL

Figure 9a: Amino acid sequence of the full-length gene encoding the protein we named SKAR (isoform α). The RNA recognition motif (RRM) is highlighted in grey, amino acids absent in isoform β are italicized.

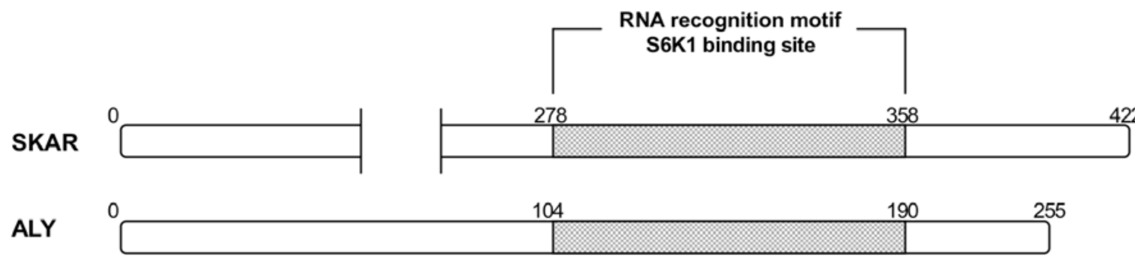


Figure 9b: SKAR and the RNA export factor ALY share 57% similarity in the RNA recognition site, which is approximately the same as the S6K1 binding site (highlighted in grey).

A major difficulty when using the Yeast-Two-Hybrid System is the elimination of false positives that activate reporter genes but not as a result of a specific interaction with the bait. To reconfirm the specificity of the interaction I retransformed #49 with full-length S6K1, two deletion mutants called S6K1ΔNT/ΔCT and S6K1CT, as well as a deletion mutant of the S6K1 homologue S6K2, S6K2ΔNT/ΔCT (Fig. 10a).

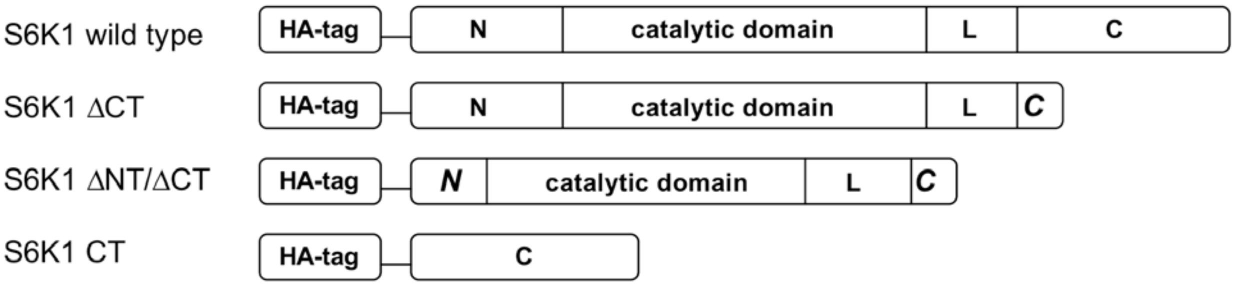


Figure 10a: S6K1 wild type and mutant constructs. Deleted parts are italicized. The mutant S6K1ΔCT (C-terminal deletion) retains kinase activity but is largely resistant to inhibition with rapamycin. The S6K1ΔNT/ΔCT mutant (N- and C-terminal deletions) is largely kinase inactive and rapamycin resistant. The S6K1 mutant consisting only of the C-terminus (S6K1 CT) is not a functionally active enzyme anymore and was used as a negative control.

S6K2 has been cloned by several groups and shares 70% overall identity and 83% identity in the catalytic domain with S6K1.

#49 protein was found to interact with full length S6K1 (Fig. 10b). The interaction was stronger with the S6K1 truncation mutant S6K21ΔNT/ΔCT consisting only of the catalytic and linker domains. Interestingly, #49 did not interact with the homologue

S6K2ΔNT/ΔCT despite its high degree of homology. There was no interaction of #49 with the isolated C-terminus of S6K1 either. None of the vector controls were positive, showing that neither one of the constructs was able to auto-activate transcription of the reporter genes.

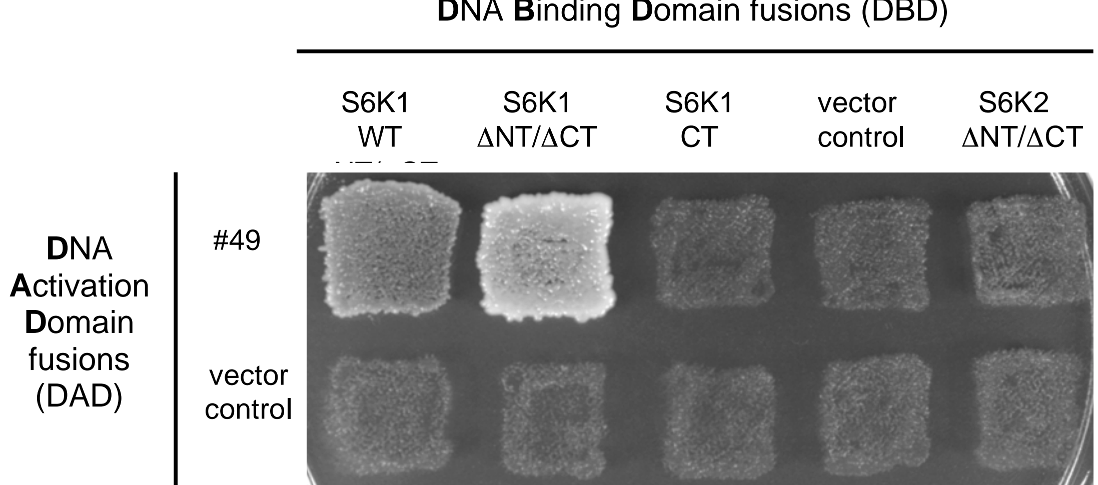


Figure 10b: Interaction in the Yeast-Two-Hybrid system. #49 was co-transformed into yeast with plasmids containing full-length (wild type) S6K1, a deletion mutant lacking N- and C-termini, S6K1ΔNT/ΔCT, a deletion mutant consisting of the C-terminus only, S6K1CT, the homologue S6K2ΔNT/ΔCT, or an empty vector as a control. Wild type S6K2 could not be used due to its ability to activate transcription on its own. The yeast was grown on minimal media lacking uracil, leucine and adenine as selection markers. Growth indicates an interaction of bait and prey proteins as a result of transcription of reporter genes. The figure shows an interaction between #49 and wild type S6K1 and even stronger with the mutant S6K1ΔNT/ΔCT. There is no interaction between #49 and the homologue mutant S6K2ΔNT/ΔCT, showing the specificity of the interaction.

Switching vectors (#49 was cloned into the vector containing the DNA binding domain and S6K1 into the vector containing the DNA activation domain) proved that the interaction works either way (data not shown).

4.2 Localization of SKAR

4.2.1 SKAR is a nuclear protein

SKAR mRNA is expressed in all tissues examined, with the highest levels being found in brain, heart, skeletal muscle and kidney (data not shown). We performed immunofluorescence assays to see where full length SKAR localizes *in vivo* and whether the location may change under various stimuli. Figure 11a shows that endogenous SKAR is localized in the nucleus (DAPI staining of chromatin as a control). It is excluded from the nucleoli. In mitotic cells it has a cytoplasmatic localization. This fits the observation that SKAR shares homology with several RNA binding proteins that are localized in the nucleus as well.

