Identification of the novel Akt2 interacting proteins RPS25 and eIF3c: characterization of interaction and physiological consequences

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1 Introduction

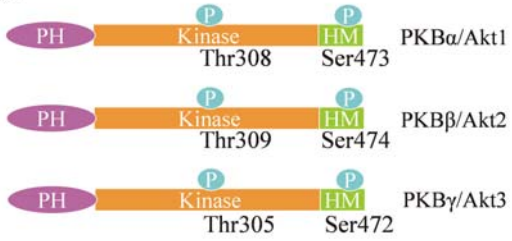
Akt is a serine/threonine protein kinase which belongs to protein kinase AGC family. This group of kinases shares structural homologies within their catalytic domain and has similar activation mechanisms. Deregulation of the kinase activity is frequently associated with human diseases including cancer and diabetes. Akt was initially identified by three independent groups based on its homology to protein kinase A (PKA) (Coffer and Woodgett, 1991), protein kinase C (PKC) (Jones et al., 1991) or as the cellular homologue of the retroviral oncogene viral Akt (Bellacosa et al., 1991). Akt is highly conserved from *C. elegans* to human, but different in yeast (Hanada et al., 2004). In mammalian cells, three closely related isoforms have been identified, named Akt1, Akt2 and Akt3 (also known as PKBα, PKBβ and PKBγ). They are encoded by three distinct genes that locate on human chromosomes 14q32, 19q13 and 1q44, respectively. Akt2 and Akt3 show 81 % and 83 % amino acid identity with Akt1. Although the Akt isoforms are ubiquitously expressed in mammalian cells, the relative isoform expression level differs among tissues. Akt1 appears to be predominantly expressed in most tissues, while Akt2 is enriched in insulin responsive tissues such as fat cells, liver and skeletal muscle (Chan et al., 1999). Akt3 is mainly expressed in brain and testis (Tschopp et al., 2005).

1.1 The structure of Akt

All three members of Akt contain a common conserved domain structure that consists of an amino-terminal pleckstrin homology (PH) domain, a central catalytic kinase domain with a regulatory threonine residue and a short carboxyl-terminal regulatory domain which contains a hydrophobic motif (HM) and a second regulatory serine residue (Fig. 1.1). The PH domain is about 80 % identical among the three isoforms and about 30 % identical to the domain in pleckstrin and other proteins. Biochemical analysis reveals that the PH domain of Akt binds to both phosphatidylinositol-3,4,5-triphosphate PI(3,4,5)P3 and phosphatidylinositol-3,4-bisphosphate PI(3,4)P2 with a similar affinity (Frech et al., 1997). The crystal structure of the PH domain of Akt bound with the inositol head group of PI(3,4,5)P3 indicates that the D5 phosphate is not physically included (Thomas et al., 2002), which has important implications regarding the regulation of the kinase activity. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) catalyzes dephosphorylation of PI(3,4,5)P3 and PI(3,4)P2 at the D3 position, thus reducing the entire pool of lipids capable of binding to Akt and negatively regulating the activation of Akt. The membrane recruitment of Akt induces a large

conformational change that enables its activation by upstream kinases (Milburn et al., 2003). In addition, the PH domain also mediates the interaction with proteins that regulate Akt kinase activity (Brazil et al., 2002). However, the PH domain is absent in the *S. cerevisiae* orthologue Sch9 (Fabrizio et al., 2001).

A.



B.

Pair	PH domain	Kinase domain	C-terminal domain
Akt1/Akt2	80%	90%	66%
Akt1/Akt3	84%	88%	76%
Akt2/Akt3	76%	87%	70%

Figure 1.1 Domain structures of Akt isoforms (A) and amino acid identity among the isoforms (B). Two phosphorylation sites critical for Akt kinase activity are shown.

The catalytic kinase domain is about 90 % identical among the Akt isoforms and is closely related to PKC, PKA and ribosomal protein S6 kinase (S6K) (Peterson and Schreiber, 1999). It contains a conserved threonine residue (Thr308 in Akt1, Thr309 in Akt2 and Thr305 in Akt3) whose phosphorylation can partially activate the enzyme. The unphosphorylated Akt is virtually inactive and the phosphorylation of Thr308 stimulates its activity by at least 100-fold (Stokoe et al., 1997). The carboxyl-terminal regulatory domain of Akt is about 70 % identical among isoforms and is closely related to PKC family proteins (Kumar and Madison, 2005). It contains a proline rich region and a hydrophobic motif with an identical conserved sequence FPQFSY in all mammalian Akt isoforms. The phosphorylation of serine in this conserved motif is very important because a deletion of the HM motif completely abolishes enzymatic activity (Andjelkovic et al., 1997). This poses the question of degree of activation of double phosphorylated Akt. Phosphorylation of Thr308 results in the partial activation of Akt while for the maximal kinase activity the phosphorylation of Ser473 is required.

The crystal structure of the PKBβ kinase domain explains how the phosphorylation of two regulatory residues contributes to kinase activation. In the inactive state, most of αC helix is disordered in the kinase domain and the activation loop adopts a conformation that sterically hinders the binding of both ATP and peptide substrates. The residues within the linker region

between the amino-terminal and carboxyl-terminal lobes of Akt contribute to the inactive conformation by partially occupying the ATP binding site (Huang et al., 2003). However, in the activated kinase domain of Akt which is in a complex with GSK3β-peptide substrate, the phosphorylation of Ser474 promotes the engagement of the hydrophobic motif with the amino-terminal lobe of the kinase domain and induces the disorder-to-order transition of the αC helix resulting in stabilization of the kinase domain. The activation loop then also becomes ordered due to interactions with the αC helix (Yang et al., 2002).

1.2 The regulation of Akt

Akt activation is induced by the lipid products of the phosphoinositide 3-kinase (PI3K) which phosphorylates the 3'-OH position of the inositol phospholipids. The generation of PI(3,4,5)P3 on the inner plasma membrane recruits Akt by direct interaction with its PH domain followed by phosphorylation on the two regulatory residues (Fig. 1.2).

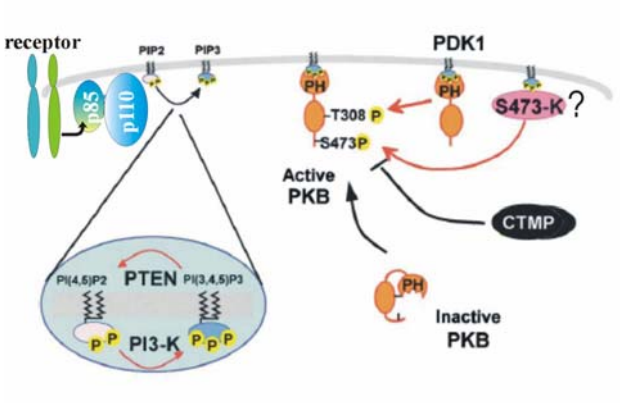


Figure 1.2 Mechanism of PKB activation. Activation of phosphoinositide 3-kinase (PI3K, p85/p110) leads to the generation of PI(3,4,5)P3 which recruits inactive PKB to the plasma membrane via PH domain binding. Residue Thr308 in the catalytic domain is then phosphorylated by PDK1 and Ser473 phosphorylated by an as yet unidentified Ser473 kinase. Activation of PKB is inhibited by the phosphatase PTEN and Akt interacting protein such as CTMP. Modified from (Brazil et al., 2002).

1.2.1 PI3K dependent activation of Akt

PI3K is a cytosolic enzyme consisting of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit that contains two Src homology 2 (SH2) domains. Activation of receptor induced recruitment of PI3K to the plasma membrane allows it to phosphorylate inositol phospholipids on the D3 position of the inositol ring (Fig. 1.3). This process is directly opposed by the phosphatase PTEN, while the SH2 domain-containing inositol phosphatase (SHIP) dephosphorylates PI(3,4,5)P3 at the D5 position to yield PI(3,4)P2. Both, PI(3,4,5)P3

and PI(3,4)P2, bind to the PH domain of Akt with a similar affinity and recruit the kinase to the plasma membrane.

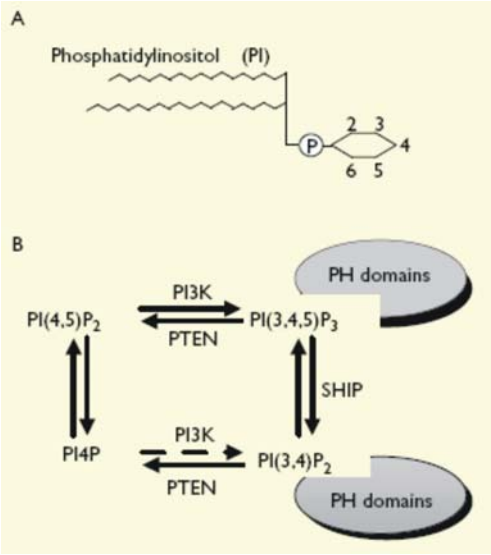


Figure 1.3 PI3K derived lipids. (A) The structure of phosphatidylinositol (PI). The carbon numbers of the inositol ring are shown. (B) The class IA PI3K generates D3 lipids by phosphorylation of the D3 position of the inositol ring. Both PI(3,4,5)P3 and PI(3,4)P2 directly bind to the PH domain and recruit PH domain containing proteins to the plasma membrane. The PTEN phosphatase directly opposes the action of PI3K by dephosphorylation of PI(3,4,5)P3 at the D3 position, while SHIP dephosphorylates PI(3,4,5)P3 at the D5 position (see below).

The upstream kinase responsible for Thr308 phosphorylation is a 67 kDa ubiquitously expressed 3'-phosphoinositide-dependent protein kinase 1 (PDK1) that contains an amino-terminal catalytic domain and a carboxyl-terminal PH domain which binds to PI(3,4,5)P3 with a high affinity. Co-localization of Akt and PDK1 at the cell membrane is necessary for the phosphorylation of Thr308. The kinase responsible for the phosphorylation of the secondary regulatory residue has not yet been determined so far. PDK1 knock-out embryonic stem cells keep the Ser473 phosphorylation that is similar to wild-type cells, whereas Thr308 phosphorylation is completely aborted (Williams et al., 2000). The finding that the level of Ser473 phosphorylation is increased by overexpression of PDK1 in transfected cells (Hill et al., 2001) indicates that PDK1 may contribute indirectly to the process of Ser473 phosphorylation. It is also reported that Akt itself autophosphorylates Ser473 under certain conditions (Toker and Newton, 2000; Laine et al., 2000). Other findings suggest that Ser473 is phosphorylated by distinct kinases such as intergrin-linked kinase 1 (ILK1) (Delcommenne et al., 1998), mitogen activated protein kinase activated protein kinase 2 (MAPKAPK-2) in neutrophils (Rane et al., 2001), protein kinase C βII (PKCβII) (Kawakami et al., 2004), DNA-dependent protein kinase (DNA-PK) (Feng et al., 2004), ataxia telangiectasia mutant (ATM) (Viniegra et al., 2005) and the rapamycin-insensitive mTOR-Rictor complex 2 (mTORC2) (Sarbassov et al., 2005). Thus, different kinases may be responsible for the phosphorylation of Ser473 in different tissues under distinct physiological conditions.

1.2.2 Akt activation in lymphocytes

T-cell activation is induced by ligation of the antigen receptor complex (TCR/CD3) as well as co-receptors such as CD28. The engagement of T-cell antigen receptor (TCR) by antigens leads to the activation of protein tyrosine kinases (PTKs) which phosphorylate the CD3ζ chain within the immunoreceptor tyrosine-based activation motifs (ITAMs). Phosphorylation of ITAMs provides docking sites for SH2-domain containing proteins which will then be activated (Osman et al., 1996). PI3K is activated by TCR via two routes, either directly by binding the p85 regulatory subunit to ITAMs or indirectly through the activation of Ras (de, I et al., 1997; Samstag and Nebl, 2005).

CD28 costimulation signals are needed for optimal cytokine production, proliferation and effector functions. CD28 directly interacts with the SH2 domain of the p85 regulatory subunit through its cytoplasmic YMNM motif (Prasad et al., 1994). Additional proline residues of CD28 mediate its binding to the Src homology 3 (SH3) domains of p56Lck and Grb2 (Holdorf et al., 1999; Kim et al., 1998) that both contribute to the activation of PI3K. The production of PI(3,4,5)P3 by PI3K leads to the membrane recruitment and activation of Akt (Cantley, 2002). Similarly, B-cell antigen receptor (BCR) engagement causes a rapid increase in the production of PI(3,4,5)P3 (Gold and Aebersold, 1994). Mice with a disrupted p85α regulatory subunit gene have a reduced number of mature B-cells and no T-cell independent antibody production (Suzuki et al., 1999). BCR-induced Akt activation requires the protein tyrosine kinase Syk and is partially dependent on Bruton's tyrosine kinase (Btk) (Craxton et al., 1999). Using an isoform-specific antibody, it has been shown that BCR ligation alters the electrophoretic mobility of Akt2 (Gold et al., 2000).

1.2.3 Negative feedback control of Akt

One way for terminating Akt activity is the removal of the activating PI(3,4,5)P3 and PI(3,4)P2 lipids. PTEN is a tumour suppressor gene that is mutated or deleted in a variety of different human tumours. Cells lacking the PTEN phosphatase have elevated levels of PI(3,4)P2 and PI(3,4,5)P3 and constitutive activation of Akt. Many T leukaemic cell lines lack the PTEN phosphatase and consequently have high basal levels of Akt activity. In B lymphocytes, the SHIP phosphatase inhibits Akt activation by binding to the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of the FcγRIIB receptor (Liu et al., 1998b). Expression of SHIP causes a reduced phosphorylation of Akt on Thr308/Ser473 residues indicating that SHIP can negatively regulate the PI3K/Akt signal pathway (Horn et al., 2004; Freeburn et al., 2002).

In addition, Akt is directly regulated through dephosphorylation of Ser473 and/or Thr308 residues by protein phosphatases PP2A and PHLPPα (PH-domain leucine-rich repeat protein phosphatase α) (Andjelkovic et al., 1996; Gao et al., 2005). Several Akt-binding proteins, such as CTMP (Maira et al., 2001), also modulate the kinase activity in a negative manner.

1.3 Akt substrates and physiological functions

The multitude of Akt functions is due to the variety of its physiological substrates (Fig. 1.4). The preferred sequence for Akt phosphorylation is RXRXXS/T where X is any amino acid and S/T is the serine or threonine residue. Database searches indicate that many proteins with this conserved sequence are expressed in lymphocytes.

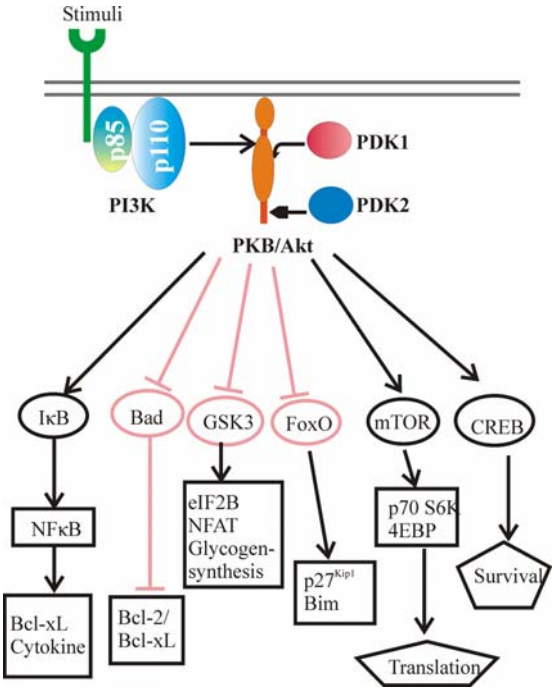


Figure 1.4 Physiological targets of Akt. Akt is recruited to the plasma membrane followed by phosphorylation on two regulatory residues. Activated Akt then phosphorylates its various targets regulating apoptosis, metabolism and protein translation.

1.3.1 Regulation of cell metabolism

Glucose cannot diffuse through biological membranes and must pass through facilitative transporters at the cell surface. In skeletal muscle and adipose tissue, insulin-stimulated glucose uptake is dependent on the translocation of insulin-responsive glucose transporter 4 (GLUT4) from intracellular storage compartments to the plasma membrane (Kanzaki, 2006). Constitutively active Akt have been shown to stimulate the transcription and the translocation of GLUT4 to the plasma membrane (Rathmell et al., 2003b; Kohn et al., 1996). In Akt2-

deficient cells, insulin-induced glucose uptake and GLUT4 translocation are reduced and neither endogenous nor overexpressed Akt1 protein can compensate for the loss of Akt2 (Bae et al., 2003). Indeed, Akt2, but not other isoforms, is directly associated with GLUT4-containing protein complexes (Calera et al., 1998). Synip, an Akt2-specific substrate involved in the regulation of GLUT4 translocation, has been identified recently (Yamada et al., 2005). Once inside cells, glucose is rapidly phosphorylated by hexokinase to form a glucose-6-phosphate, which then enters glycolysis. Akt is able to promote glycolysis through direct phosphorylation and activation of phospho-fructo kinase 2 (Deprez et al., 1997).

Stimulation of the resting T-cells with anti-CD3 and anti-CD28 antibodies leads to similar change in glucose utilization as treatment of insulin-responsive tissues with insulin. The major glucose transporter in lymphocytes is GLUT1, and its gene transcription is increased by Akt activation (Fu et al., 2004; Barthel et al., 1999). Moreover, in response to CD3/CD28 stimulation, T-cells increase the expression of GLUT1, glucose uptake and glycolysis in a PI3K dependent manner, while expression of constitutively active Akt can bypass the requirement for this stimulation (Frauwirth et al., 2002). The first identified physiological substrate of Akt was Ser9 of GSK3β and Ser21 of GSK3α (Cross et al., 1995; van Weeren et al., 1998). GSK3 was identified initially as a regulator of glycogen metabolism (Burgering and Coffer, 1995). The phosphorylation of GSK3 by Akt results in its inactivation thereby regulating glucose utilization and glycogen synthesis. Inhibition of GSK3β facilitates the export from the nucleus where it acts as a coactivator of transcription factors (Jho et al., 1999). A recent report suggests that GSK3 promotes the monocyte-derived dendritic cell (DC) differentiation and inhibits the spontaneous maturation of immature DC (Rodionova et al., 2006).

1.3.2 Regulation of cell survival and apoptosis

Overexpression of constitutively activated Akt promotes cell proliferation and inhibits apoptosis by downregulation of pro-apoptotic proteins, upregulation of anti-apoptotic proteins and change of the activity of various transcription factors.

1.3.2.1 Regulation of apoptosis

In the current understanding, an early event in apoptosis is the loss of mitochondrial integrity followed by cytochrome c release. The released cytochrome c then activates the cystine protease caspase 9 which initiates a caspase cascade (Thornberry, 1998). Human pro-caspase 9 is phosphorylated by Akt at Ser196, thus reducing its protease activity (Cardone et al., 1998). Critical regulators in the apoptotic pathway are various Bcl-2 family members

including the pro-apoptotic protein Bad (Bcl-2 antagonist of cell death protein). In the absence of survival factors, Bad is bound to Bcl-2/Bcl-xL complex and prevents their anti-apoptotic potentials (Downward, 1999). Akt phosphorylates Bad at Ser136, and the phosphorylated Bad is sequestrated in the cytoplasm in a complex with 14-3-3 proteins thereby preventing it from interacting with Bcl-2/Bcl-xL at the mitochondrial membrane (Datta et al., 1997). The final effect is the inhibition of apoptosis. Moreover, treatment of the acute myeloid leukaemia (AML) blast cells with the PI3K inhibitor LY294002 reduces Ser136 phosphorylation of Bad and induces apoptosis, indicating that the PI3K/Akt pathway plays an important role to prevent apoptosis of AML (Zhao et al., 2004).

Stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK) is an important mediator of apoptosis when the cell is exposed to various stimuli such as stress, cytokines and chemotherapeutic drug. The SAPK system consists of two groups of kinases, JNK and p38, which belong to the mitogen-activated kinase (MAPK) family (Johnson and Lapadat, 2002). Akt suppresses the SAPK signal pathway through phosphorylation of upstream kinases ASK1 (apoptosis signal-regulating kinase 1) at Ser83 (Kim et al., 2001), MLK3 (mixed lineage kinase 3) at Ser674 (Barthwal et al., 2003) and SEK1/MKKK4 at Ser78 (Park et al., 2002). In addition, the activated Akt1 associates with JNK-interacting protein 1 (JIP1) and prevents the assembly of JNK signaling cassettes (Kim et al., 2002a). Similarly, Akt2 interacts with another JNK scaffold protein, POSH (plenty of Src homology 3 domains), and this interaction prevents the formation of the POSH-MLK-MKK-JNK signaling complex (Figueroa et al., 2003). These observations suggest that Akt isoforms have distinct abilities to mediate pro-survival responses in cells where JNK activation triggers apoptosis.

In addition, Akt phosphorylates anti-apoptotic protein PED/PEA-15 (phosphoprotein enriched in diabetes/astrocytes-15) at Ser116 in a PI3K dependent manner and prevents its degradation (Trencia et al., 2003). PED/PEA-15 is a cytosolic protein. It inhibits the formation of death-inducing signaling complex (DISC) and the activation of caspase 3 which is downstream of the death-domain containing factors, including Fas and tumour necrosis factor (TNF) receptor family members. Moreover, phosphorylation of Ser116 facilitates its phosphorylation by PKC at Ser104 and only the double phosphorylated form can be recruited to the DISC to inhibit apoptotic signaling (Xiao et al., 2002).

1.3.2.2 Transcriptional control of cell survival

Recent studies have shown that Akt also stimulates cell survival through regulation of transcription factors that are responsible for the expression of pro- as well as anti-apoptotic genes. Akt either negatively regulates transcription factors that promote pro-apoptotic gene

expression or positively regulates factors that induce expression of survival genes. Several of newly identified Akt substrates such as NFκB and CREB are involved in the transcriptional regulation of cell survival.

Akt increases the activity of nuclear factor-kappa B (NFκB) which is involved in the regulation of cell proliferation, apoptosis and survival. NFκB is a heterodimeric complex composed of p50 and RelA/p65 subunits. In unstimulated cells, the majority of NFκB is found in the cytoplasm associated with a family of inhibitory molecules known as IκBs. The phosphorylation of IκB targets it for ubiquitination and proteolytic degradation thus inducing nuclear translocation of NFκB and subsequent activation of its target genes. IκB is phosphorylated and activated by IκB kinase (IKK) complex IKKα and IKKβ. Akt phosphorylates IKKα directly at Thr23 thus leading to the activation of NFκB (Ozes et al., 1999; Kane et al., 2002). In Jurkat cells, PKCθ could cooperate with Akt1 to activate NFκB (Bauer et al., 2001).

The cAMP response element binding protein (CREB) is a ubiquitously expressed nuclear transcription factor that functions in glucose homeostasis, growth-factor-dependent cell survival, and memory. In resting cells, CREB exists in an unphosphorylated state which is transcriptionally inactive but can bind to DNA. Upon cell activation, CREB becomes phosphorylated which induces the transcriptional activity by promoting its interaction with CREB-binding protein CBP. Transgenic mice with T-cell specific expression of a dominant negative CREB show impaired thymocyte proliferation and IL-2 production (Barton et al., 1996) while transgenic mice of CREB in the myeloid lineage develop myeloproliferative disease (Shankar et al., 2005), which indicates that CREB is involved in the regulation of T lymphocytes. Akt phosphorylates CREB directly at Ser133 in response to serum stimulation (Du and Montminy, 1998). In this way, Akt may play a role in regulation of lymphocyte proliferation and cytokine production.

1.3.2.3 Regulation of the p53 pathway

The tumor suppressor p53 and Akt together regulate switches between cell survival and apoptosis. Akt regulates the p53 pathway indirectly through the oncoprotein murine double minute 2 (Mdm2), an E3 ubiquitin ligase that promotes cell survival and cell cycle progression. Mdm2 inhibits the p53 pathway in two general ways. One is by binding directly to the amino-terminus of p53 and blocking its interaction with the transcriptional machinery (Thut et al., 1997). The other is by addition of ubiquitin to p53 to promote its degradation and translocation from the nucleus to the cytoplasm (Freedman and Levine, 1998). Akt interacts with Mdm2 and phosphorylates it on Ser166 and Ser186. The phosphorylation promotes

Mdm2 translocation to the nucleus to inhibit the activation of p53 (Mayo and Donner, 2001; Ashcroft et al., 2002). However, another group reported that neither Akt nor point mutants of Mdm2 at Ser166 and Ser186 had any effect on the subcellular localization of Mdm2, but did increase the ubiquitination and degradation of p53 in a PI3K/Akt-dependent manner (Ogawara et al., 2002). Taken together, Mdm2 may be an Akt target in the regulation of cell growth, but the detailed mechanism needs further investigation.

1.3.3 Regulation of cell proliferation

In addition to inhibiting cell apoptosis, the PI3K/Akt pathway is also involved in regulation of cell cycle progression through a number of mechanisms. $p27^{Kip1}$ is a well-known regulator of the G1/S transition through its cycline-dependent kinase (Cdk) inhibitory activity. The expression of $p27^{Kip1}$ is positively controlled by unphosphorylated FoxO3 transcription factor in an Akt-dependent manner (Stahl et al., 2002). Akt phosphorylates $p27^{Kip1}$ on Thr157 which resides in its nuclear localization sequences thus keeping it in the cytoplasm and preventing it from inhibiting Cdk2 in the nucleus (Liang et al., 2002). In addition, Akt regulates $p27^{Kip1}$ via direct phosphorylation of TSC2, a tumor repressor. TSC2-negative cells express lower amounts of $p27^{Kip1}$ and expressed $p27^{Kip1}$ is mislocalized in the cytoplasm (Soucek et al., 1998; Pasumarthi et al., 2000). Enhanced degradation of $p27^{Kip1}$ has become a prognostic marker for colorectal, breast and nonsmall cell lung carcinomas (Alessandrini et al., 1997).
Another target of Akt is the Cdk inhibitor $p21^{Cip1/WAF1}$, a p53-inducible protein. Two sites (Thr145 and Ser146) in the carboxyl-terminus of $p21^{Cip1/WAF1}$ are phosphorylated directly by Akt. The phosphorylation of Ser146 increases $p21^{Cip1/WAF1}$ protein stability and promotes the assembly of an active cyclin D1-Cdk4/6- $p21^{Cip1/WAF1}$ complex (Li et al., 2002). In cells overexpressing HER2, Akt phosphorylates Thr145 within its nuclear localization sequence and increases its cytoplasmic accumulation (Zhou et al., 2001). The phosphorylation of Thr145 also inhibits the binding of proliferating cell nuclear antigen (PCNA) to $p21^{Cip1/WAF1}$ thus positively regulating DNA synthesis (Rossig et al., 2001). Therefore, phosphorylation of $p21^{Cip1/WAF1}$ by Akt has negative and positive effects on the cell cycle progression. In contrast to $p27^{Kip1}$, the protein level of $p21^{Cip1/WAF1}$ is low in resting T-cells and increased upon lymphocyte activation (Appleman et al., 2000). In double knockout mice lacking both $p21^{Cip1}$ and $p27^{Kip1}$, the IL-2 sensitivity of naïve T-cells is increased and the T-cell proliferation is enhanced (Wolfraim et al., 2004).

1.3.4 Regulation of protein translation

Akt is involved in regulation of protein translation at several levels (Fig. 1.5). Akt

phosphorylates and inactivates GSK3, which in turn phosphorylates and inhibits eukaryotic initiation factor 2 binding protein (eIF2B) that is required for the recycling of eIF2. As well, activation of Akt regulates protein translation initiation through its downstream kinases.

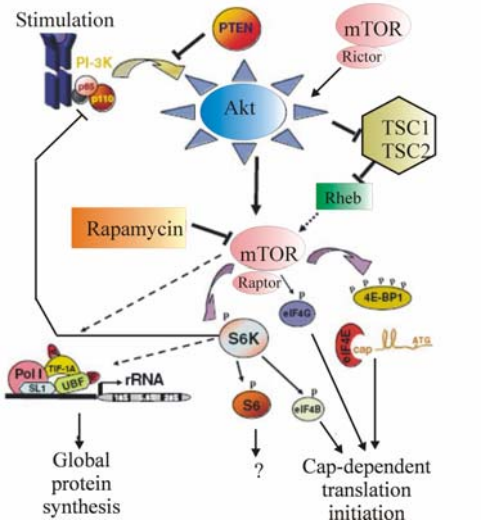


Figure 1.5 PI3K/Akt/mTOR pathway regulates protein translation initiation. Akt regulates the activity of transcription factors important for rRNA synthesis, thereby regulating ribosome biogenesis and global protein synthesis. The Akt/mTOR pathway regulates the translation of cap-dependent mRNAs through phosphorylation of 4EBP, eIF4G and S6K. The Akt/GSK3 pathway regulates the recycling of eIF2 through phosphorylation of eIF2B. Moreover, mTOR activates Akt in the complex with Rictor. Inhibition of mTOR-Raptor by rapamycin and the negative regulation of S6K by the PI3K/Akt pathway are indicated. Modified from (Ruggero and Sonenberg, 2005).

Akt regulates protein translation mainly through its downstream target, the mammalian target of rapamycin (mTOR). The mTOR enhances protein translation initiation in part by phosphorylation of two major targets eIF4E-binding proteins (4EBP) and ribosomal protein S6 kinases (S6Ks). The upstream regulators of mTOR signaling include growth factors, nutrients, energy availability and various stresses. The activity of mTOR is regulated by Akt indirectly through a heterodimeric complex of TSC1 (hamartin) and TSC2 (tuberin). The rapamycin-sensitive mTOR complex 1 (mTORC1) contains three components, mTOR, Raptor and GβL. The small GTPase protein Rheb binds directly and activates mTOR in a GTP dependent manner (Long et al., 2005), while TSC1/2 complexes act as negative regulators by inhibiting Rheb protein (Garami et al., 2003). Akt phosphorylates TSC2 directly on Ser939 and Thr1462 thereby inhibiting its property of GTPase-activating protein and activating the mTOR pathway (Inoki et al., 2002; Potter et al., 2002). Activated mTOR further phosphorylates 4EBPs and phosphorylated 4EBP dissociates with eIF4E, a limiting factor in the formation of the 43S pre-translation initiation complex. As a result, released eIF4E

increases translation initiation. In addition, the initiation factor eIF4G which recruits the translational machinery to mRNA is phosphorylated in its carboxyl-terminus through the PI3K/Akt/mTOR pathway (Raught et al., 2000).

1.4 Akt isoform specific functions

During the past few years, a variety of transgenic and knockout of Akt mice models have been generated and proven to be very helpful for studying Akt isoform specific physiological functions (Table 1.1).

Table 1.1 Physiological functions of Akt isoforms revealed by knockout mice models. Isoform means the knockout of the specific Akt gene (Yang et al., 2004).

Isoform	Function
Akt1	Placenta development and animal growth Adipogenesis
Akt2	Glucose metabolism Adipogenesis and maintenance Animal growth
Akt3	Postnatal brain growth

Akt1 mutant mice are born smaller than wild-type mice, have increased neonatal mortality (approx. 40 %) and reduced body weight (about 30 %) (Cho et al., 2001; Chen et al., 2001). Moreover, loss of Akt1 leads to placental hypotrophy with impaired vascularization (Yang et al., 2003b). Akt2 deficient mice develop severe diabetes with hyperglycaemia, glucose intolerance, and hyperinsulinaemia (Cho et al., 2000). The Akt2 null mice also exhibit a mild growth retardation and an age-dependent loss of adipose tissue (Garofalo et al., 2003). These observations indicate that Akt2 plays a critical role for glucose metabolism, adipogenesis, β cell function and animal growth, whereas both Akt1 and Akt2 are important mediators for organismal growth. Adult Akt3 knockout mice have a much smaller brain size and brain weight in all major brain regions which is caused, at least partially, by a significant reduction in both cell size and cell number (Easton et al., 2005; Tschopp et al., 2005).

Viable mice with a single Akt gene knockout suggest a mutual compensation of function among the isoforms. Given the critical functions of Akt in cell survival, the compound knockout of Akt isoform genes would have severe developmental and survival consequences. Indeed, the mice with double knockout (DKO) of Akt1 and Akt2 develop to term, but die shortly after birth. They are much smaller than wild-type mice and have impaired skin

development, skeletal muscle atrophy and abnormal bone development (Peng et al., 2003). The Akt1 and Akt3 DKO mice are embryonically lethal and the placenta is more impaired than in Akt1 knockout mice. Taken together, diverse phenotypes of Akt knockout mice suggest that there are redundant and specific physiological functions for the Akt isoforms.