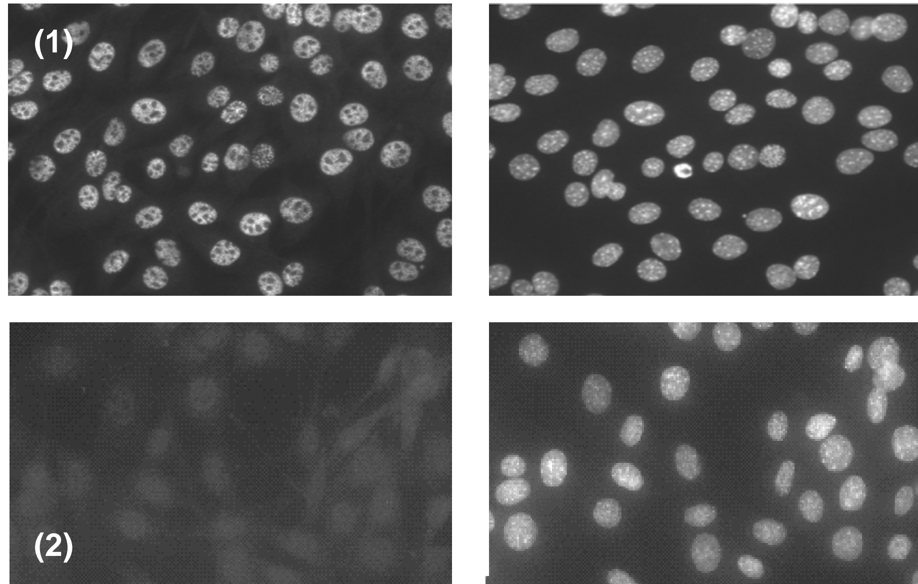
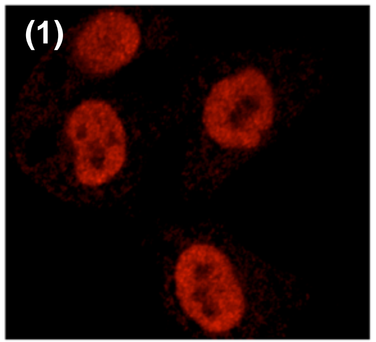
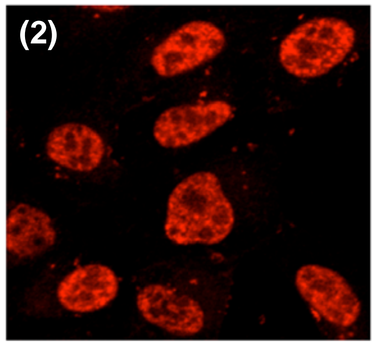


Figure 11a: Growing NIH3T3 fibroblasts were incubated with a polyclonal rabbit αSKAR antibody as a primary and a rhodamine-conjugated αrabbit antibody as a secondary antibody according to immunofluorescence protocols. As a control the SKAR antibody was blocked with SKAR peptides before the assay. (1) Endogenous SKAR is localized in the nucleus. It is excluded from the nucleoli. To the right DAPI staining as a control. (2) The SKAR antibody is specific. Cells were incubated with a SKAR peptide previous to immunostaining with the SKAR antibody.

The αSKAR staining appears to be specific as the binding could be blocked by preincubation of the antibody with the SKAR fragment it was raised against. Localization does not change upon serum starvation, stimulation with insulin or treatment with rapamycin or wortmannin. There is also no noticeable change of localization after stress such as heat or osmotic shock (data not shown). Transfected full-length SKAR is also localized in the nucleus, whereas the transfected Two-Hybrid piece (myc-49) seems to be ubiquitous, possibly due to its smaller size or the absence of a nuclear localization signal (Fig. 11b). The distribution of SKAR is the same in mouse fibroblast NIH3T3, human kidney U2OS and human HeLa cells (data not shown).





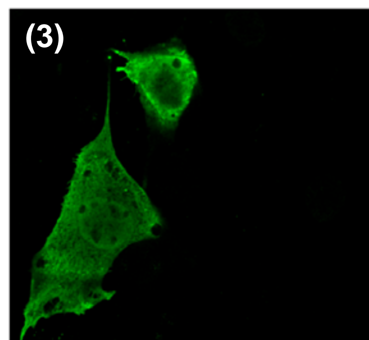


Figure 11b: Full-length endogenous (1) and transfected (2) SKAR is nuclear whereas the shorter Yeast-Two-Hybrid piece (clone #49) is not (3). Growing HeLa cells were (1) incubated with a polyclonal rabbit αSKAR as a primary and rhodamine-conjugated αrabbit as a secondary antibody to show endogenous SKAR staining. (2) HeLa cells were transfected with full length flag-tagged SKAR and incubated with polyclonal rabbit αSKAR as a primary and rhodamine-conjugated αrabbit as a secondary antibody. (3) HeLa cells were transfected with the GST-tagged Two-Hybrid clone #49 and incubated with a polyclonal rabbit αGST as a primary and FITC-conjugated αrabbit as a secondary antibody.

4.2.2 SKAR co-localizes with ALY

ALY (Ally of LEF-1 and AML-1) was identified as an activator of the T-cell receptor alpha (TCRα) enhancer complex (Bruhn et al., 1997). Studies indicate that splicing of pre-messenger RNA and export of mRNA are linked by direct interactions between ALY and the DEAD-box helicase UAP56 (Luo et al., 2001). ALY is localized in the so-called "speckles" in HeLa cells. Speckles are nuclear domains that contain components of the splicing machinery and polyA^{+} RNA (Lamond and Spector, 2003), but also other nuclear proteins involved in RNA metabolism and transcription (known as the exon junction complex) (Mintz et al., 1999; Herrmann and Mancini, 2001). A marker for those

speckles is the spliceosomal protein SC-35 (Spector et al., 1991). ALY co-localizes with SC-35 in the speckles.

Since SKAR seemed to localize in the speckles in previous assays as well and since both proteins share considerable homology in the RNA binding domain, we performed a co-localization assay of endogenous SKAR and ALY as well as SKAR and SC-35 in HeLa cells.

Fig. 12 shows a co-localization of SKAR and ALY in growing HeLa cells. Both proteins are localized in the so-called speckles. We confirmed the localization with a co-localization experiment of SKAR and the splicing factor SC-35, which is used as a marker for the speckles. We also assayed for co-localization of SKAR and endogenous S6K1 as well as several transfected truncation mutants under various conditions. However, we were unable to detect any co-localization above background (data not shown).