1.5 Akt functions in lymphocytes

Constitutively activated Akt directly affects the T-cell size, growth and activation. Transgenic mice with a constitutively active form of myr-Akt in thymocytes and peripheral T-cells have enlarged T-cells and an increased rate of glycolysis. Many aged transgenic mice display increased incidence of lymphoma. Upon activation, resting transgenic T-cells grow rapidly and secrete cytokines in the absence of CD28 ligation (Rathmell et al., 2003a). In addition, in myr-Akt transgenic mice, membrane-targeted Akt promotes a positive selection of thymocytes (Na et al., 2003). Moreover, both thymocytes and T-cells overexpressing a constitutively active form of gag-Akt display an elevated level of anti-apoptotic protein Bcl-xL and an enhanced NFκB activation (Jones et al., 2000).

A major target for Akt in T-cells are members of the Forkhead family, FKHR/FoxO1, FoxO2, FKHRL1/FoxO3 and Afx/FoxO4, which all can be phosphorylated by Akt directly on three consensus serine/threonine residues. FoxO factors reside constitutively in the nucleus in the absence of growth factor signalling where they are able to promote transcription of pro-apoptotic target genes (Burgering and Medema, 2003). Like all transcription factors, FoxO is regulated mainly at the level of nuclear entry. Activation of Akt leads to nuclear export of these transcription factors and the phosphorylated FoxO members are sequestrated in the cytoplasm in a complex with 14-3-3 proteins. Once in the cytoplasm, they are degraded via a ubiquitin-proteasome pathway (Van Der Heide et al., 2004). Akt-mediated phosphorylation of FoxO factors has also been found in HL60 cells and AML blasts (Cappellini et al., 2003; Brandts et al., 2005). A FoxO3a mutant containing no Akt phosphorylation site induces apoptosis in a Fas ligand dependent manner in Jurkat cells (Brunet et al., 1999). Stable activation of the PI3K at the immunological synapse in naïve T cells is essential for Akt-mediated FoxO1 nuclear exclusion, thereby allowing clonal expansion in response to antigen presenting cells (Fabre et al., 2005). Similarly, in a murine pre-B cell line Ba/F3, activation of FoxO3a induces apoptosis through upregulation of the expression of the pro-apoptotic protein Bim, loss of mitochondrial integrity and increase of the release of cytochrome c (Dijkers et al., 2002). Overexpression of FoxO1 and FoxO3a induces the G1 cell cycle arrest in primary B-cells and increases apoptosis which is opposed by the PI3K/Akt pathway (Yusuf et al.,

2004).

1.6 Deregulation of Akt in human diseases

The activation of PI3K/Akt pathway may result from overexpression of a growth factor receptor (Schlegel et al., 2002; Bacus et al., 2002) and/or Ras activation (Liu et al., 1998a), which both give rise to constitutive activation of Akt. Loss of PTEN function is implicated in various tumour types such as glioblastoma, endometrial tumours and Cowden's syndrome (Di and Pandolfi, 2000).

Aberrant regulation of the Akt signal pathway leads to many human diseases. One of the most investigated diseases is diabetes mellitus. Impaired activation of Akt in response to insulin has been described in insulin-resistant patients (Rondinone et al., 1999). The defective phosphorylation of Akt is associated with the defects of GLUT4 translocation and expression (Tremblay et al., 2001). Akt2 knockout mice develop hyperglycemia and hyperinsulinaemia, indicating that this isoform is specifically involved in the development of type 2 diabetes mellitus (Cho et al., 2001).

Amplification and hyperactivation of Akt have also been identified in many types of human cancers. Overexpression of Akt2 in fibroblast cells leads to malignant transformation (Cheng et al., 1997), and Akt2 gene is amplified and overexpressed in human ovarian and breast cancer (Yuan et al., 2000; Sun et al., 2001). An elevated expression of Akt2 protein, but not Akt1, has been reported in nearly 40 % of hepatocellular carcinomas. Overexpression of Akt2 has become an independent prognostic marker (Xu et al., 2004). Unlike Akt2, aberration of Akt1 in cancers is much less frequent. Akt1 amplification was initially detected in a gastric carcinoma (Staal, 1987). A more recent investigation of 103 malignant glial tumors revealed a single case with amplification and overexpression of Akt1 (Knobbe and Reifenberger, 2003). Elevated Akt3 activity has also been reported in estrogen receptor deficient breast cancer, androgen insensitive prostate cancer cell lines (Nakatani et al., 1999) and melanomas (Stahl et al., 2004). Therefore, Akt is a selective therapeutic target in drug discovery.

1.7 Akt interacting proteins

In addition to phosphorylating and regulating its downstream substrates, Akt physically interacts with many other proteins that function as modulators for regulating Akt activity and functions. This part will focus on the nonsubstrate Akt binding proteins.

1.7.1 Binding proteins for the Akt PH domain

The PH domain of Akt is crucial not only for its plasma membrane recruitment and kinase activation through binding to PI(3,4,5)P3 and PI(3,4)P2, but also for mediating protein-protein interactions. Several proteins binding to the PH domain of Akt have been identified including T-cell leukemia 1 (Tcl1). The gene of Tcl1 localizes on human chromosome 14q32.1 which is frequently involved in chromosomal translocations with one of the T-cell receptor loci in human T-cell leukemias and lymphomas. It is preferentially expressed in T- and B-lymphocytes (Virgilio et al., 1994). Tcl1 family includes three members (Tcl1, MTCP1 and Tcl1b) which are all identified as Akt1 and Akt2 binding proteins in yeast two-hybrid screens. The interaction of Tcl1 and Akt enhances kinase activity and nuclear translocation of Akt, stabilizes the mitochondrial transmembrane potential and promotes the cell survival and proliferation (Laine et al., 2000). In contrast to Akt1 and Akt2, Akt3 interacts only with Tcl1 but not with the other two family members, MTCP1 and Tcl1b. Moreover, Tcl1 enhances the heteroligomerization of Akt1 and Akt3 thus facilitating the transphosphorylation which contributes to Tcl1-induced leukemogenesis (Laine et al., 2002).

Another reported protein interacting with the PH domain of Akt is the already mentioned JIP1. Here, rather than phosphorylating downstream targets such as pro-apoptotic protein Bad and FoxO transcription factor members, Akt prevents the assembly of the JNK signaling complex thus inhibiting apoptosis. In addition to Tcl1 and JIP1, some other proteins such as myosin II (Tanaka et al., 1999) and IMPDH (inosine-5' monophosphate dehydrogenase type II) (Ingley and Hemmings, 2000) were identified to interact with the PH domain of Akt as well. However, the physiological meaning of the interaction between Akt and IMPDH or myosin II is not yet clear.

1.7.2 Binding proteins for the Akt kinase domain

Some proteins regulate the activation of Akt through binding to the central kinase domain. The mammalian homologue of tribbles (Trb3), a Cdc25 binding protein of Drosophila, was recently identified as an Akt binding protein in a two-hybrid assay. Amino acids 240-315 of Akt1 are essential for its binding to Trb3 and the interaction of Akt and Trb3 disrupts the insulin signal pathway by inhibition of Akt1 phosphorylation at both regulatory residues. Both, Akt1and Akt2, interacts with Trb3 suggesting that Trb3 performs a general role in the regulation of cellular Akt kinase activity (Du et al., 2003). However, there is no evidence to support the role of Trb3 as an Akt inhibitor in primary hepatocytes (Iynedjian, 2005). In contrast to these results, the PH domain of Akt is also required for binding to Trb3 in rat

hepatoma FGC-4 cells, and the binding of Trb3 blocked the PI(3,4,5)P3 binding site of Akt thereby preventing its membrane association and subsequent Akt-mediated signaling (He et al., 2006). It seems that there is more than one binding site of Trb3 on Akt kinase and the regulating ability of Trb3 is specific for the cell type.

APPL (Adaptor protein containing PH domain, PTB domain and Leucine zipper motif) is another Akt2 binding protein that was identified in a yeast two-hybrid screen (Mitsuuchi et al., 1999). It contains multiple important regulatory motifs including a PH domain, a phosphotyrosine binding domain and a leucine zippered coiled-coil domain. The gene of APPL is mapped to human chromosome 3p14.3-p21.1 where deletions and rearrangements have been reported in a variety of tumor types. APPL interacts with the inactive form of Akt2 and also binds to the PI3K catalytic subunit p110α. It functions as an adaptor protein by tethering inactive Akt2 to the p110α subunit in the cytoplasm and increases their recruitment to the cellular membrane in response to mitogenic stimuli. APPL increases Akt kinase activity in a PI3K dependent manner, and Akt-mediated suppression of androgen receptor (AR) transactivation is further enhanced by APPL (Yang et al., 2003a), suggesting that APPL is a positive regulator of Akt activity.

1.7.3 Binding proteins for the carboxyl-terminus of Akt

The carboxyl-terminal regulatory domain of Akt mediates specific protein-protein interactions as well. A good example is the carboxyl-terminal modulator protein (CTMP) which was identified to interact with Akt1 in a yeast two-hybrid screen (Maira et al., 2001). The binding site of CTMP lies in the carboxyl-terminus (aa411-480) of Akt1, and full length Akt1 interacts with CTMP at the plasma membrane. Overexpression of CTMP reduces the phosphorylation of both Akt regulatory sites Thr308 and Ser473, most notably on Ser473. Knockdown of endogenous CTMP enhances the Ser473 phosphorylation of Akt1. Thus, CTMP represents a novel class of protein that interacts with Akt at the plasma membrane and keeps the kinase in an inactive state.

Another example is the human protein similar to the mouse fused toes protein 1 (hFt1), which was identified in a green fluorescent protein based protein-protein interaction assay (Remy and Michnick, 2004). It interacts directly with the Akt carboxyl-terminus (aa409-480) and increases the phosphorylation of Akt at the two regulatory sites thus enhancing the kinase activity. PDK1 also interacts with hFt1 at the plasma membrane through its amino-terminal region. Since hFt1 has no conserved Akt phosphorylation motif, it may function as a scaffold protein at the plasma membrane to promote the activation of Akt by PDK1. Interestingly,

overexpression of hFt1 together with Akt and PDK1 and glucocorticoid treatment dramatically increases the apoptosis susceptibility of Jurkat cells, suggesting that the final function of hFt1 is to control the Akt-mediated apoptotic signals.

To sumarize, the activation of Akt affects a range of diverse and important cellular functions. Searching for Akt substrates has been significantly aided by the definition of the Akt consensus phosphorylation motif RXRXXS/T. Table 1.2 summarizes some of the reported Akt binding proteins. Some of them are acting as Akt substrates that are phosphorylated directly by Akt kinase, while others are identified as Akt associating proteins that regulate cellular localization and kinase activation.

Table 1.2 Examples of Akt binding proteins. Akt interacting proteins function either as substrates that can be phosphorylated by Akt at Thr/Ser residue(s) or as regulatory proteins that regulate Akt cellular localization and phosphorylation. For some Akt binding proteins the interaction has not been shown for endogenous proteins, thus the physiological meaning is not yet clear (Brazil et al., 2002).

Interacting Protein	Biological Effect	Endogenous Interaction Demonstrated?
Nonsubstrates		
TCL1	Increases PKB kinase activity and nuclear translocation	+
CTMP	Inhibits S473 phosphorylation on PKB	+
Hsp90/Cdc37	Protection from dephosphorylation, degradation of PKB, and recruiting its substrates	+
Hsp27	Activation of kinase activity	–
GLUT4 vesicles	Exact role unclear, important for insulin-stimulated glucose uptake	+
JIP1	Negative regulation of the JNK pathway	–
Axin-GSK3β-Dvl	Mediates GSK3α/β regulation in Wnt signaling pathways	+
PKC α,δ, ζ	(Functional relevance remains to be determined)	–
Keratin 10	Inhibits intracellular translocation of PKB	+
Grb 10	Translocation of PKB to the plasma membrane	+
Myosin II	(Functional relevance remains to be determined)	–
IMPDH	Regulation of GTP biosynthesis	–
APPL	(Functional relevance remains to be determined)	+
G protein βγ subunit	(Functional relevance remains to be determined)	–
Btk	Positively activates PKB in H_2O_2 signaling in B cells	+
Substrates		
Mdm2/Hdm2	Nuclear translocation of Mdm2, decreases p53 levels	+
p21Cip/WAF1	Enhances protein stability of p21 and promotes cell survival	+
EDG-1	Activation of GPCRs in Gi-independent manner	+
BAD	Phosphorylated BAD binds to 14-3-3 and is sequestered in the cytoplasm	–
TSC2	Destabilizes TSC2 and disrupts its interaction with TSC1, leading to p70S6K activation	+
Raf1	Inhibition of Raf1 signaling	+
14-3-3ζ	(Functional relevance remains to be determined)	–
Cot1/TPL2	Increase in NF-κB-dependent transcription	–
IRS-1	Positive regulation of IRS-1 function	–
Nur77	Inhibits the transactivation activity of Nur77 in a nuclear receptor pathway	–
Gab2	Negative regulation of mitogenic signaling	+

2 Aim of the doctoral thesis

The aim of the present work was to identify novel Akt2 binding proteins by using the yeast two-hybrid method. Different regions of Akt2 were cloned into pBTM116 vectors and sequential transformation of yeast L40 strain was conducted using the lithium acetate method. To determine whether candidate binding proteins identified in yeast biologically interact with Akt2, we performed co-immunoprecipitation studies by transiently overexpression of Akt and its candidate binding proteins in HEK 293 cells. The interaction of two proteins was investigated with endogenous proteins as well. To characterize the binding sites that were responsible for the interaction between two proteins, the truncation mutants and point mutants of both proteins were prepared and investigated using both a GST pull-down assay and a co-immunoprecipitation method. Since it has been reported that the activation state of Akt would affect its interaction with some proteins, we stimulated transiently transfected cells with insulin to investigate the association of Akt and its binding candidates. Considering the high amino acid identity of the three Akt isoforms, Akt1 and Akt3 were also included in the experiments to determine the isoform specific interactions. Additionally, cellular co-localization of interacting proteins was also investigated in the present work by using indirect immunofluorescence microscopy. Finally, the physiological meaning of newly identified Akt2 binding proteins was investigated in mammalian cells.

3 Materials and methods

3.1 Materials

3.1.1 Reagents

[γ–^{32}P] ATP	Amersham-Pharmacia
12-O-tetradecanoylphorbol-13-acetate (TPA)	Sigma
3-amino-1, 2, 4-triazole (3-AT)	Sigma
Adenosine 5'-triphosphate (ATP)	Sigma
Agar	Difco
Agarose	Roche
Ammonium persulfate (APS)	Sigma
Ampicillin	Roth
Aprotinin	Roche
Bisbenzimide (DAPI)	Roche
Blotting paper (3 MM)	Whatman
Bovine serum albumine (BSA)	Roche
Bradford assay reagent	BioRad
Calcimycin (A23187)	Sigma
Calf intestine alkaline phosphatase (CIP)	New England Biolabs
Dimethylsulfoxid (DMSO)	Sigma
DNase I	Boehringer
DNase, RNase A	Roche
Ethidium bromide	Roth
G418	Sigma
Glassbeads (425 – 600 µm in diameter)	Sigma
Glutathione Sepharose beads	Amersham-Pharmacia
Kanamycin	Calbiochem
Lithium acetate (LiAc)	Sigma
Lysozyme	Roche
Nitrocellulose (0.45 µm pore size)	Amersham-Pharmacia
Parafilm	Dynafilm
PermaFluor	Immunotech

Phenol	Roth
Phenylmethylsulfonyl fluoride (PMSF)	Roche
Phosphate buffered saline (PBS)	Cambrex
Polyethylene glycol 3350 (PEG 3350)	Sigma
Polyprep chromatography column	BioRad
Ponceau S	Sigma
Protein A Sepharose	Amersham-Pharmacia
Protein G Sepharose	Amersham-Pharmacia
Restriction enzymes	New England Biolabs
Sephadex G10	Amersham-Pharmacia
Sheared salmon sperm DNA (ssDNA)	5'prime – 3'prime
Sodium dodecyl sulfate (SDS)	Roth
Sterile filters (0.22 µm, 0.45 µm)	Roth
T4 DNA ligase	New England Biolabs
Taq/*Pwo* polymerase	Peqlab
Pfx polymerase	Invitrogen
Tetramethylethylenediamine (TEMED)	Roth
Thiol-Sepharose 4B	Amersham-Pharmacia
Tris-base	Sigma
Triton X-100	Roth
Trypan blue	Sigma
Trypsin	Cambrex

3.1.2 Kits

First Strand cDNA Synthesis Kit for RT-PCR (AMV)	Roche
Dual-luciferase™ reporter assay system	Promega
Enhanced Chemiluminescence (ECL)	Amersham Pharmacia
QIAquick PCR Purification Kit	Qiagen
NuclearBond® PC100	Macherey-Nagel
NucleoSpin® Extract II	Macherey-Nagel
QuikChange® Site-Directed Mutagenesis Kit	Stratagene
SuperFect Transfection Reagent	Qiagen
NucleoSpin® Plasmid QuickPure	Macherey-Nagel
Plasmid maxi-preps kit	Qiagen

3.1.3 Buffers

Ampicillin (1000 ×)

10 g Ampicillin
adding H_2O to 100 ml
stored at –20°C

BES buffered saline (BBS)

50 mM BES pH 6.97
280 mM NaCl
1.5 mM Na_2HPO_4

Cell lysis buffer

10 % Glycerol
1 % Triton X-100
1.5 mM $MgCl_2$
50 mM HEPES (pH 7.5)
150 mM NaCl
1 mM EGTA
100 mM NaF
10 mM $Na_4P_2O_7$
adding fresh: 1 mM Na_3VO_4
10 µg/ml Aprotinin
1 mM PMSF

Coomassie brilliant blue

1.25 g Comassie brilliant blue R
450 ml Methanol / H_2O (1:1)
50 ml Acetic acid
filtered through a Whatman paper

DNA loading buffer

0.05 % (w/v) Bromophenol blue
0.05 % (w/v) Xylene cyanole
30 % (v/v) Glycerol
100 mM EDTA (pH 8.0)

Glassbeads disruption buffer

20 mM Tris-HCl pH 8.0
10 mM $MgCl_2$

	1 mM EDTA
	5 % Glycerol
	0.3 M $(NH_4)_2SO_4$
	1 mM PMSF
HNTG buffer	
	20 mM HEPES (pH 7.5)
	150 mM NaCl
	10 % (v/v) Glycerol
	0.1 % (v/v) Triton X 100
	10 mM NaF
	1 mM Sodium orthovanadate
Kanamycin (1000 ×)	
	5 g Kanamycin
	adding H_2O to 100 ml
	filter sterilize, store at –20°C
KCM buffer	
	500 mM KCl
	150 mM CaCl
	250 mM $MgCl_2$
Kinase assay buffer	
	20 mM Tris pH 7.6
	5 mM β-glycerophosphate
	2 mM Dithiothreitol
	0.1 mM Sodium pervanadate
	10 mM $MgCl_2$
Laemmli loading buffer	
	10 mM EDTA pH 8.0
	20 % (v/v) Glycerin
	3 % (w/v) SDS
	0.05 % (w/v) Bromphenol blue
	3 % (v/v) β-Mercaptoethanol (freshly added)
Lithium acetate (1 M)	
	6.6 g LiAc
	adding H_2O to 100 ml

filter sterilized, pH 8.4-8.9

Lower Tris buffer (4 ×)

1.5 M Tris

0.4 % SDS, pH 8.8

NET buffer (10 ×)

1.5 M NaCl

0.5 M Tris-HCl pH 7.5

50 mM EDTA pH 8.0

0.5 % (v/v) Triton X-100

NET-gelatin buffer (NET-G)

1× NET buffer

0.25 % Gelatin

Phenol-chloroform-isoamyl alcohol (PCI)

50 % (v/v) Phenol

48 % (v/v) Chloroform

2 % (v/v) Isoamyl alcohol

Polyethylene glycol 3350 (PEG 3350, 50 %)

50 g polyethylene glycol 3350

adding H_2O to 100 ml

filter sterilized

Ponceau S

0.2 % Ponceau S

2 % Trichloroacetic acid

Stripping buffer

62 mM Tris pH 6.8

2 % SDS

0.5 % β-mercaptoethanol (freshly added)

Tris-acetate buffer (10 × TAE)

400 mM Tris-acetate (pH 8.0)

10 mM EDTA

Tris-borate buffer (10 × TBE)

0.9 M Tris-borate (pH 8.0)

20 mM EDTA

TE buffer

10 mM Tris-HCl (pH 8.0)
0.1 mM EDTA

TN buffer

10 mM Tris-HCl, pH 8.0
150 mM NaCl

Transblot buffer

48 mM Tris-base
39 mM Glycine
20 % Methanol
0.004 % (w/v) SDS

Transformation storage buffer (TSB)

1× LB-medium
10 % (w/v) PEG 3350
10 mM $MgCl_2$
10 mM $MgSO_4$

Tris glycine (TG) buffer

248 mM Tris-base
1918 mM Glycine
35 mM SDS

Upper Tris buffer (4 ×)

0.5 M Tris-base
0.4 % SDS, pH 6.8

Yeast lysis buffer

2 % (v/v) Triton X-100
1 % (w/v) SDS
100 mM NaCl
100 mM Tris-HCl, pH 8.0
1 mM EDTA, pH 8.0

Z-buffer

60 mM Na_2HPO_4
40 mM NaH_2PO_4
10 mM KCl
1 mM $MgSO_4$, pH 7.0

3.1.4 Yeast, bacteria and mammalian cell lines

Yeast	*S. cerevisiae* L40	YPD medium
	S. cerevisiae L40 LexA- Akt2 full length	UT medium
	S. cerevisiae L40 LexA-Akt2aa100-420	UT medium
	S. cerevisiae L40 LexA-Akt2aa142-481	UT medium
	S. cerevisiae L40 LexA-Akt2aa310-481	UT medium
	S. cerevisiae L40 LexA-Akt2aa360-481	UT medium
	S. cerevisiae L40 LexA-Akt2aa410-481	UT medium
Bacteria	*E. coli* 298 F'	LB + kanamycin
	E. coli DH5α	LB medium
	E. coli HB101	M9 medium
	E. coli BL21	LB medium
Mammalian cell lines	Human embryonic kidney 293 (HEK 293)	DMEM, 10 % FBS, 2 mM L-glutamine
	Murine myoblasts C2C12	DMEM, 10 % FBS, 2 mM L-glutamine
	Jurkat T cells	RPMI 1640, 10 % FBS, 2 mM L-glutamine
	L6 myotubes	DMEM, 10 % FBS, 2 mM L-glutamine

3.1.5 Media

Amino acid supplement mixture (CSM)	Bio 101 systems
Dropout base medium (DOB)	Bio 101 systems
Dulbecco's Modified Eagle Medium (DMEM)	Cambrex
Fetal bovine serum (FBS)	HyClone
L-glutamine	Cambrex
Luria broth (LB)	Sigma
RPMI 1640	Cambrex
YPD/YEPD broth	Bio 101 systems
LB medium	

15.5 g LB
9.5 g NaCl
adding H_2O to 1000 ml

YPD medium

50 g YPD broth
adding H_2O to 1000 ml

UT medium

27 g DOB
0.72 g CSM -Trp/-Ura
adding H_2O to 1000 ml

UTL medium

27 g DOB
0.62 g CSM -Trp/-Ura/-Leu
adding H_2O to 1000 ml

THULL medium

27 g DOB
0.55 g CSM -Trp/-Ura/-Leu/-Lys/-His
10 mM 3-AT
adding H_2O to 1000 ml

10× M9 salts

128 g Na_2HPO_4-$7H_2O$
30 g KH_2PO_4
5 g NaCl
10 g NH_4Cl
adding H_2O to 1000 ml

M9 minimal medium

1 mM $MgSO_4$
1 mM Thiamine
40 µg/ml Proline
0.2 % Glucose
50 µg/ml Ampicillin
100 ml 10× M9 salts

3.1.6 Antibodies

Rabbit polyclonal antisera were raised against a synthetic peptide corresponding to the amino-

terminal 15 amino acids of human RPS25 ($_1$MPPKDDKKKKDAGKS$_{15}$) and against a glutathione-S-transferase (GST) fusion protein with the carboxyl-terminal 72 amino acids (aa 410-481) of human Akt2. The rabbit antibodies to phosphorylated Thr 308 and Ser473 of Akt were purchased from Cell Signaling Technology. The polyclonal rabbit antibody against LexA was purchased from Upstate and polyclonal rabbit antibody against 14-3-3γ purchased from Santa Cruz. The monoclonal mouse antibody against phosphorylated tyrosine (4G10) was purchased from Upstate, mouse hemagglutinin (HA) epitope tag antibody HA11 purchased from Babco and rabbit c-myc epitope tag A14 antibody purchased from Santa Cruz. Anti-vesicular stomatitis virus (VSV) antibody was produced from hybridome P5D4 and anti-myc antibody produced from hybridome 9E10. Horseradish peroxidase-coupled secondary antibodies for Western blotting were purchased from Sigma. For immunofluorescence, the secondary antibodies, goat-anti-rabbit antibody coupled to Alexa Fluor® 488/546 and goat anti-mouse antibody coupled to Alexa Fluor® 488/546, were purchased from Molecular Probes.

3.1.7 Oligonucleotides

pACT2-5’	forward: GATTACGCTAGCTTGGGTGGTC
preyseq	reverse: CACGATGCACAGTTGAAGTGA
PLZF	forward CCTACGAGTGTGAGTTCTGTGGCAG
PLZF	reverse TCACACATAGCACAGGTAGAGGTACG
Akt2aa1	forward: GAGAATTCATGAATGAGGTGTCTGTCATC
Akt2aa100	forward: GAGAATTCATGCGGGCCATCCAGATGGTC
Akt2aa142	forward: GAGAATTCAAGGCACGGGCTAAAGTGACC
Akt2aa310	forward: GAGAATTCTTCTGTGGGACCCCGGAGTAC
Akt2aa360	forward: GAGAATTCGAGCTCATCCTCATGGAAGAG
Akt2aa410	forward: GAGAATTCCTCAGCATCAACTGGCAGGAC
Akt2aa141	reverse: GAGGATCCTCAGCTGACCGCCACTTCCATCTC
Akt2aa420	reverse: GAGGATCCTCACTTCTGGACCACGTCCTGCCA
Akt2aa481	reverse: GAGGATCCTCACTCGCGGATGCTGGCCGA
Akt2-A382S	forward: GAGGCCAAGTCCCTGCTTTCTGGGCTGCTTAAGAAGGAC
	reverse: GTCCTTCTTAAGCAGCCCAGAAAGCAGGGACTTGGCCTC
Akt2Δ377-385	forward:CGCACGCTCAGCCCCGAGAAGAAGGACCCCAAGCAGAGGC
	reverse: GCCTCTGCTTGGGGTCCTTCTTCTCGGGGCTGAGCGTGCG
Akt2Δ387-392	forward: CTGCTTGCTGGGCTGCTTAAGCTTGGTGGGGGGCCCAGCG
	reverse: CGCTGGGCCCCCCACCAAGCTTAAGCAGCCCAGCAAGCAG

Akt2Δ389-390 forward: GCTGGGCTGCTTAAGAAGGACCAGAGGCTTGGTGGGGGG
reverse: CCCCCCACCAAGCCTCTGGTCCTTCTTAAGCAGCCCAGC
Akt2Δ391-392 forward: GCTGCTTAAGAAGGACCCCAAGCTTGGTGGGGGGCCCAGC
reverse: GCTGGGCCCCCCACCAAGCTTGGGGTCCTTCTTAAGCAGC
RPS25aa70 reverse: GAGAATTCTCATGGGGTTATAAGTTTATAGTT
RPS25aa88 reverse: GAGACTCGAGTCAAAGGGCTGCCCTGGCCAGG
RPS25aa106 reverse: GACTCGAGTCATTGAGCTCTGTGCTTTGAAACCAG
RPS25aa109 reverse: GACTCGAGTCAGTAAATTACTTGAGCTCTGTGC
RPS25aa112 reverse: GACTCGAGTCAATT TCTGGTGTAAATTACTTGAGC TC
shRNA-6 of RPS25
AAAAAAGCGTCCTTCCTCTTCCTGTCGCCCTTAAGCAAGCTTCCCTAAGGACGAC
AAGAAGAAGAAGGACGCGGTGTTTCGTCCTTTCCACAA
shRNA-345 of RPS25
AAAAAAGCACCTTCACCAGCAACTGGAACACCTCCCAAGCTTCGGAGATGCTCCA
GCTGCTGGTGAAGATGCGGTGTTTCGTCCTTTCCACAA

3.1.8 Vectors and expression plasmids

Vectors	**Reference**
pACT2	Clontech
pACT2-RPS25	This work (isolated from a human skeletal muscle cDNA library)
pBTM116	Paul Bartel and Stanley Fields
pBTM-Akt2 full length	This work
pBTM-Akt2aa100-420	This work
pBTM-Akt2aa1-141	This work
pBTM-Akt2aa142-481	This work
pBTM-Akt2aa310-481	This work
pBTM-Akt2aa360-481	This work
pBTM-Akt2aa410-481	This work
pCR –Zero	Invitrogen
pCR-Zero-U6prom-shRNA-6 of RPS25	This work
pGEX5×-1, -2, -3	Amersham-Pharmacia
pGEX-Akt2aa410-481	This work
pGEX-RPS25aa1-70	This work

pRK_5	Genentech
pRK_5- PKBβ/Akt2aa364-481- myc(5×)	This work
pRK_5- PKBβ/Akt2aa377-481- myc(5×)	This work
pRK_5- PKBβ/Akt2aa385-481- myc(5×)	This work
pRK_5- PKBβ/Akt2aa394-481- myc(5×)	This work
pRK_5- PKBβ/Akt2Δ377-385-myc-NT	This work
pRK_5-RPS25aa1-106-HA	This work
pRK_5-RPS25aa1-109-HA	This work
pRK_5-RPS25aa1-112-HA	This work
pRK_5-RPS25aa1-88-HA	This work
pRK_5-RPS25-HA	This work
pRK_5-RPS25Δ70-88-HA	This work
pRK_{RS}	R. Lammers
pRK_{RS}- PKBβ/Akt2Δ385-394- myc(5×)	This work
pRK_{RS}- PKBβ/Akt2Δ387-392- myc(5×)	This work
pRK_{RS}- PKBβ/Akt2Δ389-390- myc(5×)	This work
pRK_{RS}- PKBβ/Akt2Δ391-392- myc(5×)	This work
pRK_{RS}-PKBα/Akt1	M. Melzer
pRK_{RS}-PKBβ/Akt2	M. Melzer
pRK_{RS}-PKBβ/Akt2-myc(5×)	D. Neuscheler
pRK_{RS}-PKBβ/Akt2-myc-NT	This work
pRK_{RS}-RPS25-VSV	This work

3.2 Methods

3.2.1 Yeast two-hybrid system

The yeast two-hybrid screening system requires expression of two fusion proteins from specially designed vectors: one encodes a sequence specific DNA-binding domain, such as that from bacteria repressor protein LexA or from yeast transcription factor GAL4; the other encodes a transcription activation domain, usually from either GAL4 or herpes simplex virus protein VP16. In yeast two-hybrid experiments, the protein of interest is cloned into a DNA-binding domain containing vector, called ‘bait’, while the cDNA library is constructed with a vector containing the activation domain, called ‘prey’. After co-transformation of bait and prey into a yeast host strain, both fusion proteins are targeted to the yeast nucleus (Silver et

al., 1984). If bait and prey protein interact with each other, they will reconstitute a functional transcription factor thus driving transcription of reporter genes. The rapid screening procedure often uses a simple growth selection which is based on the nutritional reporter gene *HIS3* and a β-galactosidase assay for blue/white screening based on the secondary reporter gene *LacZ*. In this way, novel interacting proteins can be identified by screening a single protein against a special cDNA library.