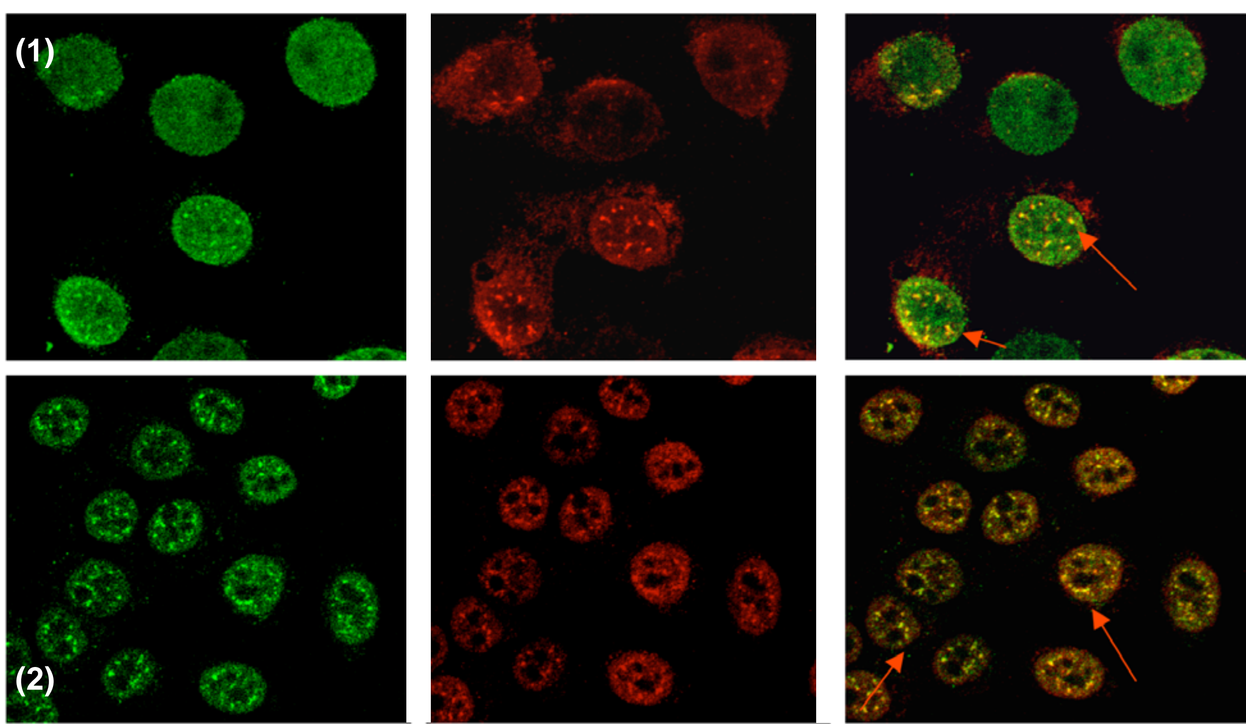


Figure 12: SKAR co-localizes with ALY and SC-35 in the speckles. (1) SKAR co-localizes with ALY in the speckles. Growing Hela cells were incubated with rabbit αSKAR and a FITC-conjugated secondary antibody (left upper side) and a human αALY antibody (Wichmann et al., 1999) and a rhodamine-conjugated secondary antibody (upper middle). Both proteins seem to enrich in little dots. The overlay shows a co-localization (yellow marks) exclusively in the little dots. (2) αSKAR antibody with a FITC-conjugated secondary antibody (left lower side) and a human αSC-35 antibody and a rhodamine-conjugated secondary antibody (lower middle). SKAR co-localizes with SC-35 in the nuclear speckles.

4.3 Interaction of SKAR with S6 kinases

4.3.1 *In vitro* interaction

To verify the result of the Yeast-Two-Hybrid screen the interaction between clone #49 and S6K1 was tested in a GST-pull down assay and analyzed by Western blotting.

Fig. 13 shows that #49 interacts specifically with the hyperphosphorylated (=activated) form of both p70-S6K1 (predominantly cytosolic) and p85-S6K1 (predominantly nuclear) that is achieved upon stimulation with insulin or growth factors. Since the hyperphosphorylated form is equivalent to the active, phosphorylated kinase the experiment implies that binding to GST-49 requires either kinase activity, a phosphorylated kinase or simply access to the binding site in the catalytic center of p70-S6K1 and p85-S6K1.

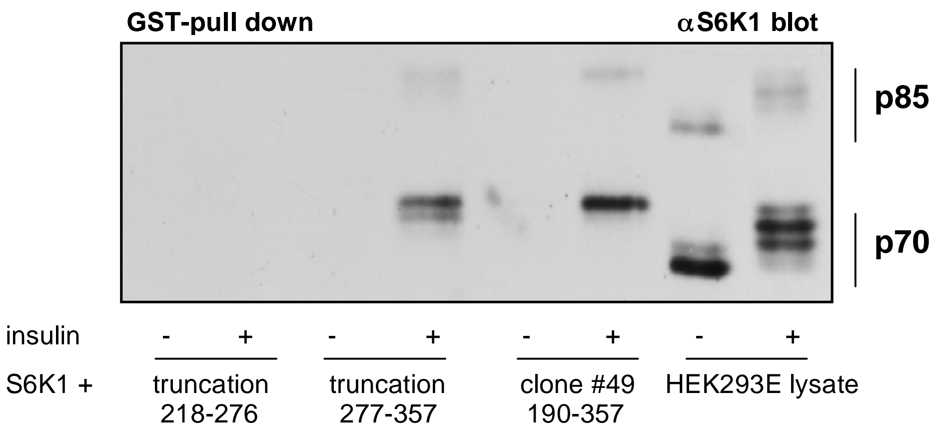


Figure 13: Interaction of #49 with S6K1 isoforms. Various truncation mutants of recombinant GST-#49 fusion proteins were isolated from bacteria and bound to glutathione sepharose beads. The beads were incubated with cellular lysate from HEK293E (human embryonic kidney) cells that had been either starved in serum-free media for 24h or starved and stimulated with insulin, conditions that inhibit or activate S6 kinases. The lysate lanes to the right (positive controls) show the upper hyperphosphorylated/active and the lower basally phosphorylated/inactive S6K1 isoforms p70 and p85. The Yeast-Two-Hybrid piece (clone #49, amino acids 191-357) and the C-terminal truncation mutant containing amino acids 277-357, but not the N-terminal mutant containing amino acids 218-276, interact with both S6K1 isoforms (p70 and p85) *in vitro* upon insulin stimulation. The interaction occurs between the hyperphosphorylated form of S6K1 and the RNA binding domain of SKAR (amino acids 277-357).

The interaction domain with S6K1 seems to be located within or close to the putative RNA binding domain of our novel protein (amino acids 277–357), since the N-terminal piece of clone #49 does not bind the kinase.

However, further deletions of the RNA binding domain abolished binding altogether (data not shown); an intact RNA binding domain thus seems to be necessary for binding of SKAR to S6K1.

4.3.2 *In vivo* interaction

To analyze the *in vivo* interaction co-immunoprecipitation assays were performed after co-transfection of GST-tagged #49 and HA-tagged S6K1 in HEK293E cells. HEK293E cells were used because transfection efficiency and protein expression in these cells are very high. Fig. 14a shows an interaction of GST-#49 with S6K1 wild type that is stimulated by insulin and inhibited by rapamycin.

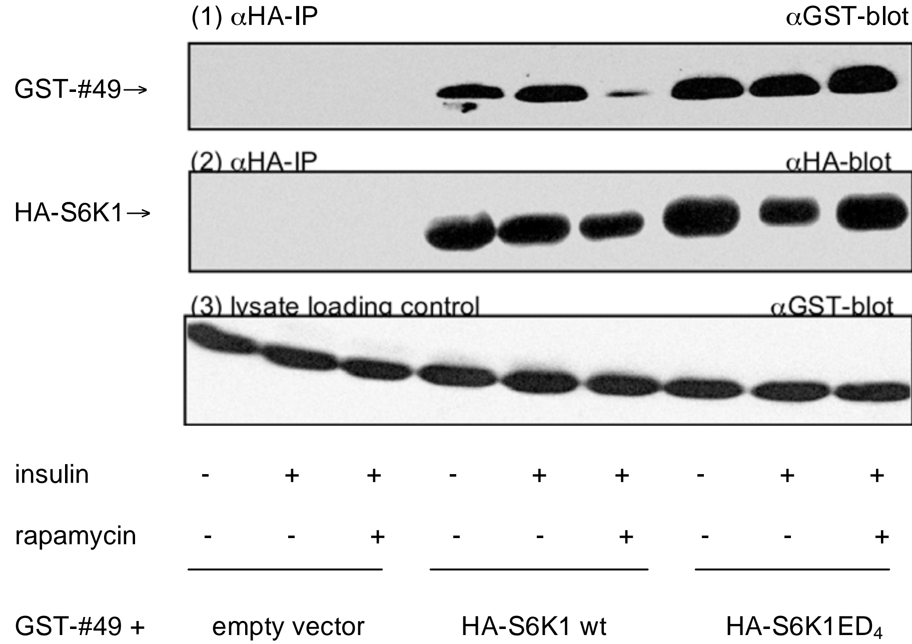


Figure 14a: Clone #49 interacts with S6K1 in an insulin- and rapamycin-dependent manner. HEK293E cells were co-transfected with GST-#49 and either an empty vector as a negative control, HA-tagged wild type S6K1 or a rapamycin resistant, constitutively active mutant of S6K1 (HA-S6K1ED$_4$). Immunoprecipitation was performed using a monoclonal mouse αHA antibody according to the protocol. Immunoprecipitates were run on SDS-PAGE gel and blotted onto a nitrocellulose membrane. The Western blot was stained with a polyclonal rabbit αGST antibody to visualize the interaction of GST-#49 with HA-S6K1 (1). The interaction between wild type S6K1 and #49 is stimulated by insulin and inhibited by rapamycin. The interaction between #49 and the constitutively active mutant S6K1ED$_4$ is stronger and independent of treatment with insulin or rapamycin compared to the wild type kinase. There is no unspecific interaction between #49 and other proteins (vector lane, negative control). (2) shows the levels of immunoprecipitated kinases (immunoprecipitates blotted with an αHA antibody, (3) shows and the levels of GST-#49 in the cellular lysate before immunoprecipitation.

The interaction is stronger with the constitutively active kinase mutant S6K1ED$_4$. S6K1ED$_4$ contains four mutated sites (three serines/ threonines at the C-terminus and one serine in the linker domain) to mimic a phosphorylated (=active) state of the kinase. According to the stronger interaction with the mutant S6K1ΔNT/ΔCT in the Yeast-Two-Hybrid screen I expected a stronger interaction *in vivo* as well. Since S6K1ΔNT/ΔCT is resistant to rapamycin treatment the interaction with GST-#49 does not change upon treatment. I included the homologue S6K2 and the deletion mutant S6K2ΔCT in the co-immunoprecipitation assay, a kinase that shares about 70% overall homology with S6K1 and also phosphorylates S6 *in vitro.* Neither S6K2 nor S6K2ΔCT interacted with GST-#49 in nutrient-starved or in insulin-stimulated cells, which shows the specificity of the interaction (Fig. 14b).

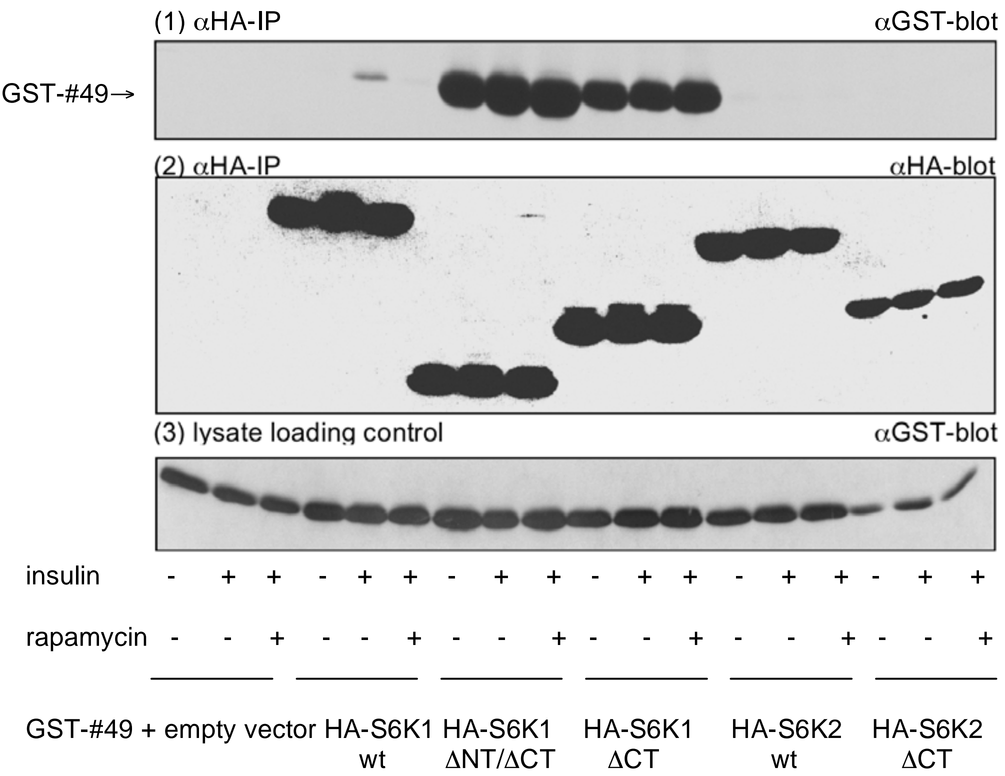


Figure 14b: #49 interacts specifically with S6K1, but not with the homologue S6K2. HEK293E cells were cotransfected with GST-#49 and HA-tagged wild type and mutant S6K1 and S6K2. Immunoprecipitation was perfomed using a mouse monoclonal αHA antibody and Western blot using a rabbit polyclonal αGST antibody. (1) GST-#49 co-immunoprecipitates with activated S6K1 wild type (thin band) and truncation mutants S6K1ΔNT/ ΔCT and S6K1ΔCT, but not with the homologue S6K2 and its mutant S6K2ΔCT. The interaction of GST-#49 with the S6K1 deletion mutants is noticeably stronger than with wild type kinase and insensitive to treatment with insulin or rapamycin. (2) loading control of immunoprecipitated kinases. (3) lysate control of transfected GST-#49.

Also other kinases like wild type RSK (Ribosomal S6 kinase) or the transcription factor binding protein 4e-BP1 did not bind GST-#49 (data not shown).

To assess whether phosphorylation of S6K1 or access to the catalytic center of the enzyme are necessary for the interaction I blotted transfected HEK293E cell lysates with phosphospecific antibodies (Fig. 14c). Upon rapamycin treatment, S6K1ΔNT/ΔCT was not dephosphorylated, whereas S6K1ΔCT did undergo dephosphorylation.