In the present work different regions of Akt2 cDNA were cloned into a pBTM116 bait vector in a way that the fusion protein between LexA and the target Akt2 was generated. Likewise, human cDNA libraries, constructed in pACT2 vectors and containing the activation domain of GAL4, of brain, liver, placenta, pancreatic and skeletal muscle were purchased from Clontech. We conducted a sequential transformation of a yeast host strain L40 with bait plasmids and human cDNA libraries. Briefly, yeast L40 was first transformed with the specific bait plasmid using the lithium acetate (LiAc) method (Gietz et al., 1995) to establish a yeast bait strain which expressed the LexA-Akt2 bait fusion protein while it did not activate the reporter genes autonomously. Distinct human cDNA library were then transformed into the established yeast bait strain in a large scale and yeast transformants were selected using a histidine auxotrophy and a β-galactosidase assay. Since the *HIS3* gene has a leaky expression in L40, this basal expression was inhibited by including 10 mM of 3-amino-1,2,4-triazole (3-AT), an inhibitor of the *HIS3* gene product, during selection on THULL plates. The *HIS+/LacZ+* positive yeast transformant contains two different two-hybrid plasmids, one encoding the bait and another containing library inserts. The library plasmid was isolated by retransformation of yeast DNA to bacteria HB101 that are unable to grow in media lacking leucine. Only by transformation of the library plasmid pACT2 that contains the *LEU2* gene, the bacteria are rescued. The pACT2 plasmid with the cDNA insert was then isolated from HB101 and sequencing analysis performed. Finally, the specific interaction was confirmed in intact mammalian cells using co-immunoprecipitation. The scheme for the yeast two-hybrid screen is shown in Fig. 2.1.

3.2.1.1 Yeast culture

The *Saccharomyces cerevisiae* host strain L40 (MATa ade2-101 his3-200 trp1-901 leu2-3,-112 gal4 gal80 Lys2::(LexO)4-Gal1-His3 Ura3::(LexO)8-GAL1-lacZ) was cultivated in an Erlenmeyer flask with YPD medium at 30°C with shaking, and the yeast bait strain was grown in UT (-Trp/-Ura) medium.

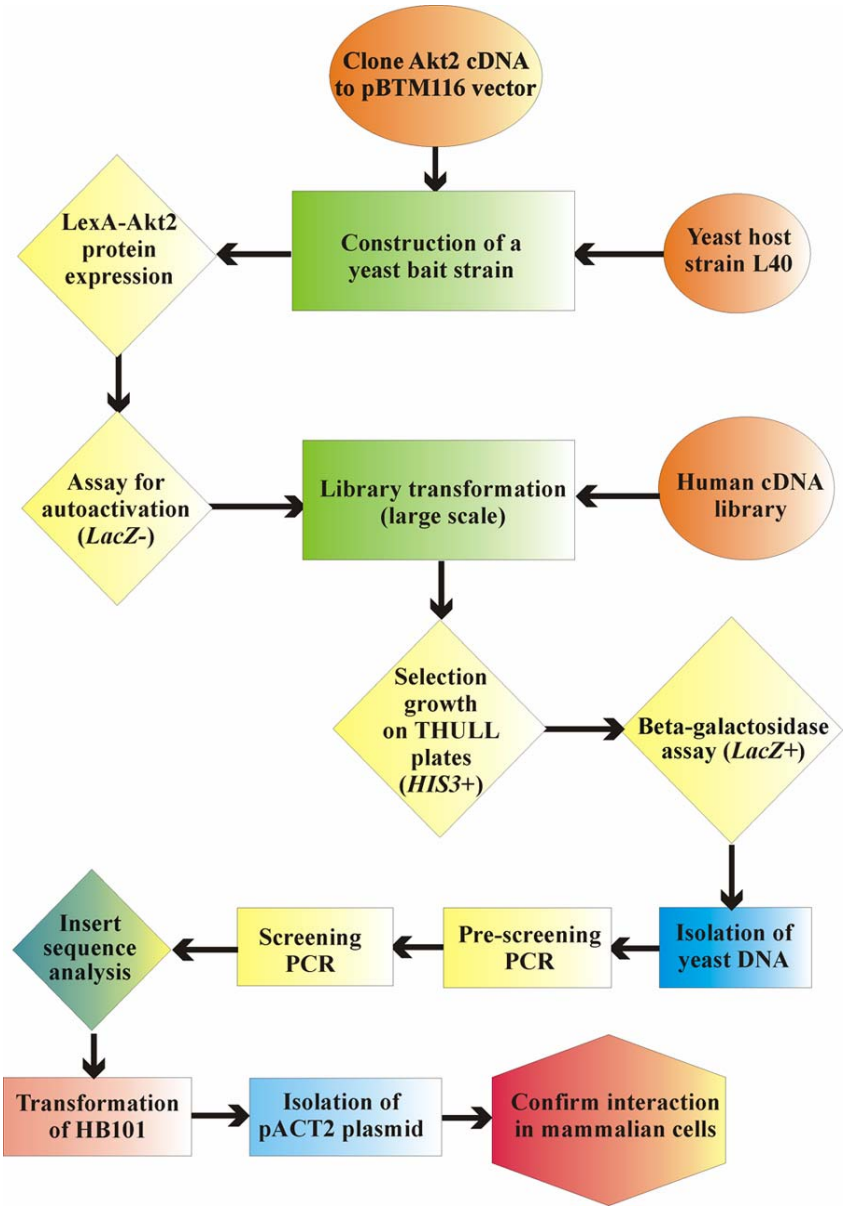


Figure 2.1 Scheme of yeast two-hybrid screen. Different regions of the Akt2 cDNA were cloned into the LexA containing pBTM116 vector. Yeast L40 was first transformed with a specific bait plasmid to establish the bait strain. Human cDNA libraries constructed in pACT2 vectors were then transformed into the bait strain and *HIS*3+/*LacZ*+ positive transformants were selected. Library plasmids were enriched through transfromation into *E. coli* HB101. The interaction of proteins was confirmed in mammalian cells.

3.2.1.2 Construction of yeast bait strain

To prepare competent yeast cells, a large single colony of yeast L40 was grown in 10 ml of YPD medium for at least 20 hours at 30°C. The primary culture was diluted to OD600 = 0.5 (total volume of 50 ml) and cultivated for another 3 hours. Yeast were collected by centrifugation for 5 min at RT, 2500 g, washed once with 40 ml of TE and incubated with 2 ml of 100 mM LiAc / 0.5× TE for 10 min at RT. Then 100 µl of yeast competent cells were resuspended in 700 µl of freshly prepared solution 100 mM LiAc / 1× TE / 40 % PEG 3350

together with the DNA mixture (1 µg of bait plasmid and 100 µg of denatured ssDNAs), vortexed vigorously and incubated in a 30°C water bath for 30 min. After incubation, 88 µl of DMSO were added and the yeast solution heat shocked in a 42°C water both for 7 min. Aliquots were then plated on UT (-Trp/-Ura) plates for selection of transformants containing the introduced bait plasmid.

3.2.1.3 Detection of the expression of LexA-Akt2 bait fusion protein

Before proceeding with library screening, it is necessary to verify the actual expression of LexA-Akt2 bait fusion protein in yeast. A single colony of yeast bait strain was grown in 2 ml of UT medium at 30°C until OD600 = 3.5. Yeast culture was centrifuged for 10 min at RT, 4000 g and pellets were resuspended in 150 µl of glass bead disruption buffer together with about 150 µl of acid washed glass beads (425 - 600 µm in diameter). The mixture was vortexed vigorously for 1 min and then cooled down for another minute on ice and the process was repeated for 5 to 7 times. The yeast debris was then pelleted by centrifugation for 5 min at RT, 13000 g. Finally, aliquots of the supernatant were resolved by SDS-PAGE (10 %) and analyzed using Western blotting with anti-LexA antibody.

3.2.1.4 Autoactivation assay of the bait plasmid

One of the important pre-requirements for a successful yeast two-hybrid screen is to make sure that the bait protein cannot autonomously induce reporter gene activity. The *LacZ* reporter gene is regulated more stringent than *HIS3* in yeast host strain L40. To test the autoactivation capacity of our bait plasmids, a β-galactosidase assay was performed with the yeast transformants. Colonies of yeast transformants and control strains were transferred to a nitrocellulose filter with a toothpick. With the colony-side up, the filter was put on a pre-cooled aluminium boat for 30 sec which was floating on liquid nitrogen. The filter was immersed in liquid nitrogen together with the boat for about 5 sec, then layered on a piece of Whatman paper that had been soaked with Z-buffer containing 0.4 mg/ml of 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and incubated in a 37°C humidified chamber for 1 h. The expression of the bait plasmid should not induce the reporter gene *LacZ*.

3.2.1.5 Human cDNA library transformation

Once the yeast bait strain was established, human cDNA libraries purchased from Clontech were transformed in a large scale for screening interacting proteins. Briefly, a single colony of yeast bait strain was cultivated in 50 ml of UT medium until OD600 = 0.8. Yeast were centrifuged for 5 min at RT, 2500 g, washed once with sterile H_2O and resuspended in 4 ml of a freshly prepared solution of 100 mM LiAc / 0.5× TE. After incubation for 15 min in a 30°C

water bath, yeast were centrifuged for 10 min at RT, 4500 g and the following solutions were added in the order as listed below:

a) 7.2 ml PEG-3350 50 % (w/v)
b) 1.08 ml LiAc 1 M
c) 0.15 ml ssDNA (10 mg/ml, boil before each use for 5 min and cooled on ice)
d) 15 µl library DNA (1 µg/µl)
e) 2355 µl H_2O

After vortexing for 1 min vigorously, the mixture was incubated in a 30°C water bath for 30 min, followed by a heat shock in a 42°C water bath for 30 min (the tube was inverted once every 5 min). Finally, yeast were collected by centrifugation for 5 min at RT, 4500 g and resuspended in 8 ml of sterile H_2O. Aliquots were then plated on one UTL (-Trp/-Ura/-Leu) plate to determine the transformation efficiency and on ten THULL (-Trp/-Ura/-Leu/-Lys/-His) plates for selection of transformants inducing the activation of reporter gene *HIS3*.
Colonies from THULL plates were transferred to nitrocellulose filters with a toothpick and *LacZ* expression tested. Strong interactions yielded a detectable color in less than 30 min.

3.2.1.6 Isolation of yeast DNA

A single colony of *HIS3+/LacZ+* positive yeast transformant was grown in 2 ml of YPD medium until saturation and yeast collected by centrifugation for 5 min at RT, 4500 g. The pellet was resuspended in 200 µl of yeast lysis buffer followed by mixing with about 200 µl of acid washed glass beads and 200 µl of phenol-chloroform-isoamyl alcohol (PCI). The mixture was vortexed vigorously for 5 min and the supernatant extracted two more times with PCI. Finally, the DNA was precipitated with 200 µl of isopropanol and washed three times with 70 % ethanol. After drying at 65°C, the DNA was dissolved in 30 µl of distilled H_2O.

3.2.1.7 Screening PCR

The promyelocytic leukemia zinc finger (PLZF) protein is a specific DNA binding transcription factor, which constitutively localizes in the nucleus and is often identified with several unrelated bait constructs. In the present work, a pre-screening PCR was conducted to eliminate false positive yeast transformants. The isolated yeast DNA was used as a DNA template and synthetic oligonucleotides specific for PLZF as primers. The PCR products were separated on an agarose gel. Only when there was no product of PLZF, the corresponding prey plasmid was used as a template for identifying the cDNA insert by PCR. This PCR was done with *Taq* polymerase and synthetic oligonucleotides specific for the pACT2 vector flanking the insert. The PCR products were further analyzed by sequencing and database

searching.

3.2.1.8 Retransformation of HB101 with the yeast DNA

To enrich the prey plasmid containing a cDNA insert which encoded an Akt2 candidate interacting protein, about 1 µl of yeast DNA was retransformed into *E. coli* HB101 competent cells following the standard transformation protocol. HB101 cannot grow in M9 medium which lack leucine. Only upon the presence of pACT2 vector transformed bacteria could be rescued on M9 minimal agar plates.

3.2.2 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is used to amplify cDNA fragments by using oligonucleotide primers (Saiki et al., 1988). Reactions were performed with the thermostable DNA polymerase *Taq*, *Pfx* or *Pwo*. PCR programs were set depending on the primer length for annealing temperature and the product fragment length for elongation time. In general, the following program was applied: (1) Denaturing at 94°C for 1 min. (2) Annealing at 40 - 60°C for 1 min. (3) Elongation at 72°C (*Taq* and *Pwo*) or 68°C (*Pfx*), assuming an efficiency of about 700 nucleotides per minute. The reaction cycle was repeated 25 or 35 times. (4) Incubation at 72°C or 68°C for 10 min. The reaction was performed in a volume of 50 µl containing 10 ng of template DNAs, 0.25 - 0.6 µM of forward/reverse primer, 2.5 U of polymerase, 5 µl of 10 × reaction buffer and distilled H_2O. For subsequent analysis, PCR products were purified using either an agarose gel or a PCR purification kit (Qiagen).

3.2.3 Isolation of plasmid DNA from *E. coli*

Plasmid isolation from bacteria in a small scale (miniprep) were performed according to (Birnboim and Doly, 1979). A single colony was inoculated in 2 ml of LB medium containing ampicillin or kanamycin and cultured overnight at 37°C. Bacteria from 1.5 ml of culture were collected by centrifugation for 1 min at RT, 13000 g. Following the resuspension in 200 µl of TE, bacteria were lysed by addition of 100 µl of 0.2 M NaOH and 1 % (w/v) SDS. The protein-SDS complex and the genomic DNA were precipitated by incubation with 50 µl of KOAc (3 M) for 5 min on ice. The mixtures were centrifuged for 5 min at RT, 13000 g and the residual proteins extracted from the supernatant with an equal volume of phenol. Finally, DNA was precipitated with 0.6 volumes of 100 % isopropanol, centrifuged for 10 min at RT, 13000 g. After washing twice with 70 % ethanol, the plasmid DNA was dried at 65°C and dissolved in 50 µl of TE buffer containing 10 µg/ml of RNase A.

When a higher quality of DNA was required (e.g. for sequencing), it was prepared with a

plasmid DNA purification kit (Macherey-Nagel) according to the manufacturer's instructions. A large amount of high quality DNA for transfection of mammalian cells was prepared with a plasmid midi-preparation kit (Qiagen) or the cesium chloride gradient method.

3.2.4 Cloning procedures and transformation of *E. coli*

Full length Akt2 and RPS25 cDNAs were digested with restriction nucleases (New England Biolabs) for 1 - 2 h in recommended buffers according to the manufacturer's instructions. Linearized vectors (pRK or pGEX) were dephosphorylated with calf intestine phosphatase for 30 min at 37°C. Then the digested DNA fragments and 20 ng of cut-vectors were ligated in a volume of 10 µl with 1 U of T4-DNA ligase and incubated overnight at 4°C. In case of the adhesive-end ligation, two times molar excess of the insert DNA were used, while 10 times molar excess of the insert DNA was used for blunt-end ligation.

The preparation of competent *E. coli* was conducted according to (Chung and Miller, 1988). Briefly, a single colony of *E. coli* was cultured in 2 ml of LB medium for 16 h at 37°C. To start the main culture, 1 ml of bacteria culture was transferred into an Erlenmeyer flask containing 100 ml of the appropriate medium. Bacteria were grown until they reached the logarithmic growth phase (OD600 = 0.4 - 0.6) and collected by centrifugation for 5 min at 4°C, 2500 g. Pellets were taken up in 1/10 volume of ice-cold TSB buffer and the suspension was kept on ice for 10 min. Aliquots of cells were then transferred to pre-cooled Eppendorf tubes and frozen in liquid nitrogen.

For transformation of *E. coli*, the ligation solution (10 µl) was mixed with 20 µl of KCM, 70 µl of sterilized H_2O and 100 µl of competent bacteria and incubated on ice for 30 min. The cells were heat shocked in a 42°C water bath for 40 sec and 800 µl of LB medium was then added to the mixture. After incubation at 37°C for 50 min, aliquots were plated on three LB agar plates containing appropriate antibiotics.

3.2.5 Site-directed mutagenesis

In vitro site-directed mutagenesis is an invaluable technique for studying protein structure-function relationships and gene expression. The QuikChange® Site-Directed Mutagenesis kit (Stratagene) allows site-specific mutation in virtually any double-stranded plasmid. The basic procedure utilizes a supercoiled double stranded DNA (dsDNA) vector with an insert of interest and two synthetic oligonucleotide primers containing the desired mutation or deletion. *PfuTurbo* DNA polymerase replicates both plasmid strands with high fidelity and without displacing the mutant oligonucleotide primers. The oligonucleotide primers are extended during temperature cycles. The mutant synthesis reaction was conducted according to the

manufacturer's instructions and the parameter of the PCR program was set as listed below: pretreatment at 95°C for 1 min; 18 cycles of heating at 95°C for 50 sec, annealing at 60°C for 50 sec and elongation at 68°C assuming an efficiency of 1 kb of plasmid length per minute; the elongation reaction was stopped by incubation at 68°C for 7 min.

Following synthesis the PCR product was digested directly with 1 µl of endonuclease *Dpn I* (10 U/µl) for 1 h at 37°C. The nicked vector DNA incorporating desired mutations was then transformed into *E. coli* 298 and the mutations were confirmed by sequencing analysis.

3.2.6 DNA gel electrophoresis

Electrophoresis uses an electric current to separate different sized molecules in a porous matrix. Smaller DNA molecules move more easily through the gel pores than larger ones. Depending on the size of the DNA fragments, various concentrations of agarose gel (0.9–2 %, w/v) were prepared by adding agarose to 1 × TAE buffer and boiling for several minutes in a microwave oven. DNA samples were mixed with 1/6 volume of DNA loading buffer (6 ×) and the electrophoresis conducted in 1 × TAE buffer with a current of 50 - 70 mA. Finally, DNA stained with ethidium bromide was visualized under UV light.

Denaturing polyacrylamide gels were used for separation and purification of single strand DNA which migrated through the gel almost independent of its base composition. The gel was polymerized in the presence of urea that suppresses base pairing in nucleic acids. To purify oligonucleotide longer than 60 base pairs, 8 % (w/v) of polyacrylamide (effective separation range of 60-400 base pairs) was used. Isolation of DNAs from the polyacrylamide gel was performed with the 'crush and soak' method (Maxam and Gilbert, 1977).

To isolate DNA fragments from agarose, the gel pieces were cut out with a razor blade, frozen in liquid nitrogen and centrifuged through silanized glass wool for 10 min at RT, 13000 g. The solution was extracted once with phenol and DNA precipitated with 3 volumes of 100 % ethanol and 1/10 volume of NaAc (3 M). Finally, the DNA was washed twice with 70 % ethanol and dissolved in TE buffer after drying at 65°C.

3.2.7 Glutathione S-transferase fusion protein purification

The use of pGEX vector allows expression of a protein in *E. coli* as a fusion to glutathione-S-transferase (Smith and Johnson, 1988). To obtain a GST fusion protein DNA sequence encoding a protein of interest was cloned into a pGEX plasmid. The expression of GST fusion protein was induced by addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) during the logarithmic growth phase of bacteria. Briefly, the plasmids for pGEX-Akt2aa410-481 or pGEX-RPS25aa1-70 were transformed into the component *E. coli* BL21 and a single colony

was inoculated in 100 ml of LB medium containing ampicillin (LB/Amp), grown overnight at 37°C. Next day, the overnight culture was diluted to 1 l of LB/Amp medium and grown until OD600 = 0.5 - 0.8. Then IPTG (100 µM) was added to the culture to induce the expression of the GST fusion protein and incubated for 3 h with shaking. Bacteria were centrifuged for 5 min at RT, 8000 g, resuspended in 18 ml of PBS containing 1 ml of lysozyme solution (90 mg/ml), EGTA (1 mM), aprotinin (2 µg/ml) and Triton X-100 (1 %), and lysed with several freeze-thaw cycles until the solution shown increased viscosity. After reducing the viscosity by addition of DNase I (10 mg/ml, 20 µl) and PMSF (100 mM, 200 µl), the solution was centrifuged for 20 min at 4°C, 10000 g and supernatants were incubated with glutathione-Sepharose 4B beads on a rotating wheel for 15 min at RT. Finally, beads were transferred to a Biorad polyprep chromatography column, washed with 10 ml of PBS and eluted with 2 ml of 50 mM Tris (pH 8.0) containing freshly added reduced glutathione (5 mM). The size and the concentration of the purified GST fusion protein were determined by SDS-polyacrylamide gel electrophoresis (10 %).

3.2.8 Affinity purification of antibodies

Affinity purification is based on the specific binding interaction between molecules. A particular ligand is chemically immobilized to a solid support so that when a complex mixture is passed through the column, only those molecules having specific binding affinity to the ligand are purified. Affinity purification of antibodies generally involves the following steps:

(1) Incubate crude sample with the immobilized ligand support material to allow the target molecules in the sample to bind to immobilized ligand.
(2) Wash away unbound sample components from solid support.
(3) Elute the target molecule from immobilized ligand by altering the buffer conditions so that the binding interaction no longer occurs.

Disulfide bridge reduction for cysteine containing peptide

Peptide containing one or more cysteine residues is particularly unstable. If the monomeric form of the peptide is needed for coupling to Thiol-Sepharose, reduction of the disulfide bridge is necessary. In the present work, the synthetic peptide of the amino-terminus of RPS25 (2 mg) was dissolved in 0.5 ml of $NaHCO_3$ (0.1 M) and 3.4 µl of DTT (1 M). The solution was bubbled with nitrogen for 2 min and incubated on a rotating wheel for 45 min at RT. To prepare the desalting column gel, Sephadex G10 (12 ml) was swollen in 30 ml of H_2O for 10 min and washed three times to remove the turbid supernatant. Beads were then equilibrated with acetic acid (0.1 M) until pH 4 - 5 in a Biorad polyprep chromatography column and the peptide solution was applied on this Sephadex G10 column. Finally, the

peptide was eluted with acetic acid (0.1 M) in 6 aliquots of 1 ml. The collected fractions were immediately neutralized with Tris-HCl (pH 7.5) and the OD280 measured.

Affinity purification of anti-RPS25 peptide antibody

To prepare the affinity matrix, Thiol-Sepharose 4B (0.5 g) was swollen in 15 ml of Tris-HCl (20 mM, pH 7.0) for 30 min and equilibrated with a solution of Tris-HCl (100 mM, pH 7.5), NaCl (500 mM) and EDTA (1 mM). The reduced peptide was incubated with these Thiol-Sepharose 4B beads for 2 h at RT followed by an equilibration with TN buffer. The original serum containing anti-RPS25 antibodies (13 ml) was mixed with the peptide-coupled Thiol-Sepharose 4B beads and incubated on a rotating wheel overnight at 4°C. Next day, after washing beads with 10 volumes of TN buffer the RPS25 antibody was eluted with (1) 10 ml of acidic solution (100 mM glycine, 300 mM NaCl, pH 2.5), (2) 10 ml of basic solution (freshly prepared 100 mM triethanolamine, pH 10.3) and (3) 10 ml of chaotropic solution (0.5 M NaI). All elutions were collected in the same 50 ml Falcon tube on ice).

Concentration of affinity purified antibody

To concentrate purified anti-RPS25 antibody, protein A Sepharose (1 ml) was equilibrated with TN buffer and incubated with the dialyzed peptide-affinity purified antibody on a rotating wheel for 2 h at 4°C. The mixture was transferred to a Biorad polyprep chromatography column and washed with 15 - 20 bead volumes of TN buffer. Antibodies were then eluted with 2.5 ml of acidic solution (100 mM glycine and 150 mM NaCl, pH 2.5). The first fraction was collected in a volume of 1.5 ml in a fresh Eppendorf tube and the second fraction of 1.0 ml in another tube. Both fractions were neutralized immediately by addition of Tris-HCl (pH 8.8). To determine the protein concentration the OD280 was measured in a spectrophotometer (Spectronic instruments). Assuming that 1 unit of OD280 corresponds to 0.75 mg/ml of IgG the concentration of purified anti-RPS25 antibody was calculated.

3.2.9 Cell culture

Mammalian cells were incubated in a FunctionLine type Heraeus incubator at 37°C in an atmosphere saturated with H_2O and 7 % CO_2. All manipulations were performed under a sterile bench according to manufacturer's recommendations (Herasafe, Heraeus). HEK 293 and C2C12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 2 mM L-glutamine. Jurkat cells were maintained in RPMI1640 medium supplemented with 10 % FBS and 2 mM L-glutamine.

For storage of cells, subconfluent cells were detached from the surface of culture dishes with trypsin (0.5 % w/v) / EDTA (2 %), centrifuged for 1 min at RT, 1200 g and resuspended in

freezing medium (10 % DMSO in FBS) at a concentration of about 10^7/ml. Aliquots of cell suspension (1 ml) were then transferred to labeled cryovials and left standing in a -70°C freezer for at least 24 h before putting them into liquid nitrogen.

Cells were tested regularly for contamination of mycoplasma using a DNA fluorescent staining. Briefly, adherent cells were grown on sterile glass coverslips for 2 days. After washing with PBS, cells were fixed with cold (-20°C) methanol for 5 min and incubated with the DNA specific dye bisbenzimide (DAPI, 0.1 mg/ml in PBS) for 15 min at 37°C. After six washes with PBS, the coverslips were mounted with 50 % glycerol : PBS (1:1) and inspected with the fluorescence microscope. In case of Jurkat cells, about 100 µl of cell suspension was added into wells with coverslips on which adherent cells had been seeded. After growing for another 2 days, cells were fixed with methanol, stained with DAPI, mounted and inspected with the fluorescence microscope.

3.2.10 Cell treatment

For activation of Akt kinase, cells were serum starved for 20 h and stimulated with insulin (final concentration 100 nM) for 10 min at 37°C before harvesting. Peroxovanadate (POV) is a potent inhibitor for protein tyrosine phosphatases (PTPs). POV was prepared 10 min before using by incubating 0.02 M sodium orthovanadate (Na_3VO_4) with 0.067 % H_2O_2 in distilled H_2O. For detection of tyrosine phosphorylation sites on RPS25 protein, transfected 293 cells were serum starved for 20 h and incubated with POV at a dilution of 1:100 for 15 min at 37°C before harvesting. To analyze the activity of the interleukin-2 (IL-2) promoter, transiently transfected Jurkat cells were first serum starved for 20 h and then stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA, 0.1 µM) in combination with calcimycin A23187 (1 µM) for 6 h at 37°C before harvesting.

3.2.11 Transfection of mammalian cells

To introduce DNA into cultured adherent mammalian cells, a modified calcium phosphate mediated transfection method was used (Chen and Okayama, 1987). Depending on the cell type, up to 80 % of cells will take up DNA through a yet unknown mechanism. There are often two types of transfections. One is transient transfection which generally requires harvesting cells between 1 - 4 days after transfection. Another is permanent or stable transfection. Approximately one in 10^3 to 10^4 cells will stably integrate DNA in a transfection. The stable transfected cell lines could be established with the co-transfection of a dominant selectable marker which induces neomycin resistance of cells (e.g. on the plasmid pSV2neo).

Transient transfection of HEK 293 cells

Twenty-four hours before transfection, subconfluent HEK 293 cells were seeded at about 225000 /ml with 2 ml of DMEM / 10 % FBS in each well of a six-well culture dish. Next day, a DNA mixture containing 4 µg of plasmid DNA in total, 10 µl of $CaCl_2$ (2.5 M), 86 µl of H_2O and 100 µl of BBS was prepared and left for 15 min at RT to allow formation of precipitates. The mixture was then slowly added into the well of a culture dish and cells incubated for 15 - 18 h at 37°C, 3 % CO_2. After one wash with DMEM / 0.5 % FBS, cells were serum starved in DMEM / 0.5 % FBS medium for another 20 h at 37°C, 7 % CO_2.

Stable transfection of Jurkat cells

To generate stable cell lines with a reduced expression of RPS25, a plasmid encoding antisense of RPS25 (pCRZeroU6prom-shRNA-6) and the selectable plasmid pSV2neo were co-transfected into Jurkat cells with the SuperFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. Briefly, Jurkat cells were split on the day before transfection to get logarithmic growing cells. Next day, cells were centrifuged for 1 min at RT, 1200 g and washed once with PBS. About 5×10^6 cells were seeded in a culture dish (10 cm in diameter) with 4 ml of RPMI 1640 / 10 % FBS. The antisense plasmid of RPS25 (shRNA-6; 4.5 µg) and pSV2neo (0.5 µg) were diluted with RPMI 1640 medium to a total volume of 150 µl. After addition of 20 µl of SuperFect Transfection Reagent, the solution was vortexed for 10 sec and left at RT for 8 min. Following addition of 1 ml of RPMI 1640 / 10 % FBS to the tube containing transfection complexes, the mixture was slowly added to Jurkat cells and cells incubated in a 37°C, 7 % CO_2 incubator.

Selection of stable transfectants

Forty-eight hours posttransfection, transfected Jurkat cells were selected by addition of RPMI 1640 / 10 % FBS containing G418 medium which was changed every three days. After 2 weeks, most nontransfected cells had died and, by 3 weeks, G418-resistant cells were visible. The G418-resistant cells were then centrifuged, diluted and placed into 96-well culture dishes with 200 µl of RPMI 1640 / 10 % FBS at about a single G418-resistant cell in each well. After incubation for several days, the clones were expanded in 12-well culture dishes and used either for protein isolation to determine the expression level of RPS25 or stored in liquid nitrogen.

3.2.12 Cell lysis and co-immunoprecipitation

In this work, cells were lysed in a Triton X-100 (1 %) containing cell lysis buffer. Cells were collected from culture dishes and centrifuged for 1 min at RT, 13000 g, washed once with PBS and incubated for 5 min with 200 µl of ice-cold lysis buffer for each well of a six-well

dish. All further steps were done on ice. Lysates were centrifuged for 5 min at 4°C, 13000 g and supernatants transferred to fresh pre-cooled Eppendorf tubes. Twenty microliters of lysate were taken out, mixed with 10 µl of Laemmli buffer and stored at 4°C as whole cell extract samples. The rest of the lysates were used either for co-immunoprecipitation or for immunokinase activity assay.

Both, A and G Sepharose beads, bind to the conserved region of immunoglobulins and the difference lies in their affinity to different classes of IgG. Here, protein A was used for antibodies raised in rabbits and protein G for antibodies from mice. For each co-immunoprecipitation reaction, about 180 µl of lysates were incubated with 200 µl of HNTG buffer, 20 µl of protein A or G Sepharose (1:1 in HNTG) and 2 µg of antibodies on a rotating wheel for at least 3 h in a cold room. The immune complex was collected by centrifugation for 10 sec at 4°C, 5000 g and washed three times with 500 µl of ice-cold HNTG buffer. For each wash step, reaction tubes were incubated on ice for 2 min and then centrifuged for 5 sec at 4°C, 5000 g. Finally, immunoprecipitated proteins were mixed with 25 µl of Laemmli buffer.

3.2.13 SDS polyacrylamide gel electrophoresis

In an SDS-polyacrylamide gel electrophoresis (SDS-PAGE), proteins are separated largely on the basis of polypeptide length. In the present work, we used a Laemmli discontinuous gel which contains a top stacking gel (pH 6.8) and a lower separation gel (pH 8.8). Electrophoresis was performed in a vertical chamber with 1× TG as a running buffer. The used gel (20 × 20 cm, 1.5 mm thick) was prepared as follow:

Stacking gel (a total volume of 15 ml)

2 ml acrylamide (30/0.8), 9.25 ml H_2O, 3.75 ml Upper Tris buffer (4 ×), 150 µl APS (10 %), and 25 µl TEMED

Separation gel (a total volume of 40 ml)

9 %: 12 ml acrylamide (30/0.8), 18 ml H_2O, 10 ml Lower Tris buffer, 270 µl APS (10 %) and 66 µl TEMED

10 %: 13.3 ml acrylamide (30/0.8), 16.7 ml H_2O, 10 ml Lower Tris buffer, 270 µl APS (10 %) and 66 µl TEMED

11 %: 14.8 ml acrylamide (30/0.8), 15.2 ml H_2O, 10 ml Lower Tris buffer, 270 µl APS (10 %) and 66 µl TEMED

As a molecular weight marker, the mixture of following proteins was used:

- myosin (200 kDa);

- β-galactosidase (116 kDa);
- phosphorylase B (97.4 kDa);
- bovine serum albumin (66 kDa);
- chicken egg white ovalbumin (43 kDa);
- carbonic anhydrase (31 kDa);
- Trypsin inhibitor (21.5 kDa);
- α-lactalbumin (14.5 kDa)

Staining protein gels with Coomassie Brilliant Blue (CBB)

Staining solution was made up by mixing of methanol (45 %), acetic acid (10 %) and CBB (0.02 %). The solution was further filtered through a Whatman paper and stored at RT for future use. The gel of interest was covered with the staining solution on a rocker platform, incubated overnight in a cold room and then destained with several changes of destaining buffer until the background was transparent. To make a permanent record, the gel was dried on a gel dryer at 60°C for two hours.

3.2.14 Immunokinase activity assay

After incubation of cell lysates with the Akt2 antibody for 3 h on a rotating wheel in a cold room, the immune complexes were collected by centrifugation, washed three times with 500 µl of HNTG buffer and twice with 500 µl of kinase assay buffer. The reaction was initiated by addition of 50 µl of kinase assay buffer, 2 µg of GST-fused Akt substrate-peptide (GRPRTTSFAES), 100 µM of cold ATP and 10 µCi [γ-^{32}P]ATP, and incubated on a 30°C shaker for 15 min. After addition of 10 µl of glutathione-Sepharose 4B beads to the reaction tubes, the mixtures were incubated for another 15 min and washed three times with 500 µl of HNTG buffer. The phosphorylated Akt substrate-peptide were then analyzed by SDS-PAGE and observed with autoradiography.

3.2.15 Western blotting

Proteins resolved by SDS-PAGE were transferred to nitrocellulose filters using a semidry blot chamber. The transfer was performed for at least 3 h in a cold room at a current of 0.8 mA/cm^2. Filters were then stained with Ponceau S, the molecular weight standard marked with a ball-point pen, unspecific binding sites blocked in NET-G buffer for 2 × 60 min and incubated with primary antibodies (at a dilution of 1:1000 for antiserum or 0.1 - 1 µg/ml of affinity-purified antibody) for at least 1 h at RT or overnight in a cold room with agitation. After washing in NET buffer for 3 × 10 min, filters were further incubated with the secondary

horseradish peroxidase (POD) coupled goat-anti-mouse/rabbit IgG (at a dilution of 1:15000) for 1 h at RT. Finally, filters were washed in NET buffer for 3 × 10 min followed by a detection of proteins using an enhanced chemiluminescence (ECL) kit according to manufacturer's instructions. If reprobing was needed, antibodies were first stripped off the filters by incubation with stripping buffer for 30 min at 55°C and then incubated with a different antibody.

3.2.16 GST pull-down assay

Lysates of HEK 293 cell overexpressing of RPS25 were incubated with 2 µg of purified GST-Akt2aa410-481 or GST alone, 10 µl of washed glutathione-Sepharose 4B beads (1:1 in PBS) and 200 µl of HNTG buffer for at least 3 h on a rotating wheel in a cold room. Following the incubation, beads were washed three times with 500 µl of ice-cold HNTG buffer, protein complexes resolved by SDS-PAGE and further analyzed by Western blotting. In a similar way, 2 µg of GST-RPS25aa1-70 were used to pull-down full length Akt2 that was overexpressed in HEK 293 cell.

3.2.17 Determination of protein and DNA concentrations

A Bradford assay was used to determine protein concentrations for gel electrophoresis (Bradford, 1976). Cell lysates (5 µl) were first diluted in 45 µl of H_2O (1:10) and 10 µl of diluted solution further diluted in 790 µl of H_2O (1:100) which were then mixed with 200 µl of Bradford assay reagent. After incubation at RT for 2 min, absorption of OD595 was measured in a spectrophotometer (Spectronic instruments). For determination of protein concentration, a calibration curve with BSA at concentrations from 2 - 20 µg/ml was carried out.

DNA concentration was determined using a UV spectroscopy. DNA solution (5 µl) was diluted in 995 µl of TE buffer (1:200) in a quartz cuvette. Absorptions of OD260 and OD280 were determined on a spectrophotometer. The concentration of DNA was calculated using the following formula:

DNA concentration (µg/ml) = (OD260) × 200 × 50 µg/ml per 1 OD260 unit

The purity of prepared DNA was estimated by figuring the ratio of OD260/OD280. Pure DNA should have a ratio of 1.8 at least.

3.2.18 Immunofluorescence

On the day before transfection, subconfluent C2C12 cells were seeded at about 37000 /ml with 1 ml of DMEM/10 % FBS in each well of 12-well culture dishes which contained sterile

glass coverslips. The DNA mixture containing plasmids for RPS25 (0.5 µg), Akt2-myc (0.1 µg), Akt1-myc (0.1 µg) and/or eIF3c-VSV (1.0 µg) as indicated was transfected into cells using the modified calcium phosphate method. Thirty-six hours posttransfection, cells were washed twice in PBS and fixed with cold (–20°C) methanol for at least 20 min. Fixed-cells were washed in PBS for 2 × 5 min, blocked with PBS/10 % FBS for 20 min at RT with agitation and incubated with primary antibodies overnight in a cold room. Rabbit anti-RPS25 antibody, rabbit c-myc polyclonal antibody and monoclonal VSV / HA / myc antibodies were used at a dilution of 1:50 or at a concentration of 1 ng/µl. After washing in PBS/10 % FBS for 3 × 10 min, cells were further incubated with secondary antibodies, Alexa Fluor® 488/546 conjugated goat anti-mouse/rabbit IgG (1:400 dilution in PBS/10 % FBS), for 1.5 h at RT with agitation. Finally, cells were washed in PBS/10 % FBS for 3 × 10 min and in PBS for 3 × 10 min, briefly rinsed with H_2O and coverslips mounted with PermaFluor (Immunotech). Images were taken with a Leica TCSNT laser scanning software connection with an inverted Leica DM IRBE microscope (63× HCX PL APO immersion oil objective).

3.2.19 Determining cell growth curve using trypan blue staining

The parental Jurkat cells and the stably reduced expression of RPS25 cell lines (clones 8, 9, 12 and 24) were seeded at the same concentration (5000 /ml) with 1 ml of RPMI 1640/10 % FBS medium in each well of 24-well plates. Aliquots of the cell suspension were taken out to count cell numbers with trypan blue staining every other day. The number of live cells was used to determine the growth rate and experiments repeated three times in duplicate. Results are shown as means + S.D. and analyzed with Student's t-Test. $p<0.05$ indicates a significant difference.

3.2.20 Luciferase activity assay

For investigation of the effect of Akt2 and RPS25 on the activity of the IL-2 promoter, a combination of plasmids for IL-2 promoter controlled *firefly* luciferase (0.5 µg), RPS25 (0.2 µg), shRNA-6 of RPS25 (0.2 µg) and Akt2 (0.1 µg), as indicated, were transiently transfected into Jurkat cells using the SuperFect Transfection Reagent. As an internal control of the transfection efficiency, pRL-TK (0.1 µg) was included in each transfection. Sixty-six hours posttransfection, cells were stimulated simultaneously with TPA (0.1 µM) and A23187 (1 µM) for 6 h at 37°C and lysed with passive lysis buffer (PLB, Promega). Luciferase activity was measured by using 5 µl of cell lysates on a Magic Lite Analyzer (Chiron Diagnostics, Fernwald) according to the manufacturer's instructions. Normalized relative luciferase unit (RLU) was calculated as following: value of IL-2 *firefly* luciferase / value of pRL-TK *Renilla*

luciferase of the same sample. Experiments were repeated three times in duplicate.

For investigating the effect of Akt and RPS25 on protein translation, the combination of pRL-TK luciferase plasmid (0.5 µg), Akt1 (0.5 µg), Akt2 (0.5 µg), RPS25 (0.5 µg) and/or shRNA-6 of RPS25 (1.0 µg), as indicated, were transiently transfected into HEK 293 cells using the modified calcium phosphate method. Seventy-two hours posttransfection, cells were harvested with the PLB buffer and protein concentrations of lysates determined with the Bradford assay. The same amount of lysate was used to measure the luciferase activity according to the manufacturer's instructions (Promega). Relative luciferase unit (RLU) was normalized to the amount of measured protein. Experiments were repeated three times in duplicate. Results are shown as means + S.D. and analyzed with Student's t-Test. $p<0.05$ indicates a significant difference.

4 Results

4.1 Identification of proteins interacting with Akt2

4.1.1 Yeast two-hybrid screen

To identify novel Akt2 binding proteins, we employed the yeast two-hybrid method in this work. Different Akt2 fragments, the PH domain of Akt2 (aa1-141); the truncated Akt2ΔPH (aa142-481); the central catalytic kinase domain of Akt2 (aa100-420); the carboxyl-terminus of Akt2 (aa310-481; aa360-481 and aa410-481) and full length Akt2 (Fig. 4.1A), were cloned into the bait vector pBTM116 that contains a LexA DNA-binding domain. To establish yeast bait strains, bait plasmids were transformed into the yeast host strain L40 and the expressions of the LexA-Akt2 bait fusion proteins were confirmed by Western blotting (Fig. 4.1B). Yeast expression of bait proteins was further analyzed with the β-galactosidase assay to confirm that the expression of the bait protein did not autonomously induce the reporter gene *LacZ*. In a second step, different human cDNA libraries (brain, pancreas, placenta, liver, and skeletal muscle) were transformed to each yeast bait strain. Aliquots of primary yeast transformants were grown on UTL plates to determine the transformation efficiency, and on THULL plates for selection of *HIS3+* colonies which were further selected with the β-galactosidase assay to get finally *HIS3+/LacZ+* positive yeast transformants. More than 2.5×10^6 primary transformants were achieved for each human cDNA library. Since the *HIS3+/LacZ+* positive yeast clone contains two types of plasmids (the bait and the prey plasmids), prey plasmids encoding the Akt2 candidate interacting proteins were prepared by retransforming *E. coli* HB101 with isolated yeast DNA.

False positives, which are *HIS3+/LacZ+* but do not contain a bait interacting protein, often arise during the yeast two-hybrid screen. To exclude the false positives, we first conducted a pre-screening PCR by using the isolated yeast DNA as a template and oligonucleotide primers which were specific for promyelocytic leukemia zinc finger protein (PLZF), a transcription factor frequently found with some bait constructs. Colonies which produced no product of PLZF were further used as templates to perform screening PCRs with oligonucleotide primers specific for the pACT2 prey plasmid (Fig. 4.1C). By using all seven bait proteins, 279 positive yeast transformants from different human cDNA libraries were finally analyzed with the screening PCRs.

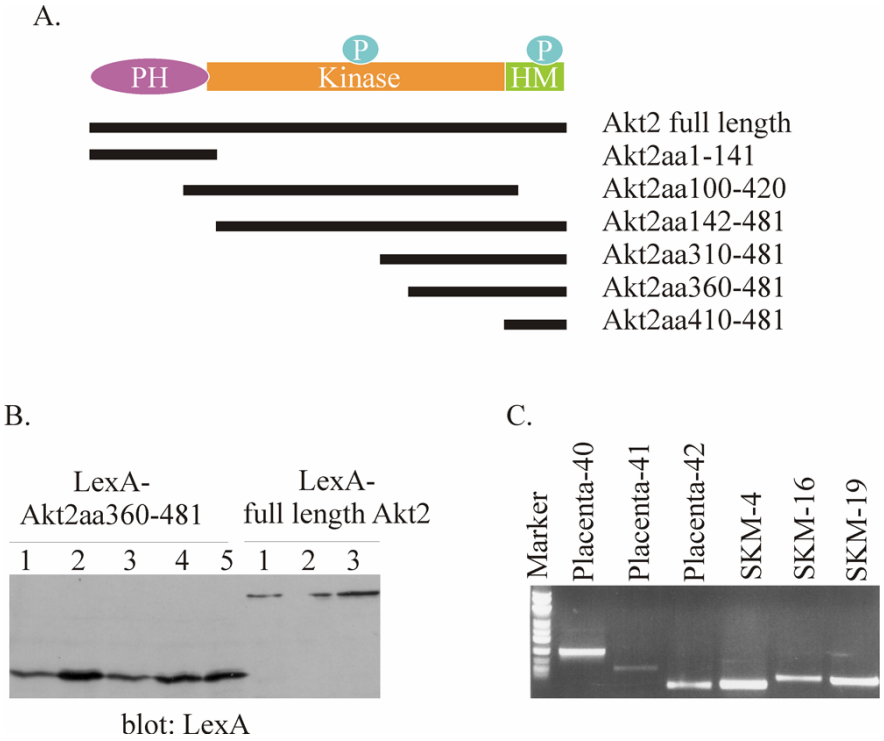


Figure 4.1 Identification of Akt2 binding proteins using the yeast two-hybrid method. (A) A schematic representation of the Akt2 domain structure is shown. The cDNA fragments used as baits in the two-hybrid system are depicted. (B) Western blotting to confirm the expression of the LexA-Akt2 bait fusion proteins. Proteins from the selected yeast bait strains were isolated and resolved by SDS-PAGE, transferred to nitrocellulose and the filter probed with anti-LexA antibody. Here, LexA-full length Akt2 and LexA-Akt2aa360-481 fusion proteins are shown. (C) Screening PCRs for *HIS3+/LacZ+* positive yeast transformants. PCR products were separated on an agarose gel (0.9 %). Here, six screening PCR results are shown as examples.

Only with the full length Akt2 and Akt2aa360-481 we were able to get thirty-six *HIS3+/LacZ+* positive yeast transformants. Sequencing and BLAST database analysis revealed that these thirty-six colonies were either complete or part of cDNAs of six different proteins as summarized in Table 4.1. Methyl-CpG binding domain protein 1 (MBD1) was the only protein identified with both Akt2 constructs as bait. Putative NFκB activating protein (NFκBAP) and eukaryotic translation initiation factor 3c (eIF3c, also named eIF3S8, eIF3 p110) were found in different human cDNA libraries only with the full length Akt2 as a bait, while GABA(A) receptor-associated protein (GABARAP), thyroid autoantigen 70 kDa protein (Ku70) and human ribosomal protein S25 (RPS25) were found in various human libraries only with the Akt2aa360-481 as a bait. Notably, RPS25 was found sixteen times in human brain, skeletal muscle, and pancreas cDNA libraries, and eIF3c was found four times in human liver, placenta, pancreas and skeletal muscle cDNA libraries.

Table 4.1 Akt2 binding proteins identified in the yeast two-hybrid system. Six Akt2

candidate binding proteins were identified using either full length Akt2 or Akt2aa360-481 as bait. SKM: skeletal muscle.

Akt2 full length				Akt2aa360-481			
Candidates	cDNA library	Primary Transformants	Positive Colonies	Candidates	cDNA library	Primary Transformants	Positive Colonies
NFkBAP	Placenta	$3{,}60 \times 10^6$	1	MBD1	Placenta	$3{,}60 \times 10^6$	1
	SKM	$3{,}55 \times 10^6$	2	GABARAP	Brain	$2{,}53 \times 10^6$	3
eIF3c	Placenta	$3{,}60 \times 10^6$	1		SKM	$3{,}55 \times 10^6$	2
	SKM	$3{,}55 \times 10^6$	1	**RPS25**	Brain	$2{,}53 \times 10^6$	1
	Liver	$7{,}50 \times 10^6$	1		SKM	$3{,}55 \times 10^6$	3
	Pancreas	$2{,}54 \times 10^6$	1		Pancreas	$2{,}54 \times 10^6$	12
MBD1	Placenta	$3{,}60 \times 10^6$	1	Ku70	Pancreas	$2{,}54 \times 10^6$	2
					Placenta	$3{,}60 \times 10^6$	3
					Brain	$2{,}53 \times 10^6$	1

NFkBAP: putative NFkB activating protein
eIF3c: eukaryotic translation initiation factor 3 subunit 8, molecular weight 110 kDa.
MBD1: methyl-CpG binding domain protein 1
GABARAP: GABA(A) receptor-associated protein
RPS25: ribosomal protein S25
Ku70: thyroid autoantigen 70kDa (Ku antigen)

4.1.2 Interaction of Akt2 and new candidate binding proteins

In general, the yeast two-hybrid results need to be further confirmed by other independent methods such as pull-down assays or co-immunoprecipitations. Most of these assays often use the epitope-tagged version of the proteins involved. In this work, we used a co-immunoprecipitation method to test the potential interactions of Akt2 and its candidate binding proteins in intact mammalian cells. The library insert of RPS25 included the full length cDNA coding sequence, thus we directly cut it from the prey plasmid and cloned it into the cytomegalovirus (CMV) immediate early promoter based expression vector pRK. However, the inserts of the other five Akt2 binding candidates in the prey plasmids included only part of the coding sequences. To get the full length cDNAs, we first cloned the 5'- or 3'-end of the cDNAs from various available EST clones and then combined them with the partial coding sequence from prey plasmids. Finally, the full length cDNA was cloned into the mammalian expression vector pRK. To enable the detection of Akt2 candidate binding proteins, constructs tagged with a hemagglutinin (HA) or vesicular stomatitis virus (VSV) epitope at either amino- or carboxyl-terminus were prepared as well.

Considering the high sequence similarity among Akt isoforms, Akt1, Akt2 or Akt3 were transiently transfected into HEK 293 cells alone or together with one of the candidates, NFκBAP-VSV, GABARAP-VSV, Ku70-VSV, RPS25-VSV or MBD1-HA. The eIF3c protein was not included here because it had been found as an Akt candidate interacting protein in a yeast two-hybrid screen in our group before. Transfected cells were harvested,

lysates incubated with the VSV or HA antibodies, and the co-immunoprecipitated proteins in immune complexes analyzed with the Akt antibody. As shown in Fig. 4.2, NFκBAP (calculated MW, 105 kDa), Ku70 (calculated MW, 70 kDa), MBD1 (calculated MW, 65 kDa) and GABARAP (calculated MW, 14 kDa) did not interact with any of the three Akt isoforms in intact mammalian cells. VSV-tagged NFκBAP, GABARAP and Ku70 were a little bigger than the calculated molecular sizes which might be caused by posttranslational modification. However, HA-tagged MBD1 was much smaller than its calculated molecular weight of 65 kDa which might be due to protein degradation.

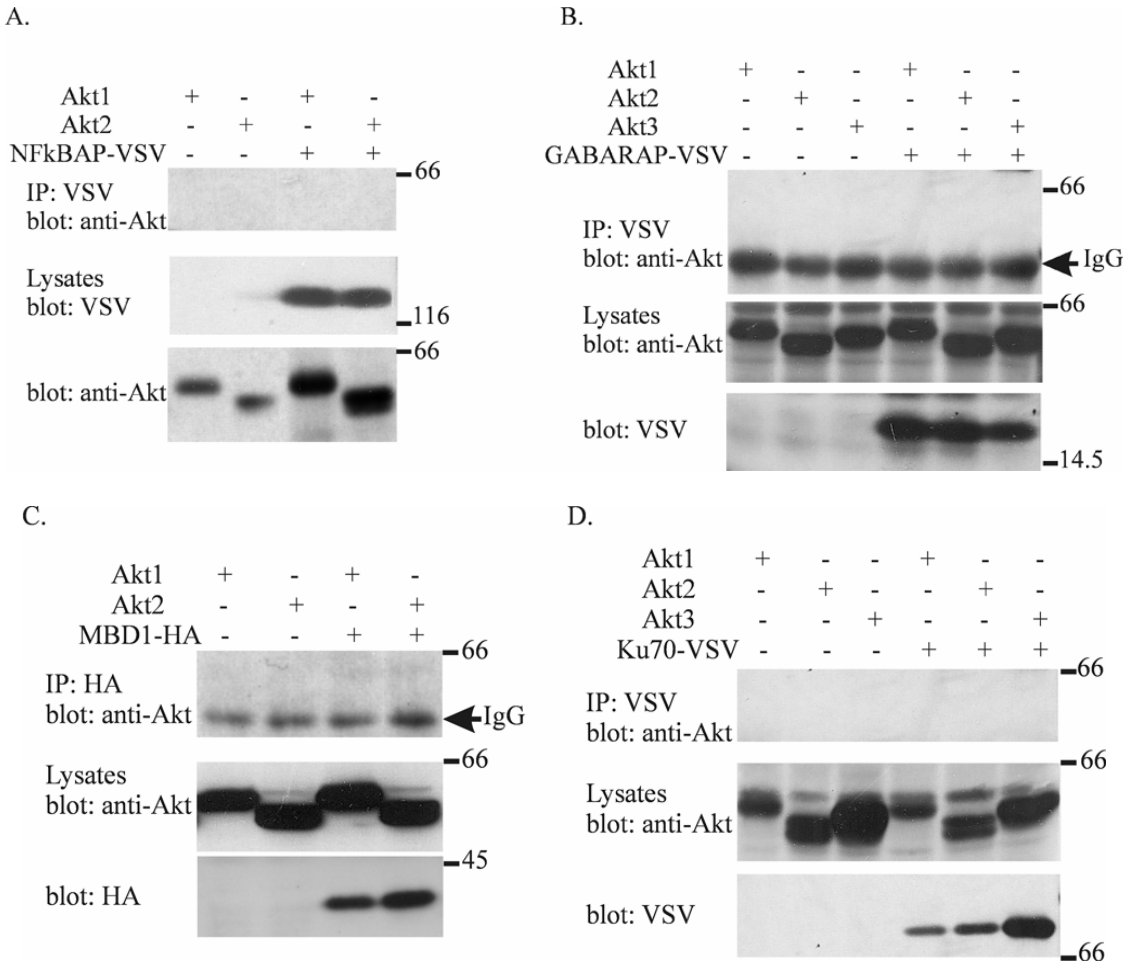


Figure 4.2 Co-immunoprecipitation of Akt2 and its candidate binding proteins. Akt1, Akt2 or Akt3 were transiently transfected into HEK 293 cells alone or together with VSV-tagged NFκBAP, GABARAP, Ku70 or HA-tagged MBD1 as indicated. Thirty-six hours posttransfection, cells were harvested and lysates incubated with either VSV (A, B and D) or HA (C) antibodies. The immune complexes were resolved by SDS-PAGE (A and D, 8 %; B and C, 11 %), proteins transferred to nitrocellulose and filters probed with anti-Akt antibody. Aliquots of lysates were analyzed for the expressions of Akt and its candidate binding proteins.

The Akt2 isoform did co-immunoprecipitate with VSV-tagged human ribosomal protein S25

(RPS25) in HEK 293 cells, but Akt1 and Akt3 did not (Fig. 4.3A). Similarly, co-overexpression of HA-tagged RPS25 and Akt2 in HEK 293 cells and incubation of cell lysates with the Akt antibody led to the co-immunoprecipitation of RPS25-HA (Fig. 4.3B). These results support the interaction of Akt2 and RPS25 identified in our yeast two-hybrid system and suggest that RPS25 specifically interacts with the Akt2 isoform but not with Akt1 and Akt3 in mammalian cells. Notably, VSV- or HA-tagged RPS25 was bigger than the calculated molecular weight of 14 kDa.

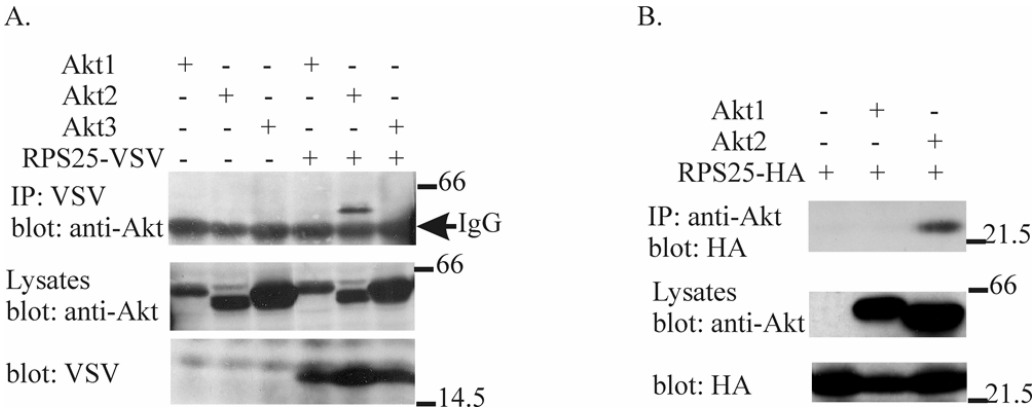


Figure 4.3 RPS25 specifically interacts with Akt2 in HEK 293 cells. VSV- or HA-tagged RPS25 were transiently transfected into HEK 293 cells alone or together with Akt1, Akt2, or Akt3 as indicated. Thirty-six hours posttransfection, cells were harvested and lysates incubated with VSV (A) or Akt (B) antibodies. The immune complexes were resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and filters probed with anti-Akt (A) or HA (B) antibodies as indicated. Aliquots of lysates were analyzed for the expressions of Akt and RPS25.

RPS25 is a highly basic protein of the 40S small ribosomal subunit (pI 10.58). It is unique to eukaryotes, 100 % identical to rat ribosomal protein S25 and 46 % identical to yeast ribosomal protein S31 (Wool et al., 1995). The gene of RPS25 is assigned to human chromosome 11q23.3 (Imai et al., 1994), and the protein consists of 125 amino acids (Li et al., 1991). Analysis of the deduced amino acid sequence of RPS25 indicated there were two putative tyrosine phosphorylation sites at Tyr55 and Tyr65 (Fig. 4.4), while there was no putative phosphorylation site for Akt kinase. Kubota et al. (1999) reported that the nuclear localization sequence of RPS25 lay in the region of amino acids 25-41.

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                                CTTCCTTTTTGTCCGACATCTTGACGAGGCTGCGGT  36