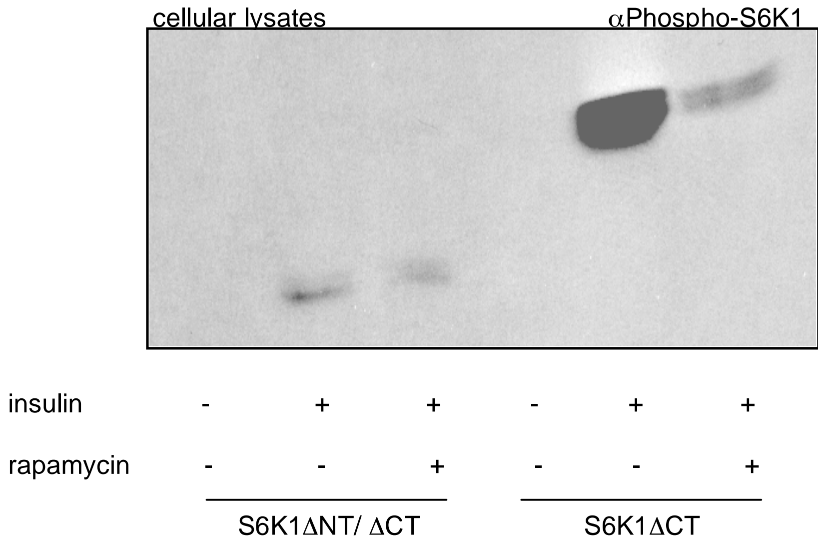


Figure 14c: Phosphorylation of S6K1 mutants. Cellular lysates of HEK293E cells transfected with S6K1 deletion mutants S6K1ΔNT/ΔCT or S6K1ΔCT were run on SDS-PAGE, blotted and probed with an αPhospho-S6K1 (phosphospecific) antibody that recognizes the phosphorylated kinase. The deletion mutant S6K1ΔCT is phosphorylated upon stimulation with insulin and de-phosphorylated upon treatment with rapamycin (lower signal with phosphospecific antibody), whereas the S6K1ΔNT/ΔCT is phosphorylated upon insulin stimulation but insensitive to rapamycin (signal of phosphospecific antibody remains unchanged). This figure shows that the strong interaction of #49 with the S6K1 deletion mutants does not depend on the phosphorylation status of the kinases.

The binding to both mutants seemed to be similar and did not change upon serum stimulation or treatment with rapamycin. Hence the binding of GST-#49 to S6K1 seems to require access to the binding site more than phosphorylation of the enzyme.

I tried to analyze binding of the endogenous protein SKAR and endogenous and transfected wild type S6K1 in co-immunoprecipitation assays as well. However, I found

that unlike the SKAR fragment #49 endogenous SKAR does not form a detectable interaction with S6K1 (data not shown).

This could be due to a different localization of the two proteins *in vivo*: For our assays we used the mostly cytosolic S6K1 isoform p70. The Two-Hybrid fragment #49 is ubiquitous. Full length SKAR might therefore simply not be available to bind to p70-S6K1 *in vivo*, since it is a nuclear protein.

To test this alternative I set up a transfection experiment with the nuclear isoform of p70-S6K1, p85-S6K1, that has an additional 23 amino acids at its N-terminus (including a nuclear localization signal) but is otherwise identical (Fig. 15). An interaction with SKAR was present that increased slightly upon insulin stimulation and that was inhibited by rapamycin. This result suggests that the nuclear p85-S6K1 isoform rather than the cytosolic p70-S6K1 might be the *in vivo* interactor with SKAR.

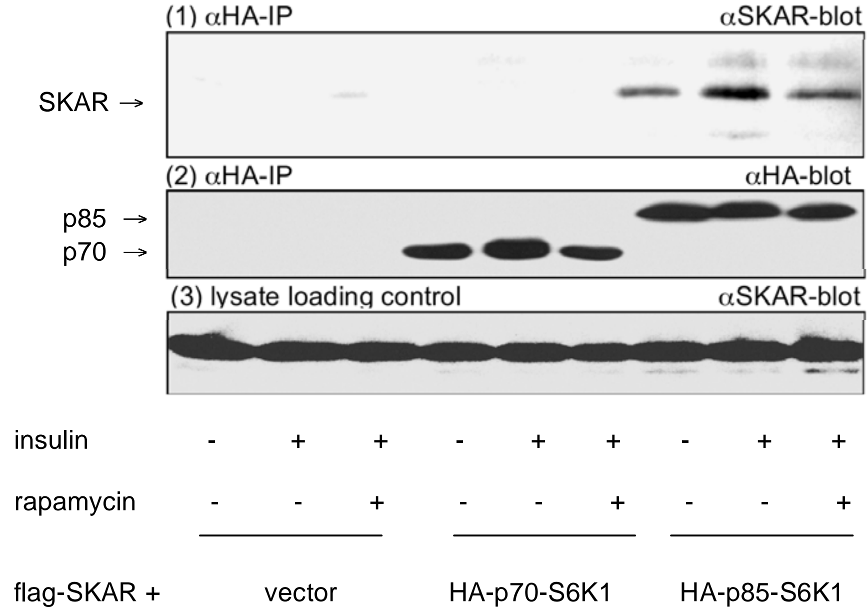


Figure 15: Full length SKAR interacts with the nuclear isoform of S6K1, p85. HEK293E cells were cotransfected with flag-tagged SKAR and the HA-tagged S6K1 isoforms p70 (predominantly cytosolic) and p85 (predominantly nuclear). Immunoprecipitation was performed using a polyclonal rabbit αHA antibody, (1) Western blot using a polyclonal rabbit αSKAR antibody. We observed an interaction between the nuclear isoform of S6K1, p85, and wild type SKAR. The interaction increased slightly upon stimulation with insulin and decreased after treatment with rapamycin. In contrast to the interaction of the truncated proteins there is no detectable interaction between full length SKAR and the cytosolic S6K1 isoform p70. (2) Loading controls of the immunoprecipitated kinases. (3) loading control of the cellular lysate before immunoprecipitation.

However, in the assays I did not consistently detect an interaction. This might be due to other influences present in the cells.
The interaction of full length SKAR with the S6K1ΔNT/ΔCT and S6K1ΔCT deletion mutants was much stronger than with wild type proteins (data not shown).

4.4 SKAR is a substrate of S6 kinase 1

I also determined whether SKAR is a substrate of S6K1, since SKAR contains the S6K1 phosphorylation motif (R-X-X-S) in the C-terminus between amino acids 380-383.
Fig. 16a shows that a SKAR fragment (amino acids 191-421) is phosphorylated upon insulin stimulation. The phosphorylation is inhibited by treatment with rapamycin. This indicates that SKAR is an *in vitro* substrate of S6K1, as no phosphorylation was observed in samples lacking S6K1 or samples with S6K2 as a control (data not shown).

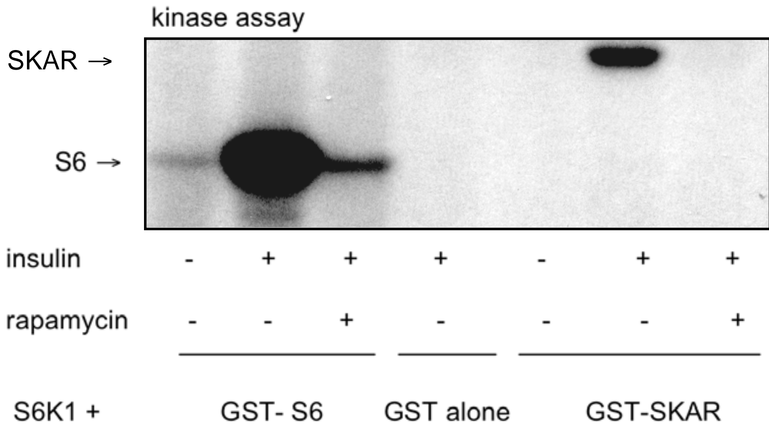


Figure 16a: *In vitro* kinase assay. SKAR is phosphorylated by S6K1 upon insulin stimulation. Bacterially expressed GST-tagged SKAR (amino acids 191-421) was purified, incubated with ^{32}P labeled ATP and immunoprecipitated S6K1 from transfected HEK293E cells treated with either insulin alone or insulin and rapamycin. Bacterially expressed GST-protein was used as a negative, GST-S6 protein as a positive control for phosphorylation by S6K1. Phosphorylation is seen by incorporation of the γ-phosphate from ^{32}P labeled ATP. SKAR is phosphorylated in an insulin- and rapamycin-dependent matter (right lanes). GST alone is not phosphorylated in the presence of S6K1 whereas S6 shows strong incorporation of the radioactive phosphate.