                                        M   P   P   K   D   D   K
GTCTGCTGCTATTCTCCGAGCTTCGCA ATG CCG CCT AAG GAC GAC AAG  84

 K   K   K   D   A   G   K   S   A   K   K   D   K   D
AAG AAG AAG GAC GCT GGA AAG TCG GCC AAG AAA GAC AAA GAC  126

 P   V   N   K   S   G   G   K   A   K   K   K   K   W
CCA GTG AAC AAA TCC GGG GGC AAG GCC AAA AAG AAG AAG TGG  168

 S   K   G   K   V   R   D   K   L   N   N   L   V   L
TCC AAA GGC AAA GTT CGG GAC AAG CTC AAT AAC TTA GTC TTG  210

 F   D   K   A   T   Y   D   K   L   C   K   E   V   P
TTT GAC AAA GCT ACC TAT GAT AAA CTC TGT AAG GAA GTT CCC  252

 N   Y   K   L   I   T   P   A   V   V   S   E   R   L
AAC TAT AAA CTT ATA ACC CCA GCT GTG GTC TCT GAG AGA CTG  294

 K   I   R   G   S   L   A   R   A   A   L   Q   E   L
AAG ATT CGA GGC TCC CTG GCC AGG GCA GCC CTT CAG GAG CTC  336

 L   S   K   G   L   I   K   L   V   S   K   H   R   A
CTT AGT AAA GGA CTT ATC AAA CTG GTT TCA AAG CAC AGA GCT  378

 Q   V   I   Y   T   R   N   T   K   G   G   D   A   P
CAA GTA ATT TAC ACC AGA AAT ACC AAG GGT GGA GAT GCT CCA  420

 A   A   G   E   D   A   *
GCT GCT GGT GAA GAT GCA TGA ATAGGTCCAACCAGCTGTACATTTGGA  468
AAAATAAAACTTTATTAAATCAAAAAAAAAAAAAAAAAAAAAAAA           514
```

Figure 4.4 The sequence of RPS25. The cDNA and the deduced amino acid sequence of human ribosomal protein S25 (RPS25). The amino-terminal nuclear localization sequence (aa25-41) is underlined, the potential tyrosine phosphorylation sites Y55 and Y65 are given in bold, and the start/stop codons are given in bold and are underlined.

4.1.3 Expression of RPS25 in mammalian cells

To demonstrate that RPS25 is expressed in our cell lines, we developed an antibody directly against RPS25 protein. First, we tried to use a GST-full length RPS25 fusion protein to generate antibody in rabbits. Unfortunately, this fusion protein was insoluble. We then generated an antibody in rabbits against an amino-terminal RPS25 peptide and affinity purified the antibody from the antiserum. To demonstrate specificity of the antibody, wild-type RPS25 was transiently overexpressed in HEK 293 cells, cell lysates resolved by SDS-PAGE and proteins analyzed with the raw antiserum, the affinity-purified anti-RPS25 antibody and the RPS25-antibody-depleted serum. As shown in Fig. 4.5A, we detected a single protein that was bigger than the calculated molecular weight of 13.7 kDa, which might be caused by posttranslational modification. Further, we overexpressed HA-tagged RPS25 in HEK 293 cells, and aliquots of cell lysates were analyzed with the HA and peptide-affinity

purified anti-RPS25 antibody. Untransfected 293 cell lysates were included as a control. The molecular size of RPS25-HA detected by the HA antibody was the same as that detected by the anti-RPS25 antibody, suggesting that our antibody is specific for the protein of RPS25 (Fig. 4.5B). We next investigated the expression of RPS25 in various mammalian cells. HEK 293, Jurkat, murine C2C12 and rat L6 cells were lysed and cell extracts incubated with the peptide-affinity purified anti-RPS25 antibody. As shown in Fig. 4.5C, RPS25 protein was expressed in all these cell lines.

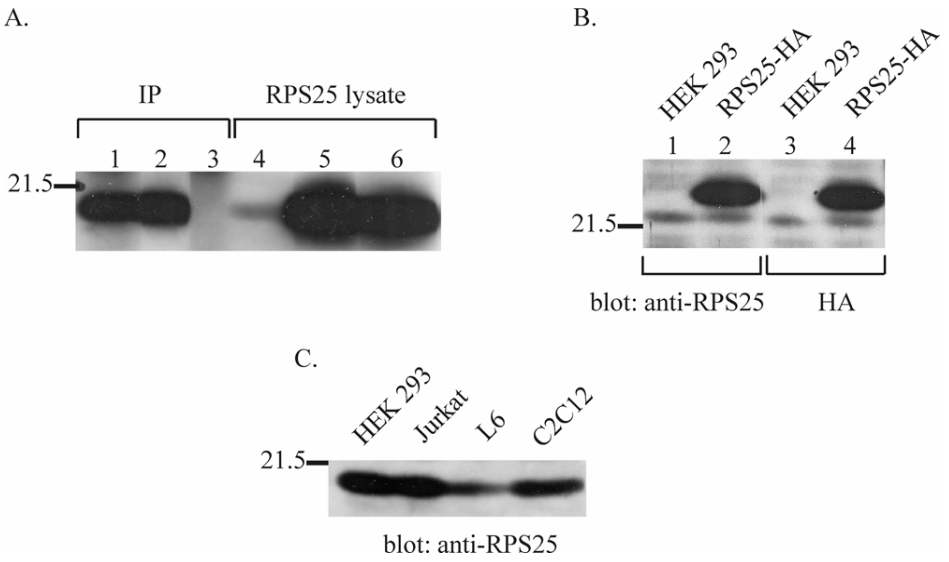


Figure 4.5 Expression of RPS25 in mammalian cells. (A) RPS25 was transiently transfected into HEK 293 cells. Thirty-six hours posttransfection, cells were harvested and lysates incubated with raw antiserum (lane 1), affinity-purified anti-RPS25 antibody (lane 2) and RPS25-antibody-depleted serum (lane 3). The immune complexes and aliquots of lysates were resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and the filter probed with raw antiserum (lanes 1 and 6), affinity-purified anti-RPS25 antibody (lanes 2 and 5) and RPS25-antibody-depleted serum (lanes 3 and 4) as indicated. (B) HA-tagged RPS25 was transiently transfected into HEK 293 cells. Thirty-six hours posttransfection, cells were harvested. Overexpressed RPS25-HA and untransfected HEK 293 cell lysates were resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose, and filters probed with anti-RPS25 (lanes 1 and 2) and HA (lanes 3 and 4) antibodies as indicated. (C) Aliquots of lysates of subconfluent HEK 293, Jurkat, murine C2C12 and rat L6 cells were resolved by SDS-PAGE (11 %), transferred to nitrocellulose and the filter probed with anti-RPS25 antibody.

4.1.4 Interaction of RPS25 with endogenous Akt2

Overexpression of proteins in cells may lead to the formation of complexes that are not formed in untransfected cells. Therefore, we tried to detect the interaction between endogenous RPS25 and Akt2 in mammalian cells. Considering that most ribosomal proteins were assembled into the ribosomal subunits under physiological conditions, we employed both untransfected cells and cells overexpressing RPS25 only. The harvested cell lysates were

incubated either with the rabbit IgG as a control or with the anti-Akt2 antibody, and the immune complexes analyzed using Western blotting with the anti-RPS25 antibody. As shown in Fig. 4.6, overexpressed RPS25 was co-immunoprecipitated with endogenous Akt2, but the endogenous RPS25 from untransfected cells was not detected by immunoprecipitation of endogenous Akt2.

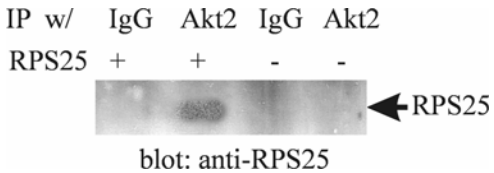


Figure 4.6 Overexpressed RPS25 interacts with endogenous Akt2. Subconfluent untransfected HEK 293 cells and cells overexpressing RPS25 were harvested and cell lysates incubated with anti-Akt2 antibody. The immune complexes were resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and the filter probed with anti-RPS25 antibody (arrow). The same lysates were incubated with the rabbit IgG as controls.

4.1.5 RPS25 associates with unphosphorylated Akt2

Akt is activated by growth factor stimulation of the cell and fully activated Akt will contain two phosphorylated residues, Thr308 in the central kinase domain and Ser473 in the hydrophobic motif. Crystal structure analysis indicates that the conformation of active Akt kinase is much more ordered than that of inactive Akt in the activation segment and in the αC helix (Yang et al., 2002). Thus, the phosphorylation state of Akt2 may influence its interaction with other proteins, as has been shown for CTMP (Maira et al., 2001). To investigate the phosphorylation state of RPS25-associated Akt2, HA-tagged RPS25 and Akt2 were transiently transfected into HEK 293 cells and cells left unstimulated or stimulated with insulin before harvesting. As shown in Fig. 4.7A, Ser473 phospho-specific antibody failed to detect the co-immunoprecipitated Akt2 when lysates were incubated with the HA antibody and analyzed by Western blotting. Reblotting of the same filter with the Akt2 antibody showed that RPS25 associated only with unphosphorylated Akt2. Moreover, the insulin stimulation was without effect on the interaction of Akt2 and RPS25 (Fig. 4.7A). A similar experiment was conducted in HEK 293 cells, and the immune complexes were analyzed by Western blotting with the Thr308 phospho-specific antibody. As shown in Fig. 4.7B, RPS25-associated Akt2 was not phosphorylated at threonine 308 and insulin stimulation did not affect the interaction of Akt2 and RPS25. Thus, we conclude that insulin stimulation does not affect the interaction of Akt2 and RPS25, and that RPS25 specifically associates with unphosphorylated Akt2 in mammalian cells.

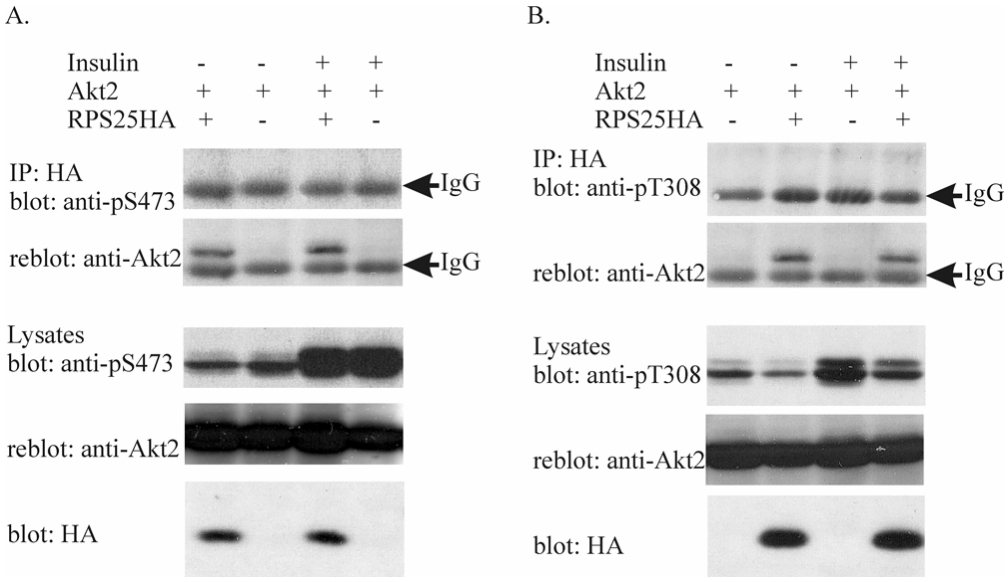


Figure 4.7 RPS25 interacts with unphosphorylated Akt2. Akt2 was transiently transfected into HEK 293 cells alone or together with RPS25-HA as indicated. Thirty-six hours posttransfection, serum-starved cells were stimulated with insulin (100 nM) for 10 min at 37°C or left untreated before harvesting. Lysates were incubated with the HA antibody, immune complexes resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and filters probed with Ser473 (A) and Thr308 (B) phospho-specific antibodies as indicated. Aliquots of lysates were analyzed for the overexpression of Akt2 and RPS25-HA. To confirm the presence of Akt2 in immunoprecipitation fractions, the nitrocellulose filters probed with phospho-specific antibodies were stripped and reprobed with anti-Akt2 antibody.

4.2 Detection of tyrosine phosphorylation of RPS25

Computer analysis of the RPS25 peptide sequence using Scansite (http://scansite.mit.edu) found two putative tyrosine phosphorylation sites, Tyr55 and Tyr65. We wanted to investigate whether RPS25 was tyrosine phosphorylated in intact mammalian cells. HA-tagged RPS25 was transiently overexpressed in HEK 293 cells and cells were treated with peroxovanadate (POV), a potent inhibitor of tyrosine phosphatases, for 10 min before harvesting. Lysates were incubated with the HA antibody and immune complexes analyzed with the phosphotyrosine specific antibody. In a control experiment, cells overexpressing RPS25-HA were directly harvested without any pretreatment. As shown in Fig. 4.8, there was no phosphorylated tyrosine residue detected on RPS25 in the immune complex fractions or in the whole cell extracts. However, when the same filter was reprobed with HA antibody, we showed the presence of RPS25-HA in both immunoprecipitate and cell lysate. Therefore, we conclude that the protein of RPS25 in HEK 293 cells is not phosphorylated on tyrosine residues.

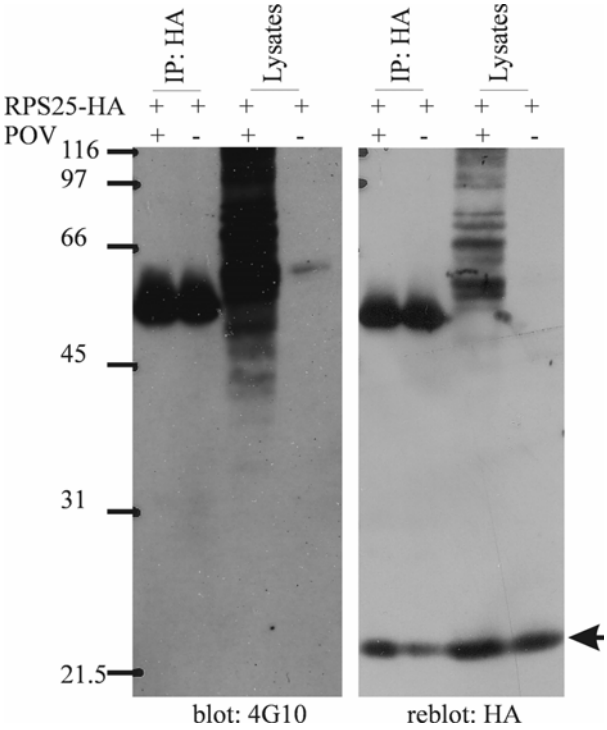


Figure 4.8 RPS25 is not tyrosine phosphorylated. HA-tagged RPS25 was transiently transfected into HEK 293 cells. Thirty-six hours posttransfection, cells were treated with peroxovanadate for 10 min at 37°C or left untreated before harvesting as indicated. Lysates were incubated with HA antibody, immune complexes resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and the filter probed with 4G10 antibody to detect tyrosine phosphorylation. To confirm the presence of RPS25-HA, the same filter was stripped and reprobed with HA antibody (arrow). POV, peroxovanadate.

4.3 Mapping the RPS25 binding site of Akt2

To define the region of protein-protein interaction, a series of Akt2 truncation mutants were used to map the RPS25 binding site of Akt2. Considering that the bait protein used in the yeast two-hybrid screen was Akt2aa360-481, we first cloned the carboxyl-terminal regulatory domain (aa410-481) of Akt2 into a pGEX vector to generate a GST-Akt2aa410-481 fusion protein. A GST pull-down assay was performed with overexpressed RPS25 from HEK 293 cell lysates. As shown in Fig. 4.9A, Akt2aa410-481 was not able to pull-down the wild-type RPS25 in an *in vitro* protein binding assay. We then generated plasmids encoding Akt2aa377-481 and Akt2aa394-481 to explore their abilities to interact with RPS25. We used Akt2aa364-481 that has four amino acids less than the bait used in the yeast two-hybrid screen as a control. The Akt2 truncation mutants were transiently overexpressed in HEK 293 cells alone or together with HA-tagged RPS25, lysates incubated with the HA antibody and immune complexes analyzed by Western blotting with the Akt2 antibody. As shown in Fig. 4.9B, Akt2aa364-481 and Akt2aa377-481 were co-immunoprecipitated with overexpressed RPS25-

HA, while Akt2aa394-481 was not. These results suggest that the amino acids 377-394 of Akt2 are responsible for its binding to RPS25 in intact mammalian cells.

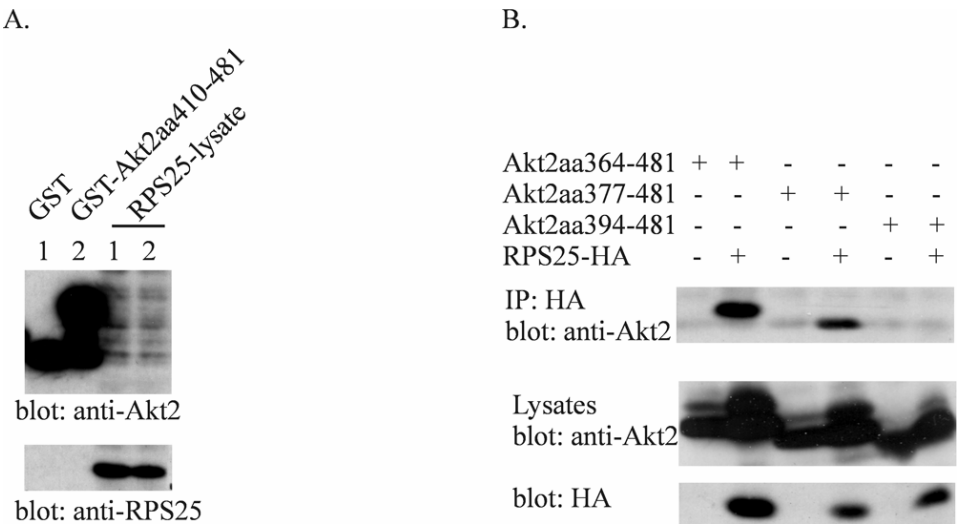


Figure 4.9 Amino acids 377-394 of Akt2 are necessary for the interaction with RPS25. (A) GST pull-down assay of overexpressed RPS25 using GST-Akt2aa410-481 fusion protein or GST alone as a control. RPS25 was overexpressed in HEK 293 cells and aliquots of lysates were used for the pull-down assay. (B) Myc-tagged Akt2aa364-481, Akt2aa377-481 and Akt2aa394-481 were transiently expressed in HEK 293 cells alone or together with RPS25-HA as indicated. Thirty-six hours posttransfection, cells were harvested and lysates incubated with HA antibody. The immune complexes were resolved by SDS-PAGE (11 %), transferred to nitrocellulose and filters probed with anti-Akt2 antibody. Aliquots of lysates were analyzed for the expressions of truncated Akt2 and RPS25-HA.

To further narrow the RPS25 binding site of Akt2, we used an Akt2aa385-481 construct. As positive controls, Akt2aa364-481 and Akt2aa377-481 were included. Similarly, Akt2aa385-481 was transiently overexpressed in HEK 293 cells alone or together with RPS25-VSV and lysates incubated with the VSV antibody. Here, immune complexes were resolved on a 9 % SDS-PAGE because we observed an unspecific protein band which had a similar molecular size as Akt2aa377-481 (see Fig. 4.9B). As shown in Fig. 4.10A, Akt2aa385-481 kept its ability to interact with RPS25, but the interaction was much weaker than that of the other two Akt2 truncation mutants, which means that the region of Akt2aa385-394 is involved in the interaction with RPS25.

We next made Akt2 deletion mutants where different amino acids in the region of amino acids 377-394 were deleted (Fig. 4.10D) to assess their binding abilities to full length RPS25. These Akt2 deletion mutants were transiently overexpressed in HEK 293 cells together with RPS25-VSV. Cell lysates were incubated with either VSV or Akt2 antibody and immune complexes analyzed by Western blotting. Akt2Δ387-392, Akt2Δ389-390 and Akt2Δ391-392 still had the

ability to interact with RPS25 (Fig. 4.10B), while Akt2Δ377-385 failed to bind to the protein of RPS25 (Fig. 4.10C). Taken together, these data suggest that amino acids 377-385 of Akt2 are the determinant for the interaction of RPS25 and Akt2 in mammalian cells.

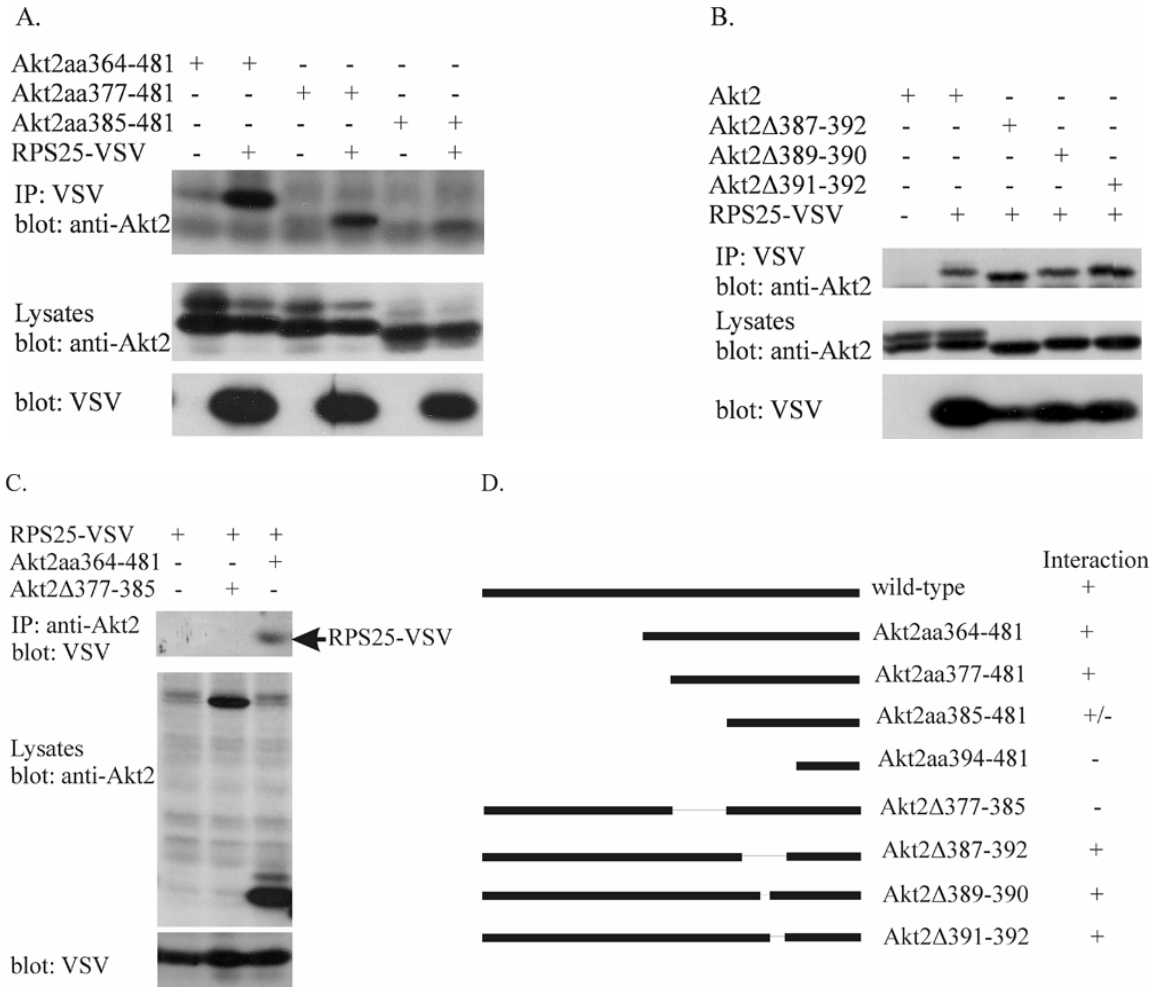


Figure 4.10 RPS25 binding site is mediated by amino acids 377-385 of Akt2. (A) Myc-tagged Akt2aa364-481, Akt2aa377-481 and Akt2aa385-481 were transiently transfected into HEK 293 cells alone or together with VSV-tagged RPS25 as indicated. Thirty-six hours posttransfection, cells were harvested and lysates incubated with VSV antibody. The immune complexes were resolved by SDS-PAGE (9 %), proteins transferred to nitrocellulose and filters probed with anti-Akt2 antibody. Aliquots of lysates were resolved by SDS-PAGE (11 %) and analyzed for the expression of proteins of interest. (B, C) Deletion mutants Akt2Δ387-392, Akt2Δ389-390, Akt2Δ391-392 and Akt2Δ377-385 were transfected into HEK 293 cells together with VSV-tagged RPS25 as indicated. Wild-type Akt2 (B) or Akt2aa364-481 (C) were included as controls. Lysates were incubated with VSV or anti-Akt2 antibody, immune complexes resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and filters probed with anti-Akt2 or VSV antibody as indicated. Aliquots of lysates were analyzed for the expressions of proteins of interest. (D) The truncation and deletion mutants of Akt2 and their abilities to interact with full length RPS25.

The catalytic kinase domain localizes in the central region of Akt2. To investigate whether the deletion of the RPS25 binding site would affect the kinase activity, one of the deletion

mutants, Akt2Δ387-392, was used to perform an *in vitro* kinase activity assay. Wild-type Akt2, kinase inactive mutant Akt2-K181A and deletion mutant Akt2Δ387-392 were transiently overexpressed in HEK 293 cells and lysates incubated with the Akt2 antibody. The immune complexes were further incubated with a GST-fused Akt substrate-peptide and [γ-^{32}P]ATP. After the reaction, protein mixtures were resolved by SDS-PAGE (11 %) and the γ-^{32}P labeled Akt substrate-peptide was detected with autoradiography. As shown in Fig. 4.11, wild-type Akt2 phosphorylated the Akt substrate-peptide upon insulin stimulation, while Akt2Δ387-392 and Akt2-K181A failed to induce the phosphorylation of substrate-peptide, suggesting that deletion of amino acids from the central region of Akt2 leads to a loss of the kinase activity. Considering that the determinant region for the binding of RPS25 to Akt2 was the amino acids 377-385 which was quite near to the amino acids 387-392, we did not further characterize this binding site because an inactive mutant Akt2 without the RPS25 binding site likely could not be employed to determine physiological activities of RPS25.

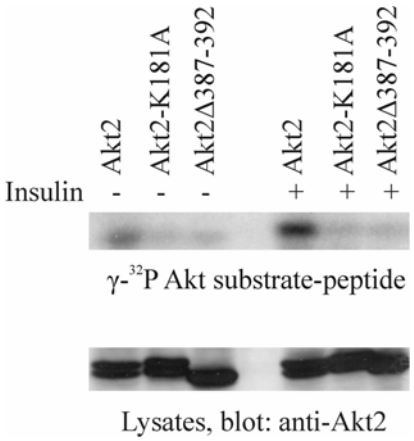


Figure 4.11 Deletion mutant Akt2Δ387-392 looses its kinase activity. The wild-type Akt2, the kinase inactive mutant Akt2-K181A and the deletion mutant Akt2Δ387-392 were transfected into HEK 293 cells and lysates incubated with anti-Akt2 antibody. The immunoprecipitated Akt kinase was further incubated with a GST-fused Akt substrate-peptide (2 μg) and [γ-^{32}P] ATP at 30°C for 30 min. After extensive washing, proteins were separated by SDS-PAGE (11 %), the resolved protein gel dried on a gel-dryer and analyzed with autoradiography. Aliquots of lysates were analyzed for the expressions of proteins of interest.

We have shown that RPS25 interacts specifically with the Akt2 isoform and that amino acids 377-394 of Akt2 are critical for the interaction with RPS25 in mammalian cells. We next wanted to investigate the structural basis for this Akt-isoform specific interaction. By comparing the deduced amino acid sequence between Akt1 and Akt2, we found only one residue difference in the region of amino acids 377-394, Ala382 in Akt2 and Ser381 in Akt1 (Fig. 4.12A). To find out whether this single amino acid difference determines the isoform-

interaction specificity, we prepared a point mutant Akt2-A382S where the Ala382 of Akt2 was changed to serine. Wild-type Akt2 and point mutant Akt2-A382S were transiently transfected into HEK 293 cells alone or together with RPS25-HA, lysates incubated with the HA antibody and immune complexes analyzed by Western blotting with the Akt2 antibody. As shown in Fig. 4.12B, both proteins, mutant Akt2-A382S and wild-type Akt2, interacted with RPS25-HA in HEK 293 cells with a similar efficiency. Therefore, we propose that there are additional binding epitopes that regulate the Akt isoform-specific interaction with RPS25.

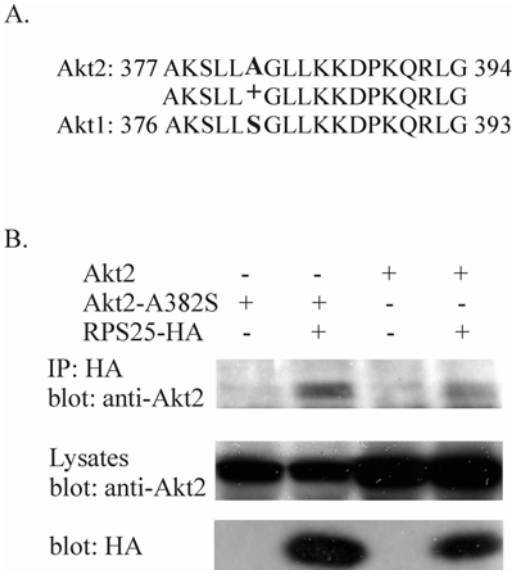


Figure 4.12 Point mutant Akt2-A382S interacts with RPS25-HA. (A) Peptide sequence similarity between Akt1 and Akt2. The different residue, Ala382 in Akt2 and Ser381 in Akt1, is shown in bold. (B) Point mutant Akt2-A382S and wild-type Akt2 were transfected into HEK 293 cells alone or together with RPS25-HA as indicated. Thirty-six hours posttransfection, cells were harvested and lysates incubated with HA antibody. The immune complexes were resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and filters probed with anti-Akt2 antibody. Aliquots of lysates were analyzed for overexpressed proteins.

4.4 Mapping the Akt2 binding site of RPS25

A series of RPS25 deletion mutants were transiently overexpressed in HEK 293 cells to map the binding domain that was responsible for the interaction with Akt2. We first performed a GST pull-down assay to investigate whether the amino-terminal region of RPS25 interacts with Akt2 or not. Wild-type Akt2 was transiently transfected into HEK 293 cells and lysates incubated with a GST-RPS25aa1-70 fusion protein. The protein complexes were resolved by SDS-PAGE, transferred to nitrocellulose and different parts of the filter detected with the Akt2 or RPS25 antibody. As shown in Fig 4.13A, RPS25aa1-70 failed to pull-down the overexpressed wild-type Akt2 in an *in vitro* protein binding assay. We then generated

plasmids encoding a HA-tagged truncated RPS25aa1-112, RPS25aa1-109, RPS25aa1-106 and RPS25aa1-88 to explore their abilities to interact with Akt2 in HEK 293 cells. To clearly demonstrate the co-immunoprecipitated Akt2 proteins which are located closely to the heavy chain of IgG on the protein gel, myc-tagged Akt2 was used instead of wild-type Akt2. Myc-tagged Akt2 was transiently transfected into HEK 293 cells alone or together with one of truncated RPS25 constructs, cell lysates incubated with the HA antibody and immune complexes analyzed by Western blotting with the myc antibody. As shown in Fig. 4.13B, all four carboxyl-terminal deletion mutants of RPS25 were co-immunoprecipitated with Akt2-myc, indicating that the central region of RPS25 is responsible for its binding to Akt2 kinase. Taken together, we conclude that amino acids 70-88 of RPS25 are critical for the interaction with Akt2 in mammalian cells.

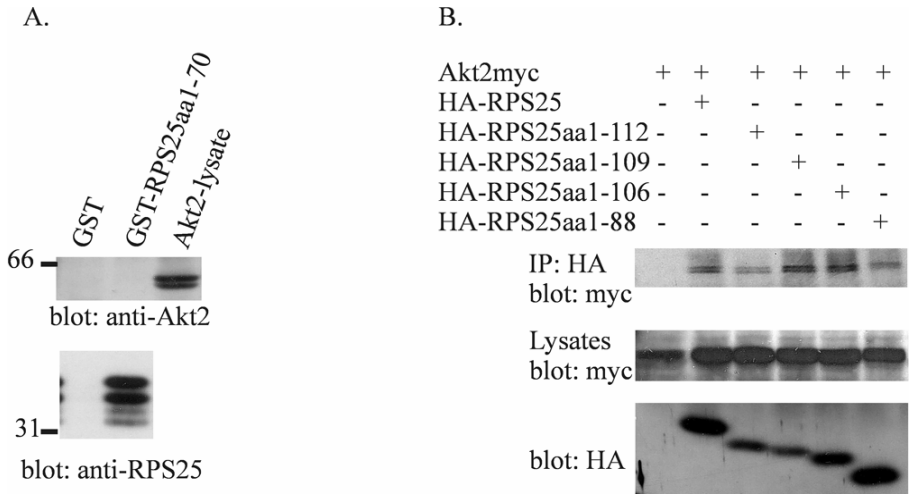


Figure 4.13 Amino acids 70-88 of RPS25 bind to the Akt2 protein. (A) GST pull-down assay of overexpressed Akt2 using GST-RPS25aa1-70 fusion protein or GST alone as a control. Akt2 was overexpressed in HEK 293 cells and aliquots of lysates were used for the pull-down assay. (B) Myc-tagged Akt2 was transiently transfected into HEK 293 cells alone or together with HA-tagged full length RPS25, RPS25aa1-112, RPS25aa1-109, RPS25aa1-106 or RPS25aa1-88 as indicated. Thirty-six hours posttransfection, cells were harvested and lysates incubated with HA antibody. The immune complexes were resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and filters probed with myc antibody. Aliquots of lysates were analyzed for the expressions of proteins of interest.

4.5 RPS25 improves the interaction of Akt2 and eIF3c

A cross-linking experiment from (Tolan et al., 1983) identified RPS25 as one of the ribosomal proteins that interacts with the eIF3 complex. The human eIF3 complex consists of at least 11 subunits, designated eIF3a-eIF3k, in the order of decreasing size from 167 kDa to 25 kDa (Browning et al., 2001). It interacts with the 40S small ribosomal subunit and plays a key role in formation of the 43S pre-translational initiation complex and mRNA binding. The eIF3c

subunit has been reported to interact with eIF5 and eIF1 factors (Phan et al., 1998; Valasek et al., 2004). In the present work, we identified eIF3c as an Akt2 interacting protein in the yeast two-hybrid screen. Sequence analysis of the eIF3c indicated that there was a potential phosphorylation site for Akt kinase at Ser711 (RRRMIS*). To investigate potential interactions among these proteins, various combinations of Akt2, eIF3c-VSV and RPS25 were transiently transfected into HEK 293 cells. Cell lysates were incubated with the Akt2 antibody and immune complexes analyzed by Western blotting with the VSV antibody. Lysates from cells overexpressing eIF3c-VSV or RPS25 alone were included as controls. Although the VSV antibody detected an unspecific protein that had a similar size as eIF3c-VSV, the presence of eIF3c-VSV, which was just above the unspecific protein band, was detected only upon the triple-overexpression of Akt2, RPS25 and eIF3c-VSV (Fig. 4.14A; lane 4), but not upon the co-overexpression of Akt2 and eIF3c-VSV (Fig. 4.14A; lane 2), indicating that eIF3c interacts with Akt2 in an RPS25-expression dependent manner.

A reciprocal immunoprecipitation experiment was conducted as well. Akt2 was co-overexpressed with eIF3c-VSV or with eIF3c-VSV and RPS25 in HEK 293 cells and lysates incubated with the VSV antibody. The immune complexes were analyzed by Western blotting with the Akt2 antibody. As shown in Fig. 4.14B, Akt2 did co-immunoprecipitate with eIF3c-VSV in an RPS25-expression dependent manner (lane 1 versus lane 2). Taken together, we conclude that the interaction of Akt2 and eIF3c might be not strong enough to survive conditions in the co-immunoprecipitation assay, while additionally overexpression of RPS25 improves the interaction of eIF3c and Akt2 in mammalian cells.

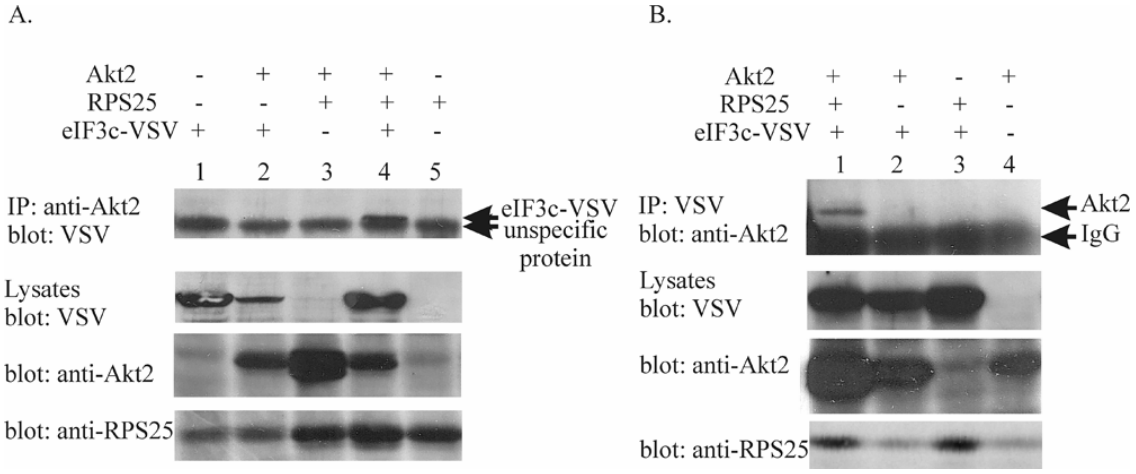


Figure 4.14 RPS25 improves the interaction of Akt2 and eIF3c. VSV-tagged eIF3c, Akt2 and RPS25 were transiently transfected into HEK 293 cells as indicated. Thirty-six hours posttransfection, cells were harvested and lysates incubated with anti-Akt2 (A) or VSV (B) antibodies. The immune complexes were resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and filters probed with VSV (A) or anti-Akt2 (B) antibodies as indicated.

Aliquots of lysates were analyzed for the expressions of eIF3c-VSV, Akt2 and RPS25.

We have shown that RPS25 specifically interacts with Akt2 in mammalian cells. To investigate whether RPS25-improved interaction of Akt2 and eIF3c is also dependent on the Akt isoforms, Akt1 or Akt2 were transiently transfected into HEK 293 cells together with eIF3c-VSV or with RPS25 and eIF3c-VSV. Cell lysates were incubated with the VSV antibody and immune complexes analyzed by Western blotting with Akt2 and RPS25 antibodies. As shown in Fig. 4.15, Akt2 interacted with eIF3c only when RPS25 protein was overexpressed simultaneously (lane 4 versus lane 5), while Akt1 did not whether RPS25 was overexpressed or not (lane 1 versus lane 2). Moreover, RPS25 was co-immunoprecipitated with eIF3c-VSV directly (lane 6), and overexpression of either Akt1 or Akt2 decreased the interaction efficiency between eIF3c and RPS25 (lanes 2, 4 versus lane 6). These results indicate that RPS25-mediated interaction of eIF3c and Akt2 is dependent on the specific association of Akt2 and RPS25 in mammalian cells.

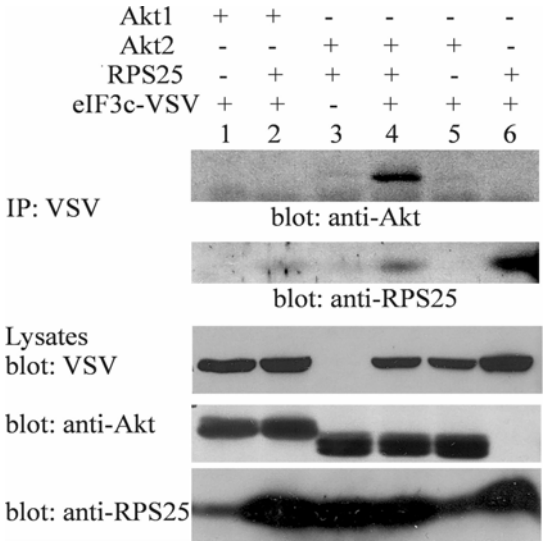


Figure 4.15 RPS25 improves the interaction of Akt2 and eIF3c. Akt1, Akt2, RPS25 and VSV-tagged eIF3c were transiently transfected into HEK 293 cells as indicated. Thirty-six hours posttransfection, cells were harvested and lysates incubated with VSV antibody. The immune complexes were resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and different parts of filters probed with anti-Akt and anti-RPS25 antibodies as indicated. Aliquots of lysates were analyzed for the expressions of eIF3c-VSV, Akt1, Akt2 and RPS25.

To detect whether the reduced interaction of eIF3c and RPS25 is due to the phosphorylation of eIF3c at Ser711 by Akt, we generated two point mutants, eIF3c-S711A mimicking the unphosphorylated serine residue, and eIF3c-S711D which mimics the phosphorylated serine residue. VSV-tagged wild-type or mutant eIF3c were transfected into HEK 293 cells together

with RPS25 or with RPS25 and Akt2, and cells stimulated with insulin or left untreated before harvesting. Cell lysates were incubated with the VSV antibody and immune complexes analyzed by Western blotting with the RPS25 antibody. As shown in Fig. 4.16A, neither eIF3c-S711A nor eIF3c-S711D had any effect on the interaction with RPS25 compared to wild-type eIF3c (lanes 2, 4 and 6), and upon the presence of Akt2 the interaction efficiency was decreased to a similar degree (lanes 3, 5 and 7). When cells were stimulated with insulin, the interaction of eIF3c and RPS25 was weaker than in unstimulated cells (Fig. 4.16B). However, the total protein amount was also lower than in the unstimulated cells. Therefore, we conclude that insulin stimulation is without effect on the interaction of eIF3c and RPS25, and that the Akt2-mediated reduced interaction of RPS25 and eIF3c is not dependent on phosphorylation of Ser711.

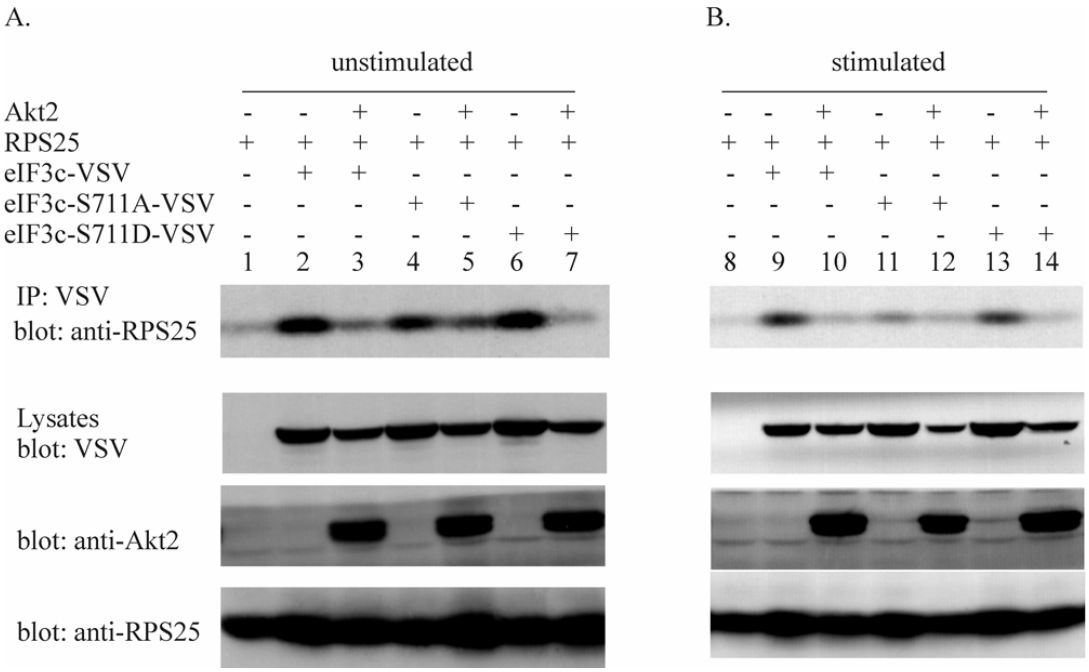


Figure 4.16 Phosphorylation state of Ser711 does not affect the interaction of eIF3c and RPS25. VSV-tagged wild-type eIF3c and mutants eIF3c-S711A or eIF3c-S711D were transiently transfected into HEK 293 cells together with RPS25 or with Akt2 and RPS25 as indicated. Thirty-six hours posttransfection, cells were left unstimulated (A) or stimulated with insulin (100 nM) for 10 min at 37°C (B) before harvesting. The cell lysates were incubated with VSV antibody, immune complexes resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and filters probed with anti-RPS25 antibody. Aliquots of lysates were analyzed for the expressions of eIF3c-VSV, eIF3c-S711A-VSV, eIF3c-S711D-VSV, Akt2 and RPS25.

4.6 Intracellular localization of RPS25

4.6.1 RPS25 localizes in the nucleus of C2C12 cells

There is no report yet on the cellular localization of wild-type RPS25. In the present work, we performed an indirect immunofluorescence experiment to detect its cellular distribution. The rabbit raised peptide-affinity purified RPS25 antibody was used to immunostain RPS25 in C2C12 cells that were fixed and permeabilized with chilled methanol. The specific cellular localization was visualized using a secondary antibody coupled to a fluorescent dye, Alexa Fluor® 488 or Alexa Fluor® 546. Untransfected C2C12 cells were stained only with secondary antibodies as control (Fig. 4.17A). As shown in Fig. 4.17B, endogenous RPS25 was predominantly found in the nucleus of C2C12 cells. C2C12 cells transiently transfected with RPS25 were also stained with anti-RPS25 antibody, and overexpressed RPS25 was again mainly found in the nucleus (Fig. 4.17C). To further confirm the specificity of RPS25 antibody, HA-tagged RPS25 was transiently overexpressed in C2C12 cells and cells stained with either HA or RPS25 primary antibodies (Fig. 4.17D and E). The nuclear localization of RPS25-HA detected by both antibodies supported the conclusion that overexpressed RPS25 mainly localized in the cell nucleus, which is consistent with the reported result that RPS25 contained an amino-terminal nuclear localization sequence (Kubota et al., 1999).

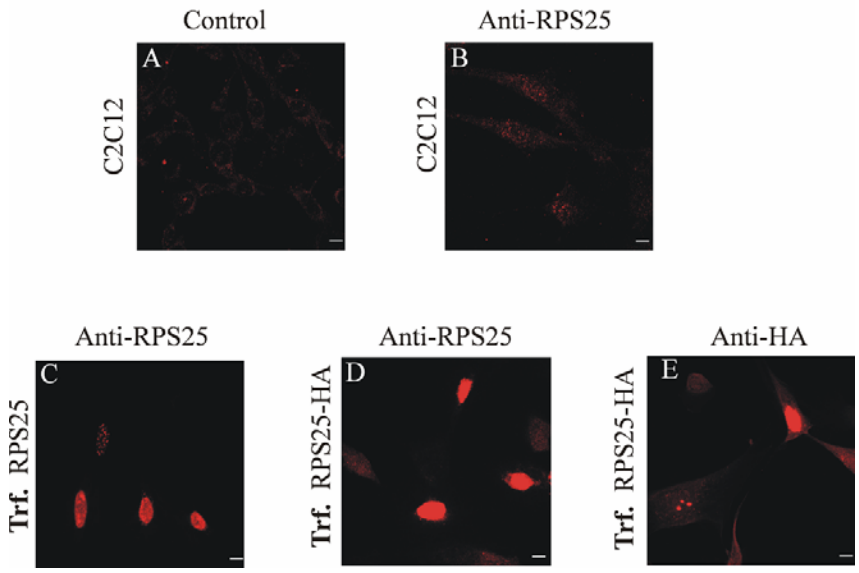


Figure 4.17 Cellular localizations of RPS25 protein in C2C12 cells. C2C12 cells were transiently transfected with RPS25 or RPS25-HA as indicated. Thirty-six hours posttransfection, immunofluorescence was performed as indicated. Untransfected C2C12 cells were stained with secondary antibody only (A) as control. Endogenous or overexpressed RPS25 were stained with rabbit anti-RPS25 antibody (B and C). RPS25-HA was stained with

either rabbit anti-RPS25 (D) or mouse monoclonal HA (E) antibodies. Images were taken with a Leica TCSNT laser scanning microscope and processed with Adobe Photoshop CS2 software. Scalebar: 10 μm. Trf: transfection.

4.6.2 RPS25 increases the nuclear translocation of Akt2

To investigate the effect of the interaction of RPS25 and Akt2 on their cellular distributions, we first detected the cellular localization of Akt2. Since both antibodies against RPS25 and Akt2 were raised in rabbits, we used myc-tagged Akt2 instead of wild-type Akt2 to transfect C2C12 cells so that the double staining of RPS25 and Akt2 was possible. As controls, untransfected C2C12 cells were stained with goat anti-rabbit IgG (Alexa Fluor® 488) and goat anti-mouse IgG (Alexa Fluor® 546) only (Fig. 4.18A and B). C2C12 cells overexpressing Akt2-myc were fixed, stained with mouse monoclonal myc antibody and secondary goat anti-mouse IgG (Alexa Fluor® 546). As shown in Fig. 4.18C, Akt2 protein mainly localized in the cytoplasm of C2C12 cells. Akt2-myc and RPS25 were then co-transfected into C2C12 cells and cells stained simultaneously with rabbit anti-RPS25 and mouse monoclonal myc antibodies. The secondary antibodies, goat anti-rabbit IgG (Alexa Fluor® 488) and goat anti-mouse IgG (Alexa Fluor® 546), were used to visualize the proteins of interest. As shown in Fig. 4.18D-F, RPS25 was still mainly found in the nucleus, while Akt2-myc was found in both the cytoplasm and the nucleus. The merged image showed that Akt2-myc colocalized with RPS25 in the nucleus. To confirm that the nuclear appearance of the co-overexpressed Akt2-myc is not due to the background fluorescence of RPS25, Akt2-myc and RPS25 were co-overexpressed in C2C12, cells stained with myc antibody only and visualized with goat anti-mouse IgG (Alexa Fluor® 546). As shown in Fig. 4.18G, Akt2-myc was evenly distributed in the cytoplasm and in the nucleus. Therefore, we conclude that co-overexpression of RPS25 results in the nuclear translocation of Akt2.

We have shown that RPS25 specifically interacts with Akt2, but not with Akt1 or Akt3 isoforms. Therefore, myc-tagged Akt1 was transfected into C2C12 cells together with RPS25 and the cellular distributions of both proteins were recorded. As shown in Fig 4.18H-J, in contrast to Akt2-myc, co-overexpressed Akt1-myc still predominantly localized in the cytoplasm while RPS25 was mainly detected in the nucleus. RPS25 failed to increase the nuclear accumulation of Akt1-myc, indicating that the enhanced nuclear translocation of Akt2-myc is dependent on its specific interaction with RPS25.

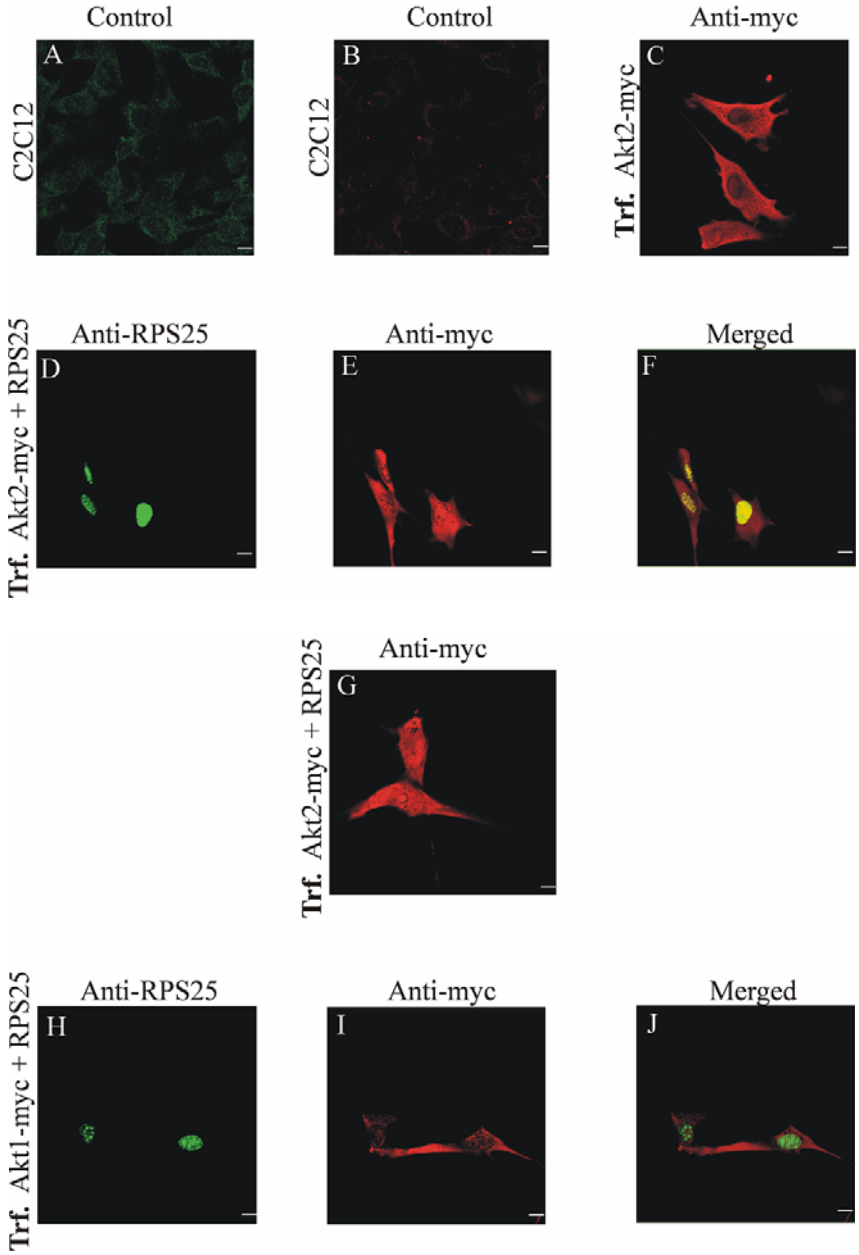


Figure 4.18 Overexpression of RPS25 increases the nuclear translocation of Akt2. C2C12 cells were left untransfected or transiently transfected with plasmids encoding Akt1-myc, Akt2-myc, or RPS25 as indicated. Thirty-six hours posttransfection, cells were fixed with chilled methanol, incubated with secondary antibody only (red, A; green, B) as controls, or incubated with mouse monoclonal myc antibody (red, C, E, G and I) or rabbit anti-RPS25 antibody (green, D and H). Alexa Fluor® 488/546 labeled goat anti-mouse/rabbit IgG were used as secondary antibodies. Images were taken and processed as described in Fig. 4.17. Scalebar: 10 µm. Trf: transfection.

4.6.3 eIF3c does not affect cellular distribution of Akt2 or RPS25

We have shown that RPS25 interacts directly with eIF3c in mammalian cells. We wanted to investigate whether the cellular localization of RPS25 would be affected by this kind of interaction. Since the anti-RPS25 antibody was generated in rabbits, a VSV-epitope tagged eIF3c was used to enable a double staining. eIF3c-VSV was first transfected alone into

C2C12 cells. Cells were fixed with chilled methanol, stained with mouse monoclonal VSV antibody and visualized with goat anti-mouse IgG (Alexa Fluor® 546) secondary antibody. As shown in Fig. 4.19A, eIF3c completely localized in the cytoplasm of C2C12 cells.

Further, RPS25 or Akt2-myc were transfected into C2C12 cells together with eIF3c-VSV. The cells were double stained with mouse monoclonal VSV and rabbit anti-RPS25 or rabbit c-myc antibodies, and visualized with goat anti-rabbit IgG (Alexa Fluor® 546) and goat anti-mouse IgG (Alexa Fluor® 488) secondary antibodies. As shown in Fig. 4.19B-G, co-overexpressed eIF3c-VSV still was found in the cytoplasm. RPS25 and Akt2-myc localized in the nucleus and the cytoplasm, respectively, similar to the pattern of single overexpressed proteins, indicating that eIF3c does not change the cellular localization of either RPS25 or Akt2 in mammalian cells.

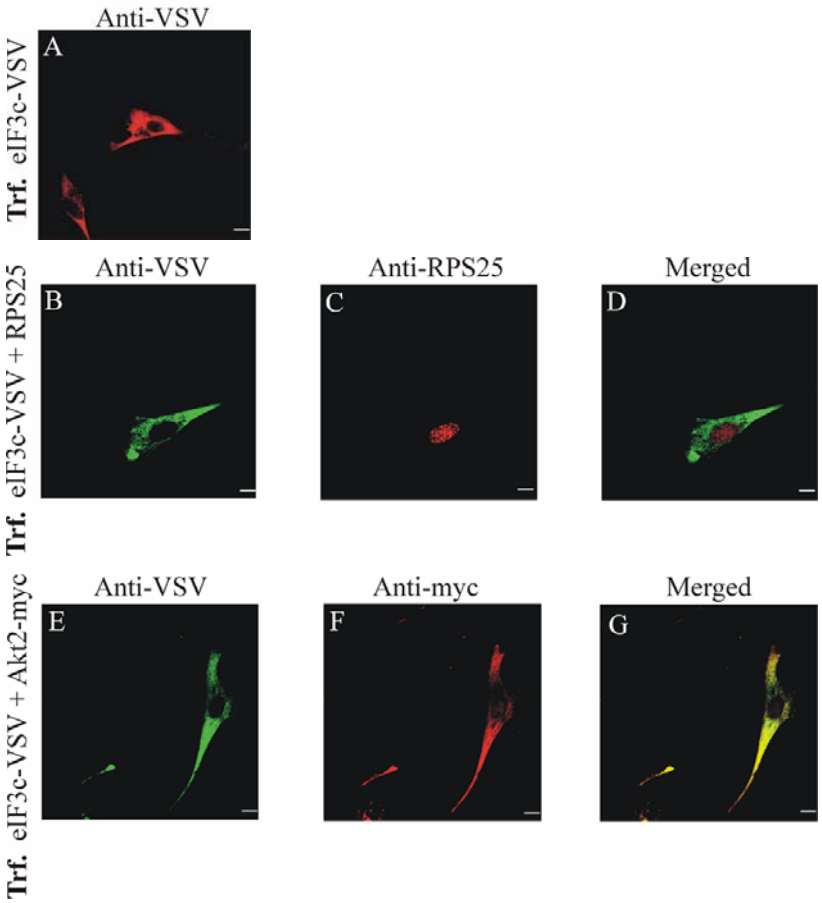


Figure 4.19 Overexpression of eIF3c does not affect the cellular distribution of Akt2 or RPS25. VSV-tagged eIF3c was transiently transfected into C2C12 cells alone or together with Akt2-myc or RPS25 as indicated. Thirty-six hours posttransfection, cells were fixed with chilled methanol. Overexpressed eIF3c-VSV was detected with mouse monoclonal VSV antibody (red, A). Co-overexpressed eIF3c-VSV and RPS25 were detected with mouse monoclonal VSV (green, B) and rabbit anti-RPS25 (red, C) antibodies. The merged image is shown in D. Co-overexpressed eIF3c-VSV and Akt2-myc were detected with mouse monoclonal VSV (green, E) and rabbit c-myc (red, F) antibodies. The merged image is shown in G. Images were taken and processed as described in Fig. 4.17. Scalebar: 10 µm. Trf:

transfection.

Based on the results that Akt2 interacts with eIF3c in an RPS25-expression dependent manner and that RPS25 increases the Akt2 nuclear translocation in mammalian cells, we wanted to know whether the cellular distributions of these proteins would be changed by the interaction. To address this question, eIF3c-VSV, Akt2-myc and RPS25 were simultaneously transfected into C2C12 cells and any two of them were stained with their specific antibodies. When cells were double stained with mouse monoclonal VSV and rabbit anti-RPS25 antibodies to visualize eIF3c and RPS25 (Fig. 4.20A-C), we did not find any change of the cellular localizations compared to the co-overexpression of these two proteins. When staining the cells with mouse monoclonal VSV and rabbit c-myc antibodies for proteins of Akt2 and eIF3c, we found Akt2-myc distributed in both the cytoplasm and the nucleus, and eIF3c still localized mainly in the cytoplasm (Fig. 4.20D-F). Similarly, the transfected cells were also stained with rabbit anti-RPS25 and mouse monoclonal myc antibodies for visualizing RPS25 and Akt2. The enhanced nuclear accumulation of Akt2-myc and the mainly nuclear localization of RPS25 were observed as well (Fig. 4.20G-I). These data indicate that overexpression of eIF3c-VSV, Akt2-myc and RPS25 did not affect the cellular distributions of proteins compared to the pattern of co-overexpression of any two of them.

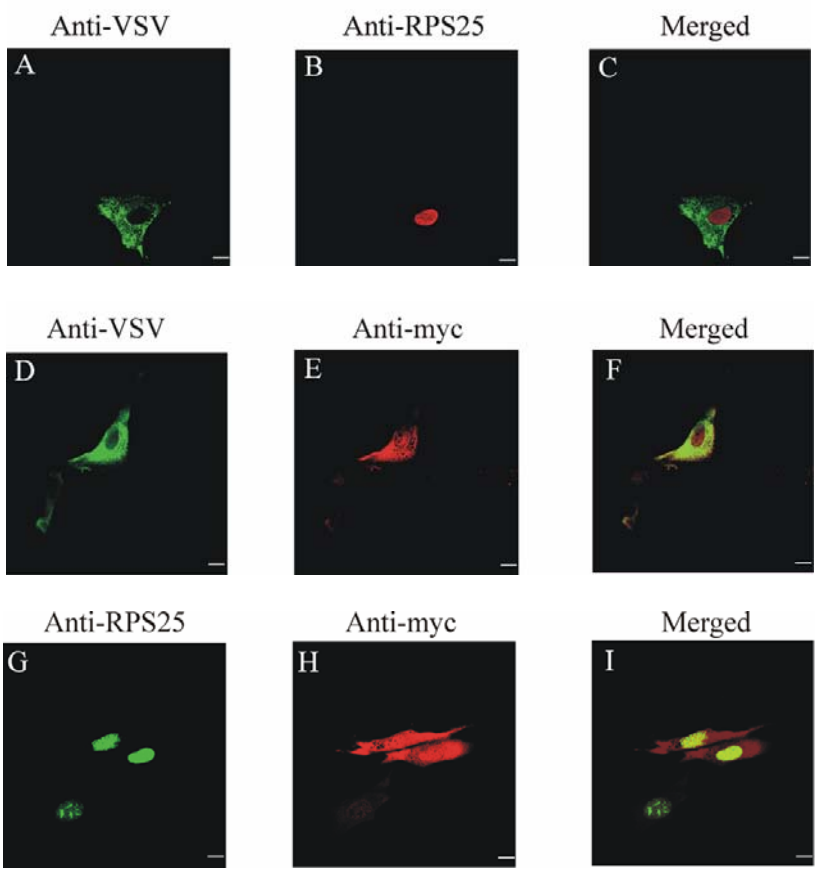


Figure 4.20 Cellular distributions of eIF3c-VSV, Akt2-myc and RPS25 upon overexpression of all three proteins. Akt2-myc, RPS25 and eIF3c-VSV were transfected simultaneously into C2C12 cells. Thirty-six hours posttransfection, cells were fixed with chilled methanol and incubated with primary antibodies as indicated. (A-C) Overexpressed eIF3c-VSV and RPS25 were detected with mouse monoclonal VSV (green, A) and rabbit anti-RPS25 (red, B) antibodies. The merged image is shown in C. (D-F) Overexpressed eIF3c-VSV and Akt2-myc were detected with mouse monoclonal VSV (green, D) and rabbit c-myc (red, E) antibodies. The merged image is shown in F. (G-I) Overexpressed RPS25 and Akt2-myc were detected with rabbit anti-RPS25 (green, G) and mouse monoclonal myc (red, H) antibodies. The merged image is shown in I. Images were taken and processed as described in Fig. 4.17. Scalebar: 10 μm.

4.7 RPS25 regulates the growth of Jurkat cells

RNA interference (RNAi) has been widely used to analyze the function of mammalian genes. The small interfering RNAs (siRNAs) bind to the target mRNA and promote its degradation, thus resulting in knockdown of the expression of specific genes. siRNA can be introduced into mammalian cells through various methods, for example, transfection of a plasmid that encodes short hairpin RNA (shRNA) (Ichim et al., 2004). In the present work, the shRNA sequences of RPS25 were designed using an shRNA Retriever software on the website of http://katahdin.cshl.org:9331/homepage/siRNA/RNAi.cgi?type=shRNA. Two sequences of RPS25 (shRNA-6 and shRNA-345) were selected for further analyses. The designed shRNA sequence of RPS25 was synthesized and cloned under the control of a U6 promoter into the pCRZero vector to generate shRNA plasmid which would lead to generation of double stranded siRNAs. To test their ability to inhibit the expression of RPS25, shRNA plasmids was transiently transfected into HEK 293 cells and lysates analyzed for the protein level of RPS25. Upon transfection, around 80 % of the 293 cells take up DNA and thus allow a direct assessment of the capability of the individual shRNA. As shown in Fig. 4.21A, shRNA-6 inhibited the expression of RPS25 (lane 3) while shRNA-345 did not (lane 2). Moreover, co-transfection of shRNA-6 and shRNA-345 did not further enhance the inhibition ability of shRNA-6 (lane 1). Therefore, we used the shRNA-6 plasmid as a tool to successfully knockdown the expression of RPS25.

The gene of RPS25 was originally isolated from a cDNA library prepared from mRNA of adriamycin-resistant HL60 cells (Li et al., 1991). The transcription of the RPS25 gene was downregulated following the stimulation with chemokines CXCL12 or CXCL10 in CXCR3-transfected Jurkat cells (Nagel et al., 2004). Thus, we used the Jurkat cell line as a parental cell to establish stable cell lines with reduced expression of RPS25. shRNA-6 and pSV2neo were co-transfected into Jurkat cells and the cells grown in the selective medium containing G418 for three weeks. G418-resistant single-colonies were further amplified and harvested.

Lysates were used to determine the expression of RPS25 by using Western blotting with the anti-RPS25 antibody. Among the analyzed more than 60 colonies, we first selected six ones which had an obviously reduced expression of RPS25 (data not shown). To confirm that the downregulation of RPS25 was stable, the colonies were re-amplified and analyzed for the protein level of RPS25. As a loading control, a different part of the same nitrocellulose filter was analyzed with anti-14-3-3γ antibody. As shown in Fig. 4.21B, five cell lines (clones 4, 8, 9, 12 and 24) had different degrees of reduced expression of RPS25, while the level of 14-3-3γ protein indicated that each lane had a similar amount of loaded protein. Among these established stable cell lines, the expression of RPS25 in clone 4 was stronger than in the others. Therefore, clones 8, 9, 12 and 24 were used for future investigations.

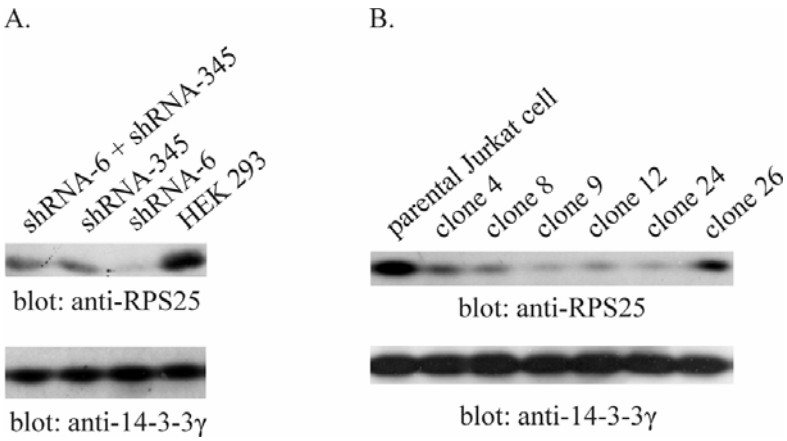


Figure 4.21 Jurkat cell lines with stably reduced expression of RPS25. (A) shRNA-6 and shRNA-345 of RPS25 were transfected into HEK 293 cells as indicated. Thirty-six hours posttransfection, cells were harvested and lysates resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and the filter probed with anti-RPS25 antibody. As a control, lysates of untransfected 293 cells were included. To verify that the same amount of protein was loaded onto the gel, 14-3-3γ protein was detected on a different part of the same filter. (B) shRNA-6 and pSV2neo were co-transfected into Jurkat cells in a ratio of 9: 1 using the SuperFect Reagent (Qiagen). Forty-eight hours posttransfection, cells were grown in the selective medium containing G418 for three weeks. Several G418-resistant single-colonies were amplified and harvested. Lysates were resolved by SDS-PAGE (11 %), proteins transferred to nitrocellulose and the filter probed with anti-RPS25 antibody. As a control, lysates of parental Jurkat cells were included. To verify that the same amount of protein was loaded onto the gel, 14-3-3γ protein was detected on a different part of the same filter.

A number of ribosomal proteins have additional functions apart from processing protein translation. A good example is ribosomal protein S19 that is involved in Diamond-Blackfan anemia (Ellis and Massey, 2006). Consistent with its role in DBA pathogenesis, depletion of S19 with specific siRNA severely alters proliferation and differentiation of erythroleukemic cells (Miyake et al., 2005). To investigate the physiological functions of RPS25, four cell

lines (clones 8, 9, 12 and 24) with stably reduced expression of RPS25 were used for analyzing cell proliferation. In order to minimize a possible influence of the selection reagent G418, two other Jurkat stable cell lines established with the same method were included as controls. By seeding cells in complete growth medium at the same and a low densitity, growing under normal conditions and counting the live cells every other day, a cell growth curve was generated as shown in Fig. 4.22. Knockdown of endogenous RPS25 strongly inhibited the growth of Jurkat cells ($p<0.05$), indicating that RPS25 plays an important role in the regulation of cell proliferation.

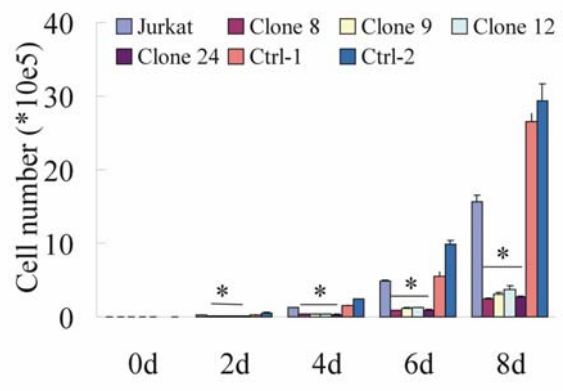


Figure 4.22 Knockdown of RPS25 expression inhibits the growth of Jurkat cells. Parental Jurkat cells and cell lines 8, 9, 12 and 24 with stably reduced expression of RPS25 were seeded at 5000 cells / ml in RPMI 1640 medium containing 10 % FBS. Live cells were counted every other day by staining with trypan blue. Two unrelated Jurkat stable cell lines established with G418 selection were included as controls. Experiments were repeated three times in duplicate. Results are shown as means + S.D. and analyzed with student's t-Test. * $p<0.05$. Ctrl: control.

4.8 Interaction of Akt2 and RPS25 has no effect on the IL-2 promoter

Interleukin-2 (IL-2) is a key growth factor of T lymphocytes whose expression is controlled at the level of transcription as well as by stability of mRNA. The luciferase system, which uses a *firefly* luciferase gene as a reporter gene, is a simple, rapid and sensitive method for the study of promoter activity in transfected cells (Williams et al., 1989). To investigate whether the interaction of Akt2 and RPS25 possibly affects the IL-2 promoter activity, a reporter plasmid pIL-2-Luc containing the minimal IL-2 promoter was transfected into Jurkat cells together with Akt2, shRNA-6 or RPS25 plasmids. A second plasmid pRL-TK, which contains a constitutively active HSV-TK promoter and a cDNA for *Renilla* luciferase, was included in each transfection as an internal control to monitor the transfection efficiency. Cells were left unstimulated or stimulated with phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) in combination with calcium ionophore A23187 for 6 h before harvesting. The light emission

was measured on a luminometer according to the manufacturer's description. In general, both *firefly* and *renilla* luciferase activities were recorded and the relative light unit (RLU) was calculated by using sample *firefly* luciferase/sample *renilla* luciferase. As shown in Fig. 4.23, a basal IL-2 transcription was observed in untransfected Jurkat cells under stimulated or unstimulated conditions. Expression of Akt2 and/or RPS25 in Jurkat cells was not sufficient to increase the activity of IL-2 promoter. When cells overexpressing RPS25 and Akt2 were stimulated with TPA and A23187, an increase of the luciferase activity was obtained. By contrast, a nearly 2-fold decrease of the luciferase transcription was observed in the presence of shRNA-6 which successfully knocked down the expression of endogenous RPS25. Overexpression of Akt2 restored the shRNA-6-induced decrease of the luciferase activity only when cells were stimulated with TPA and A23187. However, statistical analysis indicated that all these changes were not significantly different ($p>0.05$). It seems that neither RPS25 alone nor co-overexpression of RPS25 and Akt2 has an obvious effect on the activity of IL-2 promoter.

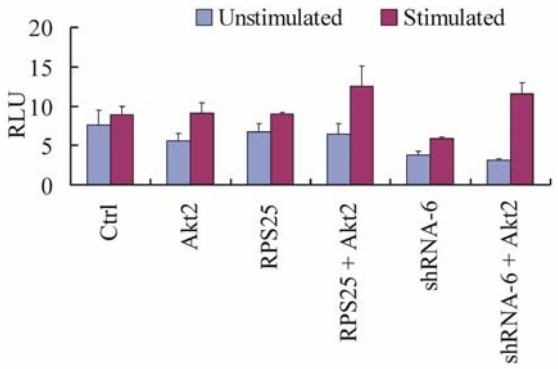


Figure 4.23 Interaction of Akt2 and RPS25 has little effect on the IL-2 promoter. pIL-2-Luc (*firefly* luciferase, 0.5 μg) was transfected into Jurkat cells together with empty vector (as control), Akt2 (0.1 μg), RPS25 (0.2 μg) or shRNA-6 of RPS25 (0.2 μg) as indicated. As an internal control for the transfection efficiency, a second plasmid of pRL-TK (*Renilla* luciferase, 0.1 μg) was included in each transfection. Seventy-two hours posttransfection, cells were left unstimulated or stimulated with TPA (0.1 μM) and A23187 (1 μM) for 6 h at 37°C before harvesting. The luciferase activities are shown as the relative light unit (RLU, value of sample *firefly* luciferase/value of sample *renilla* luciferase). Experiments were repeated three times in duplicate. Results are presented as means + S.D. and analyzed with student's t-Test. Ctrl: control. RLU: relative light unit.

4.9 Regulation of protein translation by the complex of Akt2, RPS25 and eIF3c

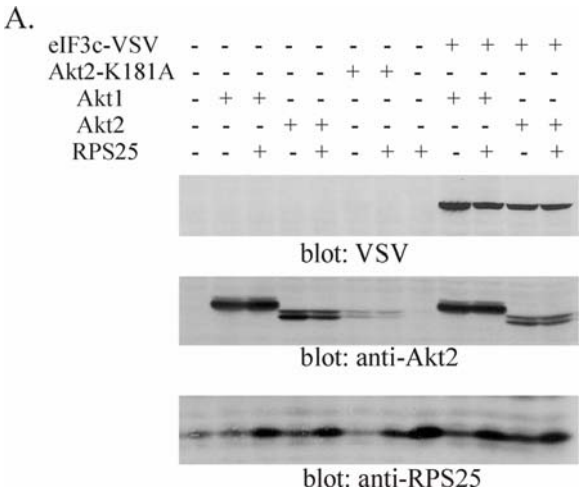
We have shown that RPS25 specifically interacts with Akt2 and improves the interaction of eIF3c and Akt2 in mammalian cells. To further investigate the physiological consequences of the ternary complex of Akt2, eIF3c and RPS25, we measured the change of protein translation

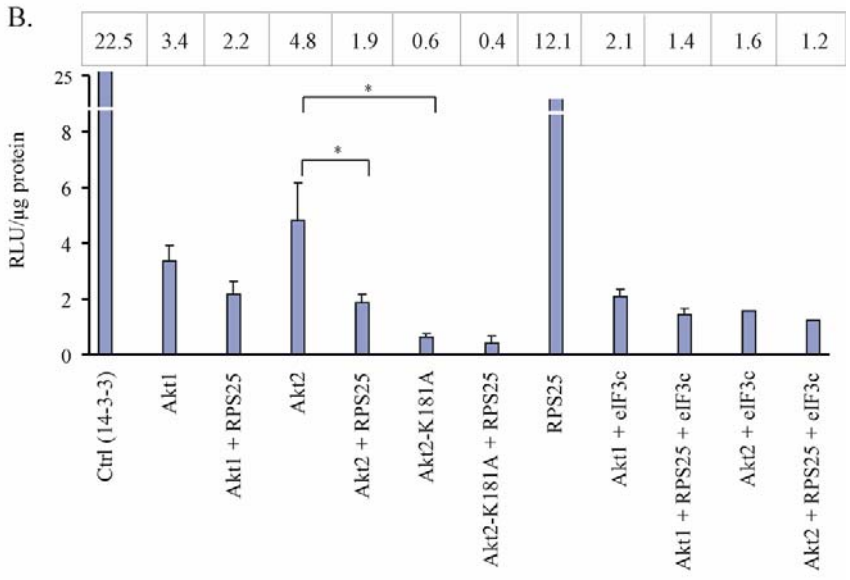
by using a luciferase reporter system. The reporter plasmid pRL-TK containing a weak, constitutively active herpes simplex virus thymidine kinase promoter and a cDNA encoding the *Renilla* luciferase was transiently transfected into HEK 293 cells alone or together with Akt1, Akt2, Akt2-K181A, RPS25, or eIF3c encoding plasmids. Because our wild-type and mutant Akt, eIF3c and RPS25 cDNAs were cloned in CMV promoter based pRK vectors, and the weak TK and the strong CMV promoter might compete with each other for recruitment of the available transcription factors, the pRK-14-3-3γ plasmid was used to complement the DNA amount in each transfection.

Seventy-two hours posttransfection, cells were harvested and lysates used for the luciferase activity assay. In parallel, the aliquots of lysates were resolved by SDS-PAGE and analyzed by Western blotting. As shown in Fig. 4.24A, the expression levels of Akt1, Akt2 and mutant Akt2-K181A were various while the expression of eIF3c-VSV was similar in all samples. For the luciferase activity assay, the read out of light emission was normalized to the amount of protein (Fig. 4.24B). HEK 293 cells transfected only with pRL-TK reporter plasmid and a plasmid encoding 14-3-3γ were included as control (Fig. 4.24B). Single overexpression of Akt1, Akt2, Akt2-K181A or RPS25 all decreased the luciferase activity, and the mutant Akt2-K181A led to the lowest expression of luciferase. When Akt2 was co-overexpressed with RPS25, it led to a prominent inhibition of the luciferase activity ($p<0.05$) compared to the single expression of Akt2. However, when Akt1 was overexpressed together with RPS25, it did not lead to an additional inhibition of luciferase expression compared to the single overexpression of Akt1 ($p>0.05$), suggesting the inhibition of luciferase activity in HEK 293 cells was dependent on the specific interaction of Akt2 and RPS25. The eIF3c was also overexpressed in HEK 293 cells together with Akt2 alone or with Akt2 and RPS25. Although the luciferase activity in cells co-overexpressing Akt2 and eIF3c was decreased to a similar level as in cells co-overexpressing Akt2 and RPS25, the presence of the ternary complex of eIF3c, Akt2 and RPS25 did not lead to a prominent inhibition. These data indicate that the interaction of Akt2 and RPS25 leads to an inhibition of protein translation, and that the ternary complex of eIF3c, Akt2 and RPS25 is not affected.

Considering the different expression levels of Akt in the distinct samples as shown in Fig. 4.24A, we wanted to know whether the observed changes of translation efficiency could be related to the different amount of Akt. To address this question, the amount of Akt protein was quantified by using ImageMaster 1D Prime (Pharmacia) software, and the luciferase activity was normalized to the amount of Akt (Fig. 4.24C). From this point of view, the mutant Akt2-K181A which had the lowest expression in this experiment led to a similar

luciferase activity as Akt1, and upon co-overexpression of Akt2-K181A and RPS25 the luciferase expression did not decrease. Thus, only for active Akt2 RPS25 can downmodulate translation. Assuming that the overexpressed RPS25 is not ribosome-associated, it could sequester Akt2 to the nucleus, indicating that Akt2 kinase-activity is required for efficient translation.





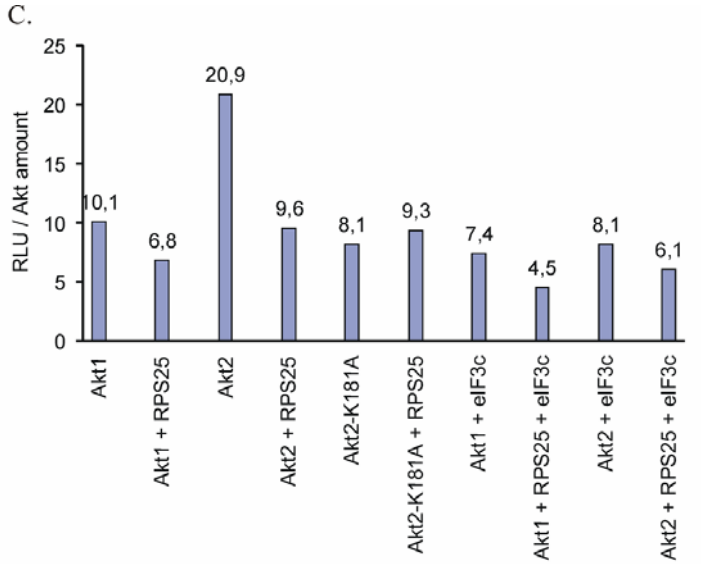


Figure 4.24 Interaction of Akt2 and RPS25 inhibits the luciferase expression in HEK 293 cells. pRL-TK was transiently transfected into HEK 293 cells together with the control plasmid encoding 14-3-3γ, plasmids encoding Akt1, Akt2, Akt2-K181A, or RPS25 as indicated. Final DNA mixtures included the same amount of TK- and CMV-promoter controlled plasmids. Seventy-two hours posttransfection, cells were harvested and the protein concentrations were determined by the Bradford method. Aliquots of cell lysates were analyzed by Western blotting to show the expression of proteins of interest (A). In parallel, lysates were used for luciferase activity assays. The values of luciferase activity (RLU) were calculated using the read out of light emission divided by the amount of measured total protein (B), or using the read out of light emission divided by the amount of measured Akt (C). Experiments were repeated four times in duplicate. Results are shown as means + S.D. and analyzed with student's t-Test. * $p<0.05$. Ctrl: control.

In the next step, we used shRNA-6 to knockdown endogenous RPS25 and monitored the translation efficiency in a similar way. Since the plasmid of shRNA-6 was under the control of U6 promoter while the plasmid of Akt1 and Akt2 was under the CMV promoter, we added unrelated shRNA-plasmid, anti-KBP, to complement the DNA amount in control-1, while pRK-14-3-3γ and anti-KBP plasmids were included in control-2. Similarly, when cells were transfected with pRL-TK together with Akt1 or Akt2, anti-KBP was also included to keep the same amount of U6 promoter. Seventy-two hours posttransfection, cells were harvested and aliquots of lysates used for Western blotting and luciferase activity assay. The result of Western blotting showed that transfection of shRNA-6 successfully reduced the expression of endogenous RPS25 (Fig. 4.25A). The combination of distinct promoters obviously affected the translation. Co-transfection of TK and U6 promoter (Fig. 4.25B, control-1) yielded two times the luciferase expression compared to co-transfection of TK and CMV promoter (Fig. 4.24B, control). When the three promoters TK, U6 and CMV were present simultaneously, the expression of luciferase (Fig. 4.25B, control-2) was also lower than upon co-transfection of TK and U6 promoter plasmids (Fig. 4.25B, control-1). Similar to the overexpression of