To delineate the phosphorylation site several SKAR mutants as well as wild type SKAR were tested in a kinase assay (Fig. 16b). Full-length SKAR was phosphorylated upon

insulin stimulation as well as the truncation mutants consisting of amino acids 191-421 and 277-421, which all contain the putative C-terminal phosphorylation site.
To our surprise a mutant consisting of the C-terminus of SKAR (amino acids 358-421) did not show any phosphorylation above the background signal. As the S6K1 binding site (between amino acids 191-357) is abrogated in this mutant it suggests the need for the kinase to bind in order to be able to phosphorylate its substrate.

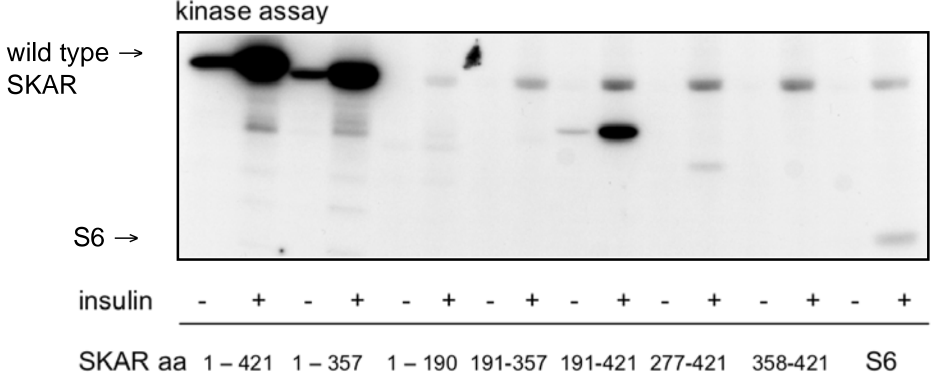


Figure 16b: *In vitro* kinase assay with S6K1 and various SKAR truncation mutants. Bacterially expressed GST-tagged SKAR mutants were incubated with ^{32}P labelled ATP and immunoprecipitated S6K1 from cells that had either been treated or not treated with insulin previous to immunoprecipitation. The SKAR fragments were subsequently run on SDS-PAGE. A putative phosphorylation site lies in the C-terminus between amino acids 380 and 383 in SKAR. Wild type SKAR as well as SKAR fragments containing the amino acids 1-357, 191-421 and 277-421 are phosphorylated *in vitro* upon insulin stimulation (to the right S6 as a positive control). The phosphorylation of fragment 1-357 suggests another N-terminal phosphorylation site. The C-terminal fragment 358-421 was not phosphorylated despite the presence of the predicted phosphorylation site.

Such docking sites have been proposed for other kinases like ERK (extracellular signal-regulated kinase) as well, but not yet for S6K1 substrate phosphorylation. The phosphorylation of the C-terminal deletion mutant 1-357 suggests an alternative phosphorylation site despite the absence of the S6K1 phosphorylation motif R-X-X-S (Fig. 16c).

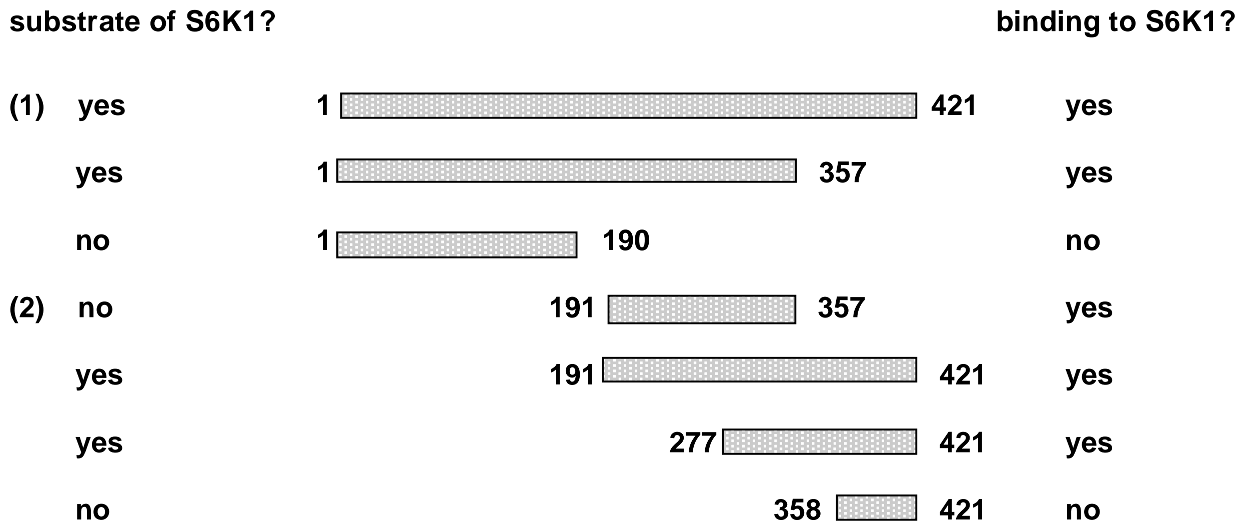


Figure 16c: The *in vitro* kinase assays suggest a docking site mechanism where phosphorylation requires a binding site. The S6K1 binding domain of SKAR without the C-terminal phosphorylation site (Yeast-Two-Hybrid clone #49, amino acids 191-357) or the C-terminal phosphorylation site without the binding domain (amino acids 358-421) are not phosphorylated. (1) Full-length SKAR, (2) Two-Hybrid clone #49.

5. Discussion

I can conclude that I isolated a novel substrate specific for S6K1. Due to the sequence homology with the mRNA export factor ALY/Ref-1 the protein was named **SKAR** (for S6 K1 ALY/Ref-like target). *SKAR* mRNA is ubiquitously expressed with the highest levels in brain, heart, skeletal muscle and kidney.

I isolated SKAR as an interactor with the mTOR- and PI3K-effector S6 kinase 1 in a genetic system, the Yeast-Two-Hybrid screen, and confirmed the interaction with both S6K1 isoforms *in vitro* and potentially also with the nuclear isoform p85 *in vivo*. SKAR did not bind to the highly homologous S6K2 or other kinases.

The interaction domains include an RNA recognition motif (RRM) in SKAR and the catalytic/linker domain of S6K1. Deletion of the N- and C-termini of S6K1 enhances the binding to SKAR significantly as compared to wild type protein. This indicates that SKAR recognizes a domain of activated S6K1 that is exposed upon phosphorylation (or truncation) of S6K1.