RPS25, knockdown of endogenous RPS25 inhibited the expression of luciferase. When the shRNA-6 plasmid was transfected into cells together with the Akt2 plasmid, the expression of luciferase was two times that of co-expression of Akt2 and control plasmid anti-KBP ($p<0.005$, Fig. 4.25A). Even though co-expression of shRNA-6 and Akt1 yielded a higher luciferase activity, it was not statistically significantly different from that of co-expression of Akt1 and control plasmid anti-KBP (Fig. 4.25A). Taken together with the inhibition of luciferase expression induced by the overexpression of RPS25 as observed in Fig. 4.24B, we conclude that the amount of RPS25 is important for the regulation of translation in HEK 293 cells.

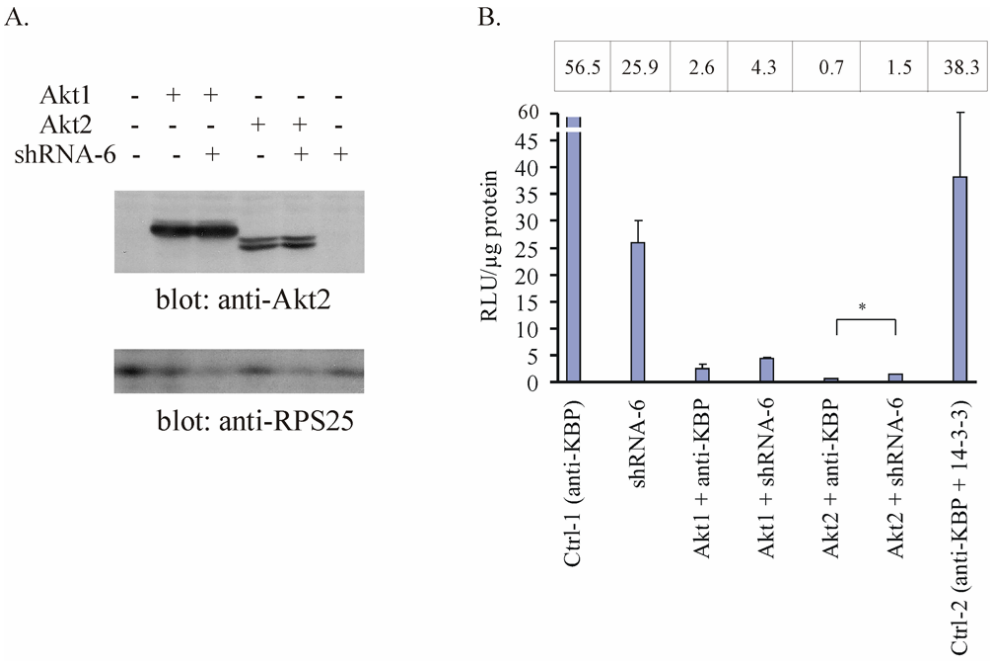


Figure 4.25 Knockdown of endogenous RPS25 inhibits the luciferase expression in HEK 293 cells. HEK 293 cells were transfected with reporter plasmid pRL-TK and the plasmids of interest as indicated. Final DNA mixtures included the same amount of TK, U6 and CMV promoter controlled plasmids. The unrelated shRNA-plasmid (anti-KBP) was included in control-1, and pRK-14-3-3γ together with anti-KBP plasmids included in control-2. Seventy-two hours posttransfection, cells were harvested and the protein concentrations were determined by the Bradford method. Aliquots of lysates were used for luciferase activity assays. In parallel, aliquots of lysates were analyzed by Western blotting to show the expression of proteins of interest (A). The values of luciferase activity (RLU) were calculated using the read out of light emission divided by the amount of measured total protein. Experiments were repeated four times in duplicate. Results are shown as means + S.D. and analyzed with student's t-Test. * $p<0.05$. Ctrl-1: control-1; Ctrl-2: control-2.

5 Discussion

To identify novel proteins that interact with Akt2, we conducted yeast two-hybrid screens using different regions of Akt2 as bait. Six Akt2 candidate interacting proteins (NFκBAP, MBD1, GABARAP, Ku70, eIF3c and RPS25) were identified but only RPS25 and eIF3c did also associate with Akt2 in intact mammalian cells. The amino acids 377-394 of Akt2 were responsible for its binding to RPS25, and the amino acids 70-88 of RPS25 were required for the interaction with Akt2. Overexpressed RPS25 mainly localized in the nucleus of C2C12 cells, and the specific binding of RPS25 to Akt2 increased the nuclear translocation of Akt2. The amount of RPS25 was crucial for efficient translation, and stable knockdown of RPS25 inhibited the Jurkat cell growth in an IL-2 promoter independent manner. Co-overexpression of wild type Akt2 and RPS25 inhibited luciferase expression whereas simultaneous presence of mutant Akt2-K181A and RPS25 had no effect on translation, indicating that the Akt2 kinase activity is required for efficient translation. The ternary complex of eIF3c, Akt2 and RPS25 did not affect the protein translation compared to co-overexpression of Akt2 and eIF3c.

5.1 Yeast two-hybrid screen for Akt2 interacting proteins

The yeast two-hybrid screen is a powerful technique for studying protein-protein interactions. The advantages of this method include that the protein interactions are detected in vivo, only one plasmid (bait plasmid) construct is required, and the cloned cDNA is immediately available. The biggest disadvantage is that the two fusion proteins are artificially targeted to the yeast nucleus, which leads to false positive colonies.

In the present work, we cloned different regions of Akt2 to a pBTM116 vector to generate bait plasmids and screened five human cDNA libraries. The cDNA within the prey plasmids encoding the candidate binding proteins were amplified by screening PCRs and analyzed with sequencing and database searching. We finally got 279 positive results in total with seven Akt2 baits. Database searches of the 279 positive colonies revealed that a number of them encoded mitochondrial proteins or extracellular proteins which localize in a cellular compartment different from Akt2. These prey plasmids therefore were not investigated any more. As a result, thirty-six prey plasmids that encoded six different proteins were selected as Akt2 candidate interacting proteins (see Table 4.1). We did not find previously reported Akt interacting proteins that had been identified in yeast two-hybrid screens, such as CTMP, Trb3

or POSH. This might be caused by different features of the yeast two-hybrid systems used. In general, the yeast transcription factor GAL4 has two distinct functional domains, the DNA-binding domain and the activation domain. The GAL4 activation domain can be fused to the DNA-binding domain of *E. coli* LexA to create a functional transcription activator in yeast (Brent and Ptashne, 1985). In our analysis system, the bait plasmid was constructed in a pBTM116 vector containing the LexA DNA-binding domain, whereas for the identification of CTMP and Trb3, the cDNAs of Akt were cloned into bait vectors that include the DNA-binding domain of GAL4 (Maira et al., 2001; Du et al., 2003). The difference of the DNA-binding domain from LexA and GAL4 might lead to the different screening results. For the identification of POSH, even though the pBTM116-Akt2 was used as a bait, the screened library was derived from mouse embryo cDNA (Figueroa et al., 2003), while the cDNA libraries used in our work were derived from various human tissues.

5.2 Confirmation of interactions outside the yeast system

A positive result in the yeast two-hybrid screen demonstrates that the two proteins have the ability to interact with each other but does not indicate that they normally interact *in vivo* in mammalian cells (Luban and Goff, 1995). Other protein-protein interaction methods are necessary to determine which interactions should be further characterized. One approach to evaluate protein interactions is the co-immunoprecipitation. In the present work, epitope tagged candidate binding proteins were co-overexpressed with full length Akt1/2/3 in HEK 293 cells and the *in vivo* interactions were documented by co-immunoprecipitation. RPS25 specifically interacted with the Akt2 isoform but not with Akt1 or Akt3 (see Fig. 4.3), confirming the interaction in the yeast two-hybrid screen. However, the other Akt2 candidate binding proteins (NFκBAP, MBD1, GABARAP and Ku70) failed to co-immunoprecipitate with any Akt isoform (see Fig. 4.2), suggesting that either the interactions identified in yeast are not biologically relevant or the protein interactions are not strong enough to survive co-immunoprecipitation conditions. Co-overexpression of eIF3c with Akt1 or Akt2 did not lead to co-immunoprecipitation, while Akt2 interacted with eIF3c upon the triple-overexpression of eIF3c, Akt2 and RPS25 (see Fig. 4.14), indicating that RPS25 mediates the interaction of Akt2 and eIF3c. The failure to detect the association of eIF3c and Akt1 upon the triple-overexpression of eIF3c, Akt1 and RPS25 (see Fig. 4.15) suggested that RPS25-mediated interaction of Akt2 and eIF3c was dependent on its specific binding ability to Akt2. To further confirm the interaction, we showed that endogenous Akt2 also interacted with overexpressed RPS25 although we did not find the interaction between endogenous Akt2 and endogenous

RPS25 in untransfected HEK 293 cells (see Fig. 4.6).

5.3 Characterization of the interaction of Akt2 and RPS25

The ribosome assembly is a complex process including various interactions of ribosomal RNAs and ribosomal proteins (Melese and Xue, 1995). Many prokaryotic ribosomal proteins have at least one globular domain, which is generally located on the surface of the ribosome, and an extended tail that is rich in basic amino acids to interact with ribosomal RNAs and penetrates the ribosome's interior (Wilson and Nierhaus, 2005). The exact position of RPS25 on the ribosome is unknown because there is no prokaryotic counterpart of this protein and the crystal structure of a eukaryotic ribosome has not been solved. In the 80S ribosome subunit, RPS25 interacts with 18S rRNA (Westermann et al., 1981) and is cross-linked to the large ribosomal protein L5 (Uchiumi et al., 1986). Analysis of the deduced amino acid sequence of RPS25 indicates that its amino-terminus is rich of basic residues (see Fig. 4.4). Considering the similarity of prokaryotic and eukaryotic ribosomal proteins, we hypothesize that the amino terminus of RPS25 lies in the inside of the 40S ribosomal subunit and is responsible for the interaction with 18S rRNA. Because the deduced amino acid sequence of RPS25 does not contain the conserved Akt substrate sequence RXRXXS/T, it does not appear to be a direct phosphorylation target for Akt2. Although sequence analysis of RPS25 indicated that there were two potential tyrosine residues that could be phosphorylated, we did not detect a phosphorylated tyrosine in RPS25 (see Fig. 4.8). Thus, RPS25 does not seem to act as a scaffold for the interaction of Akt2 and another SH2 domain containing protein. This mechanism is realized for another Akt2 binding protein, APPL, which contains a potential tyrosine-phosphorylation site recognized by SH2 domain of PI3K. It slightly binds to the regulatory subunit of PI3K when analyzed with the co-immunoprecipitation method (Mitsuuchi et al., 1999). Activation of Akt sometimes affects its interaction with other proteins. For example, carboxyl-terminal modulator protein (CTMP) has been shown to interact with Akt1 at the plasma membrane in unstimulated cells (Maira et al., 2001). In this study, we found that insulin stimulation had little effect on the interaction efficiency of Akt2 and RPS25 in HEK 293 cells, and that RPS25-associated Akt2 was not phosphorylated on its two regulatory residues (see Fig. 4.7). These results indicate that RPS25 specifically binds to the unphosphorylated Akt2.

Mapping the binding site of Akt2 indicated that it localized in the amino acids 70-88 of RPS25 because a GST-RPS25aa1-70 fusion protein failed to pull-down Akt2 while truncation mutant RPS25aa1-88 could be co-immunoprecipitated with Akt2 (see Fig. 4.13). It seems that

the ribosomal RPS25 keeps its ability for binding to Akt2 considering the report that human RPS25 resides at the interface region of the small ribosomal subunit (Westermann et al., 1980; Tolan et al., 1983), and our hypothesis that the amino-terminus of RPS25 localizes in the inside of the ribosomal subunit. Immunofluorescence studies indicated that RPS25 mainly localized in the cell nucleus (see Fig. 4.17). This result is consistent with the current view that in higher eukaryotic cells newly synthesized ribosomal proteins are rapidly transported to the nucleus, where they are incorporated into pre-ribosomal subunit particles. As a structural component of the 40S ribosomal subunit, a cytoplasmic distribution of RPS25 was expected. However, we were unable to show the cytoplasmic localization of RPS25 in our analysis system. One reason may be the RPS25 antibody used, of which the epitope was at the amino-terminus of RPS25. Considering the similarity among ribosomal proteins and the binding site of Akt2 that localized in the middle region of RPS25, it seems that most of cytoplasmic RPS25 may exist in a ribosome-bound form which would sequester the epitope of anti-RPS25 antibody during the process of ribosomal assembly, whereas the interaction of cytoplasmic RPS25 and Akt2 may take place directly on the ribosomal surface.

Various regions of Akt are responsible for the interactions with different Akt binding proteins in mammalian cells. For example, Trb3, a negative modulator of Akt kinase activity, interacts with the amino acids 240-315 of Akt1 (Du et al., 2003), and Tcl1 that contributes to Akt activation binds to the PH domain of Akt1, Akt2 and Akt3 (Laine et al., 2002). In the present work, truncation mutants Akt2aa364-481, Akt2aa377-481 and Akt2aa385-481 all interacted with RPS25 while Akt2aa394-481 and Akt2aa410-481 did not (see Fig. 4.9 and 4.10), indicating that the carboxyl-terminal regulatory domain of Akt2 is not required for its binding to RPS25. Deletion of amino acids 377-385 of Akt2 completely abrogated the interaction of RPS25 and Akt2 (see Fig. 4.10C), suggesting that this region is a determinant for the association of Akt2 and RPS25. Although Akt2aa385-481 kept the ability to interact with RPS25, the binding efficiency was lower than Akt2aa377-385 and Akt2aa364-481 (see Fig. 4.10A). This result indicates that the amino acids 385-394 of Akt2 are involved in the interaction of Akt2 and RPS25. Therefore, the binding site of RPS25 localized in the amino acids 377-394 of Akt2. Because the result of the immunokinase activity assay showed that deletion of amino acids 387-392 of Akt2, which was included in the binding site of RPS25, led to the loss of kinase activity (see Fig. 4.11), we did not further characterize this binding site.

Akt1, Akt2, and Akt3 are closely related protein kinases with a conserved phosphorylation motif. They are all activated through similar mechanisms involving upstream kinases PI3K

and PDK1. The function of each Akt isoform has been studied in knock-out mice model systems as well as in isoform specific knockdowns in cell cultures by specific siRNA. These studies reveal the existence of both redundant and distinct functions of Akt isoforms. For example, although Akt1 and Akt2 similarly inhibit FoxO1 mediated gene expression, they have different roles in the activation of CREB. Akt1 induces phosphorylation of CREB on Ser-133 and expression of CREB target genes, while Akt2 is unable to induce the Ser-133 phosphorylation and fails to activate CREB in vivo (Kato et al., 2007). In addition, it was reported that Akt2 stimulates motility of breast and ovarian cancer cells, while Akt1 actually inhibits motility in these cells (Arboleda et al., 2003; Irie et al., 2005; Yoeli-Lerner et al., 2005). The exact mechanism for the specificities of closely related Akt isoforms is not yet clear. However, the specific interacting proteins for each isoform might be one possible explanation. For instance, POSH which is a scaffold for the JNK signalling pathway interacts only with Akt2 but not with Akt1 (Figueroa et al., 2003), while JIP1 (JNK-interacting protein 1) specifically interacts with Akt1 (Kim et al., 2002) and promotes its activation (Kim et al., 2003). Our results added another protein, RPS25, to the list of specific Akt2 binding proteins (see Fig. 4.3). Moreover, we pointed out that the single amino acid difference within the RPS25 binding site between Akt1 and Akt2 (Ser381 versus Ala382) was not enough to determine the specific interaction of Akt2 and RPS25 (see Fig. 4.12). One explanation is that, except for the amino acids 377-394 of Akt2 which are responsible for binding to RPS25, other region(s) of Akt2 which are different from Akt1 also contribute to this isoform-specific interaction.

5.4 A protein complex of eIF3c, Akt2 and RPS25

The eukaryotic initiation factor 3 contains at least 11 subunits in mammals and plays an important role in the formation of a 43S pre-initiation complex. Computer analysis of the peptide sequence of eIF3c revealed an Akt phosphorylation site at Ser711. In the present yeast two-hybrid screen, eIF3c was identified as an Akt2 candidate binding protein in human liver, placenta, pancreas and skeletal muscle cDNA libraries (see Table 4.1). The direct interaction of eIF3c and Akt2 was not detected in intact mammalian cells when using the co-immunoprecipitation method (see Fig. 4.14A, lane2 and B, lane 2). However, in a transient overexpression system, we found that Akt2 and eIF3c co-localized in the cytoplasm of C2C12 cells (see Fig. 4.18 and Fig. 4.19). It seems that the interaction of eIF3c and Akt2 is too weak to survive the co-immunoprecipitation conditions. The yeast eIF3 subunits TIF32/eIF3a and NIP1/eIF3c directly interact with ribosomal protein S0A (RPS0A), which is believed to reside

on the interface side of yeast 40S ribosomal subunit (Valasek et al., 2003). In addition, TIF32/eIF3a directly associates with ribosomal protein S10A (RPS10A), whose location in the 40S ribosome is not clear (Spahn et al., 2001). In a chemical cross-linking study RPS25 was identified as one of the ribosomal proteins which interacts with the eIF3 complex (Westermann and Nygard, 1983). In our study, we found that RPS25 was co-immunoprecipitated with eIF3c upon overexpression of both proteins in HEK 293 cells (see Fig. 4.15, lanes 4 and 6). It seems that several ribosomal proteins including RPS25 are involved in the binding of eIF3c to the ribosome. Moreover, we found that eIF3c co-immunoprecipitated with Akt2 when RPS25 was overexpressed together with Akt2 and eIF3c simultaneously in transiently transfected HEK 293 cells (see Fig. 4.14A, lane 4; 4.14B, lane 1 and 4.15, lane 4). When Akt1 took the place of Akt2, the co-immunoprecipitation of eIF3c and Akt1 was not detected under the same conditions (see Fig. 4.15, lane 2). The data indicate that RPS25 has the ability to improve the interaction efficiency of eIF3c and Akt2. Since we found that Akt2 and eIF3c co-localized in the cytoplasm, and that Akt2 directly interacted with ribosomal RPS25, it seems that the interaction of Akt2 and eIF3c might take place directly on the ribosomal surface where the ribosome may act as a scaffold platform to facilitate the interaction. Therefore, we propose that a ternary protein complex of eIF3c, Akt2 and RPS25 is formed in mammalian cells, at least under overexpression conditions. As well, we found that overexpression of either Akt1 or Akt2 reduced the interaction efficiency of RPS25 and eIF3c (see Fig. 4.15, lanes 2, 4 and 6). Although there was a potential Akt phosphorylation site at Ser711, the mutation of this residue to alanine (eIF3c-S711A) or a change to aspartic acid (eIF3c-S711D) had no effect on the interaction of eIF3c and RPS25 (see Fig. 4.16). It suggests that the Akt1/2-mediated decrease of interaction efficiency occurs via a Ser711-phosphorylation independent way. Several eIF3 subunits including eIF3c have been shown to interact directly with protein kinase S6K1 (Holz et al., 2005). The interaction is potently disrupted by insulin stimulation, and pre-treatment of cells with rapamycin restores the binding of S6K1 to eIF3c. However, the mTOR/ Raptor complex binds to the eIF3c subunit in a pattern opposite to that of S6K1: it weakly associated with eIF3 under serum-starved and rapamycin-treated conditions and strongly binds to the eIF3c subunit following insulin stimulation. The eIF3 complex to which S6K1 bound appears not to be associated with the 40S ribosomal subunit, and the phosphorylation of Thr389 by mTOR (Kim et al., 2002b) is critical for release of S6K1 from eIF3 complex. Both, mTOR and S6K1 kinases, are well identified downstream targets of Akt. The ternary complex of eIF3c, Akt2 and RPS25 might bring Akt2 close enough to the mTOR kinase which was recruited to the eIF3 complex upon

mitogen stimulation (Holz et al., 2005), thus regulating the association of eIF3 complex and the 40S ribosomal subunit.

Collectively the data from these experiments indicate that there exists a ternary complex of eIF3c, Akt2 and RPS25 in mammalian cells. Although the exact molecular mechanism for the formation of this complex is not clear, we have demonstrated that overexpression of RPS25 improves the interaction of eIF3c and Akt2, while overexpression of Akt2 decreases the interaction of RPS25 and eIF3c which is independent of the phosphorylation on Ser711 of eIF3c.

5.5 Cellular localization of eIF3c, Akt2 and RPS25

To determine the co-localization of proteins is another way to validate interactions in mammalian cells. In the present work, we performed indirect immunofluorescence experiments to detect the cellular localizations of eIF3c, Akt2 and RPS25. Both, endogenous and overexpressed RPS25, were mainly found in the nucleus of C2C12 cells. We also found a nuclear localization of HA-tagged RPS25 (see Fig. 4.17). The nuclear localization of wild-type RPS25 is consistent with the identified nuclear localization sequence of RPS25 at its amino-terminus and the observed nucleolar localization of myc- and Flag-tagged RPS25 (Schmidt et al., 1995; Annilo et al., 1998; Kubota et al., 1999; Russo et al., 1997; Jakel and Gorlich, 1998; Quaye et al., 1996). Because ribosomal RPS25 might loose the antibody epitope as we discussed before, we were unable to show the cytoplasmic localization of RPS25. Although a ternary complex of eIF3c, Akt2 and RPS25 has been identified by the co-immunoprecipitation method, we only detected the cytoplasmic localization of Akt2 (see Fig. 4.18C) and eIF3c (see Fig. 4.19A) in transiently transfected C2C12 cells. Trying to generate another RPS25 antibody, which has a different epitope and recognizes the ribosomal RPS25, is necessary to support our explanation that the observed interactions occur in the cytoplasm with ribosomal RPS25.

Activated Akt phosphorylates multiple cytoplasmic and nuclear substrates. However, Akt does not possess an inherent nuclear localization sequence. The nuclear localization of Akt has specific physiological functions in disease generation because overexpression of constitutively activated Akt induces cardiac hypertrophy and ventricular dysfunction, while expression of nuclear targeted Akt results in the inhibition of hypertrophy (Tsujita et al., 2006; Shiraishi et al., 2004). The nuclear translocation of activated Akt can be increased by binding to interacting proteins such as Tcl1 and Zyxin (Pekarsky et al., 2000; Kato et al., 2005). Many transcription factors are phosphorylated by nuclear targeted Akt directly or

indirectly via its downstream kinases such as mTOR. In the present work, we observed that the specific interaction of RPS25 and Akt2 led to an increase of nuclear accumulation of Akt2 (see Fig. 4.18D-F), suggesting that the binding of RPS25 to Akt2 may regulate the process of transcription in mammalian cells.

5.6 Physiological consequences of the interaction of Akt2, RPS25 and eIF3c

Besides functioning in protein synthesis within the ribosome, some ribosomal proteins are involved in other cellular processes. For example, ribosomal proteins L5, L11 and L23 are released during ribosomal stress and modulate the MDM2-p53 interaction (Dai et al., 2004; Dai and Lu, 2004; Lohrum et al., 2003). Ribosomal protein S7 forms a ternary complex with MDM2 and p53 thus regulating apoptosis (Chen et al., 2007). For the eukaryotic ribosomal protein RPS25, a recent report (Nishiyama et al., 2007) suggested that it was important for the binding of the internal ribosome entry site (IRES) to the 40S ribosomal subunit, and the expression of RPS25 protein was found to be increased upon amino acid starvation in Fao hepatoma cells which also exhibited a p53-dependent apoptosis (Adilakshmi and Laine, 2002). To investigate the role of RPS25 in the regulation of cell proliferation, several Jurkat cell lines in which endogenous RPS25 was stably knocked down by using siRNAs (see Fig. 4.21) were plated at a low density and cell growth monitored every other day. The lack of RPS25 clearly inhibited the cell growth and the difference between stable cell lines and the controls was statistically significant from the second day on (see Fig. 4.22). This result indicates that RPS25 may play a positive role in regulating cell proliferation. However, previous studies reported that the gene expression of RPS25 was upregulated during ageing of rat liver (Lavery and Goyns, 2002) and downregulated in Jurkat cells upon cytokine stimulation (Nagel et al., 2004). Although these two groups did not further characterize the change of RPS25 protein, it gives a hint that RPS25 may regulate proliferation in a negative manner. One explanation may be the difference of investigated cell types. Lavery and Goyns used rat liver tissues for preparing the total RNAs thus investigating gene expression changes, while Nagel et al. used Jurkat cells that expressed endogenous CXCR4 and were transfected with human CXCR3 gene. In our work, the Jurkat cells were stably transfected with a specific siRNA of RPS25 and selected with G418. Although the expression levels of RPS25 were different among the four selected cell lines (see Fig. 4.21B), the rates of cell growth were similar (see Fig. 4.22). The ribosomal subunits are considered to be regulatory elements that mediate interaction between particular mRNA and components of the translation machinery.

Specific mRNA-rRNA and mRNA-ribosomal protein interactions are important in controlling translation. Mauro and Edelman (2002) put forward a 'ribosome filter hypothesis' that ribosomal subunits themselves are considered to be filters to mediate interactions between particular mRNAs and components of the translation machinery. This hypothesis emphasizes competitive interactions among various mRNA sequences for binding to ribosomal proteins or rRNAs. It implicates that the different ribosomes, which differ in rRNAs or in ribosomal proteins, may bind to particular mRNA to a different extent, thus affecting the relative translation efficiency. In this work, stable downregulation of endogenous RPS25 might induce a structural change of the 40S ribosomal subunit, thus resulting in the change of protein translation.