The low levels of *in vivo* interaction between wild type S6K1 and SKAR despite phosphorylation suggest a kinase/substrate interaction that might be too transient to be detected in a co-immunoprecipitation assay. At the same time my experiments show that protein interaction (or the interaction site) is necessary for phosphorylation to occur. In other kinases this mechanism is called a docking-site mechanism, when substrate phosphorylation requires a binding site to the substrate. Docking sites have so far been described for members of the MAP kinase family such as p38, JNK (c-Jun N-terminal kinase) and ERK (extracellular signal-regulated kinase) (Jacobs et al., 1999; Tanoue et al., 2000), but not for S6K1. These kinases target their substrates either directly (for example ERK→ RSK) or via a scaffolding protein (phosphorylation of p27 through the Cyclin D/Cdk-complex).

Consistent with this model SKAR might be targeted by S6K1 via a docking site and then released after phosphorylation. It is supported by the fact that a SKAR fragment containing the phosphorylation but lacking the S6K1 binding site is not phosphorylated. Likewise mutants (such as clone #49) that lack the phosphorylation site show significantly stronger binding to S6K1. A possible explanation is a reduced release rate in the absence of phosphorylation.

This model is further supported by the fact that the S6K1 deletion mutants that exhibit strong binding capacity to SKAR have lower kinase activity as compared to wild-type ones. Also neither kinase activity nor kinase phosphorylation are necessary for S6K1 binding to SKAR, which is confirmed by the stimulation- and rapamycin-insensitive interaction of SKAR and the deletion mutant S6K1ΔCT.

The difficulty to detect wild-type protein interaction supports a mechanism of S6K1 targeting the substrate via a docking site and releasing it directly after phosphorylation. The S6K1 docking site might increase substrate specificity, as well as phosphorylation efficiency. It may also influence the localization of the kinase: The inactive kinase is sequestered by the substrate, and after stimulation phosphorylation occurs and the kinase is released.

As the interaction with S6K1 occurs in the predicted RNA binding domain of SKAR, binding of RNA might facilitate or inhibit binding of the kinase. However, I have not been able to test this hypothesis yet.

If SKAR is only briefly sequestered by S6K1 upon stimulation and released directly after phosphorylation, it would explain the fact that I was not able to find co-localization between SKAR and S6K1 in our immunofluorescence assays. However, RNA binding and S6K1 signaling to SKAR might also be two independent functions.

Furthermore, I was able to show *in vitro* phosphorylation of wild-type SKAR by S6K1 and hence identify SKAR as a new substrate for S6K1. SKAR is the first substrate specific for S6K1 but not for S6K2. I proposed a phosphorylation site in the C-terminus of SKAR that is phosphorylated by S6K1 *in vitro* in an insulin-dependent manner (amino acid S383 of SKAR lies within an S6K1 recognition motif). As mentioned above, binding to the kinase is necessary for phosphorylation. Phosphorylation can be inhibited by the immunosuppressant rapamycin.

However, there seem to be one or more other phosphorylation sites as the C-terminal deletion mutant 1-357 was also phosphorylated *in vitro* despite the absence of the S6K1 recognition motif, R-X-X-S.

Further experiments done by our group showed that SKAR is phosphorylated *in vitro* specifically by S6K1, but not by S6K2, on either residue S383 or S385. These phosphorylation sites are regulated by insulin and rapamycin *in vivo* (Richardson et al., 2004).

However, the question of the function of SKAR and the significance of the phosphorylation of SKAR by S6K1 still remained unclear. Before starting this project several other S6K substrates had been proposed by other groups. However, none of them had been shown to be specific for S6K1.

Inhibition of S6K activation by inhibition of mTOR or PI3K-signaling with the drugs rapamycin or LY294002 (wortmannin) results in a decrease in cell size in mammalian tissue culture models. In contrast, overexpression of S6K1 results in an increase in cell size suggesting a function of S6K1 in the regulation of cell size (Fingar *et al.,* 2002). Until recently the effect of S6K1 on cell size was thought to be mediated via phosphorylation of the ribosomal protein S6 and hence translational control. This model has come under discussion because the level of S6 phosphorylation remains unchanged in S6K1 knockout mice probably due to S6K2 activity (Shima et al., 1998), even though the animals have a reduced body size.

Continuing with this project our group was able to show that SKAR plays a role in cell size control mediated by S6K1. Reducing SKAR or S6K1, but not S6K2 protein levels using RNAi, led to a reduction of cell size (Richardson et al., 2004).

SKAR contains a putative RRM (RNA recognition motif) RNA binding domain within amino acids 278–356. The RRM motif and a domain in the N-terminus are the regions of greatest homology between SKAR and ALY (Ally of LEF-1/AML-1). In contrast to ALY, which is highly conserved between species (including *S. cerevisiae*, *C. elegans* and mammals), homologues of SKAR exist in mammals, but not in other higher eukaryotes (with a possible exception of *Drosophila*) or prokaryotes.

Immunofluorescence experiments show that SKAR is a nuclear protein that is excluded from the nucleoli, where rRNA and pre-tRNA processing occur. It co-localizes with splicing and mRNA export factors in splicing factor compartments (most pre-mRNA splicing factors are enriched in domains termed speckles or splicing factor compartments/SFCs). Although S6 kinases have been proposed to control translation through S6 phosphorylation, the nuclear localization and homology of SKAR with ALY suggest a function in mRNA processing rather than translation. The nuclear localization of SKAR is consistent with the fact that wild type protein interaction could only be detected between SKAR and the nuclear S6K1 isoform, p85.

Most metazoan pre-mRNAs contain several introns and exons. Whereas the exons are comparatively short, the introns often contain between hundreds and thousands of

nucleotides. Splicing of the pre-mRNAs removes the introns in the nucleus, and the mature mRNAs are then transported to the cytoplasm where translation occurs. As a consequence of this mechanism, most of the pre-mRNA sequence is retained in the nucleus as excised introns and degraded, while only a relatively small amount of processed mature mRNA is destined for export to the cytoplasm. However, also spliced mRNA is not necessarily translated: A part of it is selectively degraded in a process called nonsense-mediated decay (NMD). NMD eliminates mRNA transcripts containing premature termination codons and thus plays a role in regulating protein synthesis on mRNA level. This process has not only physiological, but also pathological implications for example in β-thalassemia, when β-globin protein is not expressed due to NMD (for review, see Holbrook et al., 2004).

Upon splicing a multi-protein complex called the exon-junction complex (EJC) is deposited onto the exon-exon boundaries. Some of the EJC proteins remain bound to mRNA during translocation through the nuclear membrane. They are thought to recruit NMD factors to the ribosome. The EJC therefore links splicing, nuclear mRNA localization, nuclear export, NMD and translation (Tange et al., 2004).

ALY binds RNA and modulates the formation of RNA-protein and RNA-RNA complexes. It is part of the exon-junction complex and shuttles between nucleus and cytoplasm to increase the rate and efficiency of mRNA export (Reed et al., 2001; Straesser et al., 2000; Zhou et al., 2002).

In our assays the nuclear localization of SKAR did not change visibly upon stimulation with growth factors or insulin or inhibition of mitogen-activated signaling pathways. This makes a function of SKAR that involves shuttling between nucleus and cytoplasm improbable. However, shuttling might be too transient as well to be detected by immunofluorescence. More detailed experiments will be required to investigate shuttling.

ALY and SKAR are more than 50% homologous in a 30 amino acid stretch in the N-terminus as well as in a 60 amino acid-stretch, the RRM (RNA recognition) RNA binding motif, which contains also the S6K1 binding site in SKAR.

RRM motifs are highly conserved and found in eukaryotes as well as in prokaryotes and viruses. They are present in a variety of proteins that function in pre-mRNA processing, splicing, alternative splicing, mRNA stability, mRNA export, translation regulation and

degradation. The motif also appears in a few single stranded DNA binding proteins (for review, see Maris et al., 2005).

The N-terminal region of homology between SKAR and ALY contains no known functional domain. One could speculate that it might contain a nuclear localization or a targeting signal, since only the full-length protein SKAR and not the SKAR fragments such as the Two-Hybrid clone #49 are localized in the nucleus. Deletion experiments might elucidate this in the future.

I could show co-localization of ALY and SKAR in the splicing factor compartments of HeLa cells, the so-called speckles. So far I have not been able to test an effect of SKAR on the function of ALY or on mRNA export. Neither did SKAR bind RNA in later assays so far. However, the homology and the co-localization with ALY suggest a role of SKAR in mRNA processing. Whether a function in mRNA processing can be linked to S6K1 signaling and the effect on cell size remains to be determined in the future, although one could speculate that control of mRNA processing might also influence cell size: if SKAR for example would bind to specific mRNAs or be part of a protein complex depending on its phosphorylation status it could function as a positive regulator by enhancing mRNA processing and lead to an increase in cell size. Alternatively unphosphorylated SKAR would function as a negative regulator and inhibit the processing of mRNAs required for cellular growth. These effects could either take place during splicing or further mRNA processing that is mediated by the exon-junction complex. Since ALY is part of the exon-junction complex the latter seems more likely (Fig. 17).

Lately other groups investigated SKAR function: One group showed an increase in the amount of SKAR protein in an animal model of chronic paraplegia-induced muscle atrophy despite a reduction of S6K1 activity. They proposed SKAR to function as an adaptive response to prime the muscle for regeneration (Dreyer et al., 2007). However, neither SKAR phosphorylation levels nor inhibition of SKAR phosphorylation were examined so the meaning of elevated SKAR levels remained unclear. Another group identified SKAR as an *in vitro* interactor with ER (enhancer of rudimentary), a highly conserved protein implicated in control of pyrimidine metabolism and transcriptional control (Smyk et al., 2006).

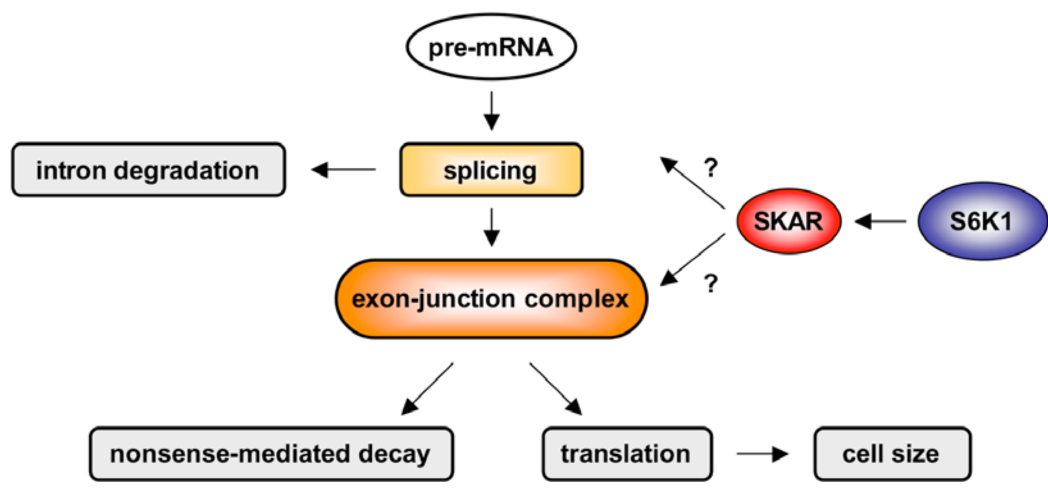


Figure 17: Hypothetical model of SKAR signaling: SKAR might function either in the spliceosome where pre-mRNA is spliced and the introns are removed, or in the large protein complex called the exon junction complex, which links splicing, nonsense-mediated decay and mRNA export to the cytoplasm. Phosphorylation through S6K1 could have a positive or negative impact on RNA processing and hereby influence cell size.

S6 kinases are downstream targets of several signaling pathways that are frequently dysregulated in cancer, but also in other diseases such as tuberous sclerosis. Inhibitors of these pathways such as the immunosuppressant rapamycin are currently in use for a variety of clinical applications. Therefore it is crucial to identify novel components of the signaling pathways in order to develop new therapeutic strategies. One study showed an increase of cell size and S6 kinase activation in a mouse model of TSC1-deficient astrocytomas (Uhlmann et al., 2004). SKAR is the first S6K1 substrate shown to be involved in regulation of cell size. Also, constitutive activation of S6K1 in murine hematopoietic cells resulted in an increase in cell size and malignant transformation, an effect that was overcome by treatment with rapamycin (Valentinis et al., 2000).

Hence one might speculate that the mTOR-S6K1-SKAR pathway might play at least a partial role in tumor formation and growth. However, regulation of SKAR in tumor cells or effects of rapamycin on SKAR activity in tumor cells have not been investigated yet, but since SKAR is a target of rapamycin action and since I was able to show an effect of rapamycin on SKAR regulation this work contributed to the elucidation of the mechanism of rapamycin action in a cell.

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Appendix

Abbreviations

4E-BP1	4E-binding protein 1
5'TOP	5' terminal oligo pyrimidine
Akt	PKB, protein kinase B
ALY	Ally of Lef-1/AML-1
BAD	BCL2-antagonist of cell death
BLAST	Basic local alignment search tool
CBP80	Cap-binding protein 80
c-Myc	Myelocytomatosis oncogene
CREB	cAMP responsive element binding protein
Cremτ	cAMP responsive element modulator
eEF2K	Eukaryotic elongation factor-2 kinase
EGF	Epidermal growth factor
EJC	Exon-junction complex
eIF3	Eukaryotic translation initiation factor 3
eIF4B	Eukaryotic translation initiation factor 4B
ERK	Extracellular regulated kinase
EST	Expressed sequence tag
FKBP12	FK506 binding protein 12
FLAIR	Fluid attenuated inversion recovery
GβL	G protein beta subunit-like
GSK	Glycogen synthase kinase
GST	Glutathione-S-transferase
GvHD	Graft versus Host Disease
IL-2	Interleukin-2
MAPK	mitogen-activated protein kinase
mLST8	Mammalian leukocyte specific transcript 8
MRI	Magnetic resonance imaging

mSin1	Mammalian stress-activated protein kinase-interacting protein 1
mTOR	Mammalian target of rapamycin
mTORC1/2	mTOR complex
NLS	Nuclear localization signal
NMD	Nonsense-mediated decay
PDGF	Platelet derived growth factor
PDK1	Phosphoinositide-dependent protein kinase 1
PI3K	Phosphatidyl-inositol-3-kinase
PRAS40	Proline-rich protein kinase B substrate 40 kD
PTCA	Percutaneous transluminal coronary angioplasty
PTEN	Phosphatase and tensin homolog
Raptor	Regulatory associated protein of TOR
Rheb	Ras homologue enriched in brain
Rictor	Rapamycin insensitive companion of TOR
RNP	Ribonucleoprotein/particle
RRM	RNA recognition motif
RSK	Ribosomal S6 kinase
S6K1/2	S6 kinase 1/2
SKAR	S6K1 ALY/Ref-like target
TSC1/2	Tuberous sclerosis complex 1/2
VSMC	Vascular smooth muscle cell

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Publications

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