IL-2 acts as an autocrine growth factor to promote the proliferation and development of T cells during immune responses as well as tumorigenesis. One consequence of T-cell activation is the expression of IL-2, due to both transcriptional upregulation and stabilization of IL-2 mRNA. The minimal IL-2 promoter region extending to -300 bp relative to the transcriptional start site is sufficient for induction of IL-2 transcription (Siebenlist et al., 1986). By using a reporter plasmid pIL-2-fLuc that contains a minimal IL-2 promoter and a cDNA encoding *firefly* luciferase, we found that neither knockdown of endogenous RPS25 nor overexpression of RPS25 and/or Akt2 affected the activity of the IL-2 promoter, at least not to an extent that was statistically significant (see Fig. 4.23). This result indicates that the interaction of Akt2 and RPS25 has little effect on the IL-2 promoter, and that RPS25 mediated regulation of proliferation is not related to a change of transcription from the IL-2 promoter.

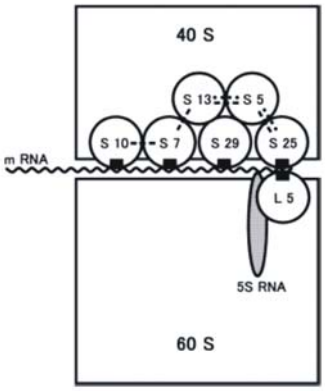


Figure 5.1 Ribosomal proteins bind to the initiator AUG of mRNA in the translation initiation complex induced by UV-irradiation. Proteins pairs are shown with a dotted line and proteins harbouring a filled box are those crosslinked to mRNA by the UV-irradiation. S25, r-protein S25; S5, r-protein S5; S13, r-protein S13; S 29, r-protein S29; S7, r-protein S7; S10, r-protein S10; L5, r-protein L5 (Takahashi et al., 2005).

As a structural and functional component of the 40S ribosomal subunit, the primary role of

RPS25 could be expected to be in the process of translation. A model of ribosomal proteins that bound to the initiator AUG of mRNA in the translation initiation complex (Fig. 5.1) was established by Takahashi et al. (2005). In this model, several ribosomal proteins including RPS25 constitute the AUG-binding domain. In 80S ribosomes RPS25 locates at the contact region of the large and small subunits (Uchiumi et al., 1986). Considering previous report (Takahashi et al., 2005) and our results of a ternary complex of Akt2, eIF3c and RPS25 in mammalian cells, the change of translation efficiency induced by these three proteins was investigated. Akt1, which shares a highly similar amino acid sequence with Akt2, was also included. The reporter plasmid used in this study contains a constitutively active HSV-TK promoter and a cDNA encoding *Renilla* luciferase. Because post-translational modification is not required for the luciferase activity, the enzyme may function as a genetic reporter immediately following translation. In a transient overexpression system the transfection efficiency might be different due to cell viability or other factors. To minimize the experimental artifacts, experiments should be repeated several times with at least two different preparations of each sample or a second plasmid encoding a different reporter gene should be included in each transfection. Thus, some people use a plasmid encoding β-galactosidase as an internal control for monitoring the transfection efficiency. However, it is reported that the interpretation of results could be affected because transcription from the internal control plasmid was suppressed by other plasmids which would overestimate the activity of the co-transfected plasmid (Farr and Roman, 1992). Therefore, in the current study we used only one reporter plasmid and repeated the experiments several times. In parallel, the aliquots of cell lysates used for luciferase activity assay were analyzed by Western blotting. It is also important to realize that *trans* effects between promoters from co-transfected plasmids can potentially affect reporter gene expression. Because all cDNA plasmids used in this work were cloned into the CMV promoter based expression vector pRK, potential competition for the available transcription factors was considered by keeping the same amount of distinct promoter controlled plasmids.

Protein translation has an established role in cell growth. Basically, an increase in protein synthesis occurs as a consequence of mitogenesis. The immunosuppressive drug rapamycin has been shown to block cell growth in part by inhibiting translation of ribosomal protein mRNA thus leading to a general decrease in protein synthesis (Terada et al., 1994). The luciferase activity in cells overexpressing RPS25 was decreased to 54 % of control (see Fig. 4.24B), and knockdown of endogenous RPS25 led to an inhibition of 46 % (see Fig. 4.25B), indicating that the amount of RPS25 is crucial for efficient translation in mammalian cells.

The observed inhibition of cell proliferation induced by stable knockdown of RPS25 m may be causal for this inefficient translation. However, various expression levels of ribosomal proteins do not always lead to a change of protein translation. For example, overexpression of wild-type RPS19 had no effect on the level of cellular translation in the human erythroleukemia cell line K562 (Cmejlova et al., 2006). Therefore, as a structural component of the ribosomal subunit, RPS25 may play an additional role in regulating translation. The notion that ribosomes might be structurally and functionally heterogeneous has led to a 'ribosome filter hypothesis' that the long rRNAs and various ribosomal proteins enable ribosomes to select among mRNAs to a novel level of translational regulation. Interestingly, to ensure the rapid presentation of viral proteins to CD8+ T lymphocytes, cells might dedicate a subset of ribosomes, termed immunoribosomes, to facilitate the antigen processing. It is believed that these immunoribosomes would translate the defective ribosomal products (DRiPs) which were preferentially degraded by proteasomes (Yewdell and Nicchitta, 2006). Consistent with this explanation, it is reported that increased translation of a viral protein resulted in a more effective display of epitopes to T cells and thereby a better immune response (Tellam et al., 2007). Thus, deciphering the intricacies of translation should enhance our understanding about the immune system.

Activation of the PI3K/Akt pathway by growth factors, hormones and mitogen stimuli controls protein synthesis at the level of translation initiation and ribosome biogenesis (Ruggero and Sonenberg, 2005). In the present study, we found that the luciferase expression was inhibited upon single overexpression of Akt1 or Akt2 (see Fig. 4.24B). The luciferase activity in cells co-overexpressing RPS25 and Akt2 was further decreased to 40 % of those only overexpressing Akt2 whereas there was no statistical difference when Akt1 was used instead of Akt2 (see Fig. 4.24C). The data suggest that the inhibition of translation induced by Akt2 and RPS25 is dependent on their specific interaction which sequesters the cytosolic Akt2 to the nucleus. Moreover, co-overexpression of RPS25 and Akt2-K181A, an inactive mutant, did not downmodulate the luciferase activity, indicating that the cytosolic Akt kinase activity is not affected by co-overexpression of these two proteins, and that the Akt kinase activity is required for efficient translation. Co-transfection of Akt2 and shRNA-6 plasmids yielded two times the luciferase activity compared to that of Akt2 and anti-KBP (see Fig. 4.25B), indicating that endogenous RPS25 which is mainly contained in ribosomal subunits in the cytoplasm also interacts with cytosolic Akt2 thus in part contributing to the inhibition of translation. Although the interaction of overexpressed Akt2 and RPS25 led to the nuclear translocation of Akt2 thus reducing the availability of cytosolic Akt2 and resulting in

inefficient translation, the ternary complex of eIF3c, Akt2 and RPS25 did not affect the luciferase activity compared to co-overexpression of Akt2 and eIF3c (Fig. 4.24B and C). The formation of a ternary complex might result in an increase in protein translation initiation because the eIF3c subunit is required for the efficient assembly of the 43S pre-initiation complexes and for the selection of the AUG start codon (Valasek et al. 2004). Although in C2C12 cells the overexpression of eIF3c was not stable (Lammers, personal communication), an overexpression of eIF3c mRNA was reported in testicular seminomas (Rothe et al., 2000). In a cell line with inducible expression of eIF3c, it was found that increases in eIF3c expression elevated cell proliferation (Scoles et al., 2006). Expression of schwannomin, a neurofibromatosis 2 tumour suppressor protein that directly interacts with eIF3c, was effective in inhibiting eIF3c-mediated cell proliferation. Schwannomin has been shown to interact with and to inhibit proliferation mediated by the transactivation response RNA-binding protein (TRBP), an activator of protein translation (Lee et al., 2004). Therefore, the interacting proteins of eIF3c might regulate cell proliferation through changing the protein translation efficiencies.

In summary we report two newly identified Akt2 binding proteins, RPS25 and eIF3c. RPS25 plays an important role in regulating protein translation in addition to its function as a ribosomal component. The identified interactions among eIF3c, Akt2 and RPS25 might modulate ribosome structure and accessibilities of mRNAs and translation initiation factors, and thereby affect protein synthesis. Numerous diseases have been attributed to both specific and global disruptions in translation (Mendell and Dietz, 2001). Some specific binding molecules for ribosomal proteins that can reverse the effects of a disrupted translation may have therapeutic applications.

6 Summary

There exist three Akt isoforms in mammals, PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3, which play important roles in regulation of cell metabolism, apoptosis and proliferation. Akt is activated upon the phosphorylation of Thr308 and Ser473 by upstream kinases PDK1 and PDK2. The deregulation of Akt kinase activity leads to diseases including carcinoma and diabetes. Identification of Akt interacting proteins is an important task to enhance our understanding of the Akt signalling pathway. This dissertation dealt with the identification of novel Akt2 binding proteins and the characterization of their physiological function.

The search for novel Akt2 interacting proteins was accomplished by doing yeast two-hybrid screens. Different regions of Akt2 were cloned into a pBTM116 bait vector and five cDNA libraries from various human tissues used for screening. Two hundred and seventy-nine HIS3+/LacZ+ positive yeast colonies were obtained and six Akt2 candidate interacting proteins, NFκBAP, MBD1, GABARAP, Ku70, RPS25 and eIF3c, finally selected. In order to confirm the results of the yeast two-hybrid, co-immunoprecipitation and GST pull-down studies were conducted. Four Akt2 candidate interacting proteins (NFκBAP, MBD1, GABARAP and Ku70) failed to co-immunoprecipitate with full length Akt1, Akt2, or Akt3 in HEK 293 cells, while RPS25 specifically interacted with Akt2. RPS25-associated Akt2 was in an unphosphorylated state, and insulin stimulation had little effect on the interaction of RPS25 and Akt2. Amino acids 377-394 of Akt2 were responsible for the interaction of Akt2 and RPS25 and the middle region of RPS25, amino acids 70-88, was a determinant for its binding to Akt2. Immunofluorescence investigation showed that both, endogenous and overexpressed RPS25, mainly localized in the nucleus, and overexpressed Akt2 was mainly in the cytoplasm. Upon co-overexpression of Akt2 and RPS25, the nuclear accumulation of Akt2 was increased.

Sequence analysis of the deduced amino acid sequence of eIF3c indicated that there was a potential Akt kinase phosphorylation site at Ser711. Although eIF3c was also identified in yeast two-hybrid studies before in our group, it failed to co-immunoprecipitate with Akt1, Akt2 or Akt3. In the present work, overexpression of RPS25 together with eIF3c and Akt2 led to the co-immunoprecipitation of Akt2 and eIF3c, while the triple-overexpression of RPS25, Akt1 and eIF3c failed to show an interaction of Akt1 and eIF3c. Therefore, we conclude that RPS25 mediates the interaction of Akt2 and eIF3c and that this process is dependent on the specific interaction of RPS25 and Akt2. Our co-immunoprecipitation results showed that RPS25 also interacted with eIF3c directly, and simultaneous expression of Akt kinase reduced

the interaction. By using the eIF3c mutants eIF3c-S711A and eIF3c-S711D, we showed that the phosphorylation of Ser711 did not contribute to the decreased interaction efficiency of eIF3c and RPS25. Immunofluorescence experiments showed that overexpressed eIF3c and Akt2 mainly localized in the cytoplasm of C2C12 cells. Based on the analysis of the property of prokaryotic ribosomal proteins, we presumed that the amino-terminus of RPS25 which is rich in basic residues was in the ribosome's interior and interacted with 18S rRNA. This may be one reason why we did not detect the ribosomal RPS25 protein in our immunofluorescence experiments since our RPS25 antibody was directed against the amino-terminal 15 amino acids. Therefore, we suggest that the ternary complex of eIF3c, Akt2 and RPS25 may be formed directly on the ribosome surface instead with free RPS25 protein.

In the last part of this work, we investigated the physiological consequences of the identified interactions among eIF3c, Akt2 and RPS25. Knockdown of endogenous RPS25 inhibited cell proliferation in an IL-2 promoter independent manner. We found that the proper amount of RPS25 was crucial for efficient protein translation, and that the binding of RPS25 to Akt2 had a negative effect on protein translation. However, a ternary complex of eIF3c, Akt2 and RPS25 could restore the decreased translation efficiency induced by specific interaction of Akt2 and RPS25. Although translation was inhibited by overexpression of only Akt1, Akt2 or Akt2-K181A, the Akt2 isoform has a less inhibitory effect as compared to Akt1 or Akt2-K181A, a kinase inactive mutant. This result indicates that Akt might have isoform-specific functions in regulation of translation, and that the kinase activity might be required to regulate protein translation.

Collectively the data presented in the current study provide evidence for two newly identified Akt2 binding proteins, RPS25 and eIF3c. Considering the 'ribosome filter hypothesis' and the idea of immunoribosomes that a set of ribosomes are specified for producing the defective ribosomal products (DRiPs) thus ensuring the rapid presentation of antigens for T cells, it will be interesting to further decipher the physiological consequences of the ternary complex of eIF3c, Akt2 and RPS25 in the immune system. Moreover, many diseases have been related to specific and/or global disruptions in translation. Specific binding molecules for ribosomal proteins that can reverse the effects of a disrupted translation may have therapeutic applications.

7 Abbreviations

µ	micro
µg	microgram
aa	amino acid
AML	acute myeloid leukaemia
Amp	ampicillin
BCR	B-cell receptor
bp	base pairs
CTMP	carboxyl-terminal modulator protein
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide 5'-triphophates
DRiPs	defective ribosomal products
DTT	1,4-dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
eIF	eukaryotic translation initiation factor
EST	expressed sequence tag
Fig.	figure
FoxO	forkhead box class O transcription factor
g	gravity
GLUT1/4	glucose transporter 1 or 4
GSK3	glycogen synthase kinase 3
GST	glutathione S-transferase
h	hour
HA	hemagglutinin
HM	hydrophobic motif
IL-2	interleukin-2
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
kDa	kilodalton
KOAc	potassium acetate
l	liter

LB	luria bertani
MAPK	mitogen activated protein kinase
m	milli
mA	milliampere
min	minute
mTOR	mammalian target of rapamycin
M	molar
MW	molecular weight
NaAc	sodium acetate
PBS	phosphate-buffered saline
PDK1/2	phosphoinositide-dependent kinase 1/2
PH	pleckstrin homology domain
PI(3,4)P2	phosphatidylinositol-3,4-bisphosphate
PI(3,4,5)P3	phosphatidylinositol-3,4,5-trisphosphate
PI3K	phosphatidylinositol 3-kinase
PKB/Akt	protein kinase B
PLZF	promyelocytic leukaemia zinc finger protein
POV	peroxovanadate
PTEN	phosphatase and tensin homolog deleted on chromosome ten
Rheb	ras homolog enriched in brain
RPS	ribosomal protein of the 40S small ribosomal subunit
RT	room temperature
S6K	ribosomal p70 S6 kinase
sec	second
SH2/SH3	Src homology 2 or 3
SHIP	SH2 domain containing inositol phosphatase
shRNA	short hairpin RNA
TCR	T-cell receptor
U	unit
UV	ultraviolet
VSV	vesicular stomatitis virus
Y2H	yeast two-hybrid

8 References

Adilakshmi,T. and Laine,R.O. (2002). Ribosomal protein S25 mRNA partners with MTF-1 and La to provide a p53-mediated mechanism for survival or death. J. Biol. Chem. *277*, 4147-4151.

Alessandrini,A., Chiaur,D.S., and Pagano,M. (1997). Regulation of the cyclin-dependent kinase inhibitor p27 by degradation and phosphorylation. Leukemia *11*, 342-345.

Andjelkovic,M., Alessi,D.R., Meier,R., Fernandez,A., Lamb,N.J., Frech,M., Cron,P., Cohen,P., Lucocq,J.M., and Hemmings,B.A. (1997). Role of translocation in the activation and function of protein kinase B. J Biol. Chem. *272*, 31515-31524.

Andjelkovic,M., Jakubowicz,T., Cron,P., Ming,X.F., Han,J.W., and Hemmings,B.A. (1996). Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors. Proc. Natl. Acad. Sci. U. S. A *93*, 5699-5704.

Annilo,T., Jelina,J., Pata,I., and Metspalu,A. (1998). Isolation and characterization of the mouse ribosomal protein S7 gene. Biochem. Mol. Biol. Int. *46*, 287-295.

Appleman,L.J., Berezovskaya,A., Grass,I., and Boussiotis,V.A. (2000). CD28 costimulation mediates T cell expansion via IL-2-independent and IL-2-dependent regulation of cell cycle progression. J. Immunol. *164*, 144-151.

Arboleda,M.J., Lyons,J.F., Kabbinavar,F.F., Bray,M.R., Snow,B.E., Ayala,R., Danino,M., Karlan,B.Y., and Slamon,D.J. (2003). Overexpression of AKT2/protein kinase Bbeta leads to up-regulation of beta1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. Cancer Res. *63*, 196-206.

Ashcroft,M., Ludwig,R.L., Woods,D.B., Copeland,T.D., Weber,H.O., MacRae,E.J., and Vousden,K.H. (2002). Phosphorylation of HDM2 by Akt. Oncogene *21*, 1955-1962.

Bacus,S.S., Altomare,D.A., Lyass,L., Chin,D.M., Farrell,M.P., Gurova,K., Gudkov,A., and Testa,J.R. (2002). AKT2 is frequently upregulated in HER-2/neu-positive breast cancers and may contribute to tumor aggressiveness by enhancing cell survival. Oncogene *21*, 3532-3540.

Bae,S.S., Cho,H., Mu,J., and Birnbaum,M.J. (2003). Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B. J Biol. Chem. *278*, 49530-49536.

Barthel,A., Okino,S.T., Liao,J., Nakatani,K., Li,J., Whitlock,J.P., Jr., and Roth,R.A. (1999). Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. J. Biol. Chem. *274*, 20281-20286.

Barthwal,M.K., Sathyanarayana,P., Kundu,C.N., Rana,B., Pradeep,A., Sharma,C., Woodgett,J.R., and Rana,A. (2003). Negative regulation of mixed lineage kinase 3 by protein kinase B/AKT leads to cell survival. J. Biol. Chem. *278*, 3897-3902.

Barton,K., Muthusamy,N., Chanyangam,M., Fischer,C., Clendenin,C., and Leiden,J.M. (1996). Defective thymocyte proliferation and IL-2 production in transgenic mice expressing a dominant-negative form of CREB. Nature *379*, 81-85.

Bauer,B., Krumbock,N., Fresser,F., Hochholdinger,F., Spitaler,M., Simm,A., Uberall,F., Schraven,B., and Baier,G. (2001). Complex formation and cooperation of protein kinase C theta and Akt1/protein kinase B alpha in the NF-kappa B transactivation cascade in Jurkat T cells. J. Biol. Chem. *276*, 31627-31634.

Bellacosa,A., Testa,J.R., Staal,S.P., and Tsichlis,P.N. (1991). A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science *254*, 274-277.

Birnboim,H.C. and Doly,J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. *7*, 1513-1523.

Bradford,M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248-254.

Brandts,C.H., Sargin,B., Rode,M., Biermann,C., Lindtner,B., Schwable,J., Buerger,H., Muller-Tidow,C., Choudhary,C., McMahon,M., Berdel,W.E., and Serve,H. (2005). Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. Cancer Res. *65*, 9643-9650.

Brazil,D.P., Park,J., and Hemmings,B.A. (2002). PKB binding proteins. Getting in on the Akt. Cell *111*, 293-303.

Brent,R. and Ptashne,M. (1985). A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. Cell *43*, 729-736.

Browning,K.S., Gallie,D.R., Hershey,J.W., Hinnebusch,A.G., Maitra,U., Merrick,W.C., and Norbury,C. (2001). Unified nomenclature for the subunits of eukaryotic initiation factor 3. Trends Biochem. Sci. *26*, 284.

Brunet,A., Bonni,A., Zigmond,M.J., Lin,M.Z., Juo,P., Hu,L.S., Anderson,M.J., Arden,K.C., Blenis,J., and Greenberg,M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.

Burgering,B.M. and Coffer,P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature *376*, 599-602.

Burgering,B.M. and Medema,R.H. (2003). Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J. Leukoc. Biol. *73*, 689-701.

Calera,M.R., Martinez,C., Liu,H., Jack,A.K., Birnbaum,M.J., and Pilch,P.F. (1998). Insulin increases the association of Akt-2 with Glut4-containing vesicles. J Biol. Chem. *273*, 7201-7204.

Cantley,L.C. (2002). The phosphoinositide 3-kinase pathway. Science *296*, 1655-1657.

Cappellini,A., Tabellini,G., Zweyer,M., Bortul,R., Tazzari,P.L., Billi,A.M., Fala,F., Cocco,L., and Martelli,A.M. (2003). The phosphoinositide 3-kinase/Akt pathway regulates

cell cycle progression of HL60 human leukemia cells through cytoplasmic relocalization of the cyclin-dependent kinase inhibitor p27(Kip1) and control of cyclin D1 expression. Leukemia *17*, 2157-2167.

Cardone,M.H., Roy,N., Stennicke,H.R., Salvesen,G.S., Franke,T.F., Stanbridge,E., Frisch,S., and Reed,J.C. (1998). Regulation of cell death protease caspase-9 by phosphorylation. Science *282*, 1318-1321.

Chan,T.O., Rittenhouse,S.E., and Tsichlis,P.N. (1999). AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. Annu. Rev. Biochem *68*, 965-1014.

Chen,C. and Okayama,H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell Biol. *7*, 2745-2752.

Chen,D., Zhang,Z., Li,M., Wang,W., Li,Y., Rayburn,E.R., Hill,D.L., Wang,H., and Zhang,R. (2007). Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. Oncogene.

Chen,W.S., Xu,P.Z., Gottlob,K., Chen,M.L., Sokol,K., Shiyanova,T., Roninson,I., Weng,W., Suzuki,R., Tobe,K., Kadowaki,T., and Hay,N. (2001). Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev. *15*, 2203-2208.

Cheng,J.Q., Altomare,D.A., Klein,M.A., Lee,W.C., Kruh,G.D., Lissy,N.A., and Testa,J.R. (1997). Transforming activity and mitosis-related expression of the AKT2 oncogene: evidence suggesting a link between cell cycle regulation and oncogenesis. Oncogene *14*, 2793-2801.

Cho,H., Mu,J., Kim,J.K., Thorvaldsen,J.L., Chu,Q., Crenshaw,E.B., III, Kaestner,K.H., Bartolomei,M.S., Shulman,G.I., and Birnbaum,M.J. (2001). Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science *292*, 1728-1731.

Chung,C.T. and Miller,R.H. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res. *16*, 3580.

Cmejlova,J., Dolezalova,L., Pospisilova,D., Petrtylova,K., Petrak,J., and Cmejla,R. (2006). Translational efficiency in patients with Diamond-Blackfan anemia. Haematologica *91*, 1456-1464.

Coffer,P.J. and Woodgett,J.R. (1991). Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. Eur. J Biochem *201*, 475-481.

Craxton,A., Jiang,A., Kurosaki,T., and Clark,E.A. (1999). Syk and Bruton's tyrosine kinase are required for B cell antigen receptor-mediated activation of the kinase Akt. J. Biol. Chem. *274*, 30644-30650.

Cross,D.A., Alessi,D.R., Cohen,P., Andjelkovich,M., and Hemmings,B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785-789.

Dai,M.S. and Lu,H. (2004). Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. J. Biol. Chem. *279*, 44475-44482.

Dai,M.S., Zeng,S.X., Jin,Y., Sun,X.X., David,L., and Lu,H. (2004). Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. Mol. Cell Biol. *24*, 7654-7668.

Datta,S.R., Dudek,H., Tao,X., Masters,S., Fu,H., Gotoh,Y., and Greenberg,M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

de,A., I, Metzger,M.H., Exley,M., Dahl,C.E., Misra,S., Zheng,D., Varticovski,L., Terhorst,C., and Sancho,J. (1997). Tyrosine phosphorylation of the CD3-epsilon subunit of the T cell antigen receptor mediates enhanced association with phosphatidylinositol 3-kinase in Jurkat T cells. J. Biol. Chem. *272*, 25310-25318.

Delcommenne,M., Tan,C., Gray,V., Rue,L., Woodgett,J., and Dedhar,S. (1998). Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc. Natl. Acad. Sci. U. S. A *95*, 11211-11216.

Deprez,J., Vertommen,D., Alessi,D.R., Hue,L., and Rider,M.H. (1997). Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. J Biol. Chem. *272*, 17269-17275.

Di,C.A. and Pandolfi,P.P. (2000). The multiple roles of PTEN in tumor suppression. Cell *100*, 387-390.

Dijkers,P.F., Birkenkamp,K.U., Lam,E.W., Thomas,N.S., Lammers,J.W., Koenderman,L., and Coffer,P.J. (2002). FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. J. Cell Biol. *156*, 531-542.

Downward,J. (1999). How BAD phosphorylation is good for survival. Nat. Cell Biol. *1*, E33-E35.

Du,K., Herzig,S., Kulkarni,R.N., and Montminy,M. (2003). TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. Science *300*, 1574-1577.

Du,K. and Montminy,M. (1998). CREB is a regulatory target for the protein kinase Akt/PKB. J. Biol. Chem. *273*, 32377-32379.

Easton,R.M., Cho,H., Roovers,K., Shineman,D.W., Mizrahi,M., Forman,M.S., Lee,V.M., Szabolcs,M., de,J.R., Oltersdorf,T., Ludwig,T., Efstratiadis,A., and Birnbaum,M.J. (2005). Role for Akt3/protein kinase Bgamma in attainment of normal brain size. Mol. Cell Biol. *25*, 1869-1878.

Ellis,S.R. and Massey,A.T. (2006). Diamond Blackfan anemia: A paradigm for a ribosome-based disease. Med. Hypotheses *66*, 643-648.

Fabre,S., Lang,V., Harriague,J., Jobart,A., Unterman,T.G., Trautmann,A., and Bismuth,G. (2005). Stable activation of phosphatidylinositol 3-kinase in the T cell immunological synapse stimulates Akt signaling to FoxO1 nuclear exclusion and cell growth control. J.

Immunol. *174*, 4161-4171.

Fabrizio,P., Pozza,F., Pletcher,S.D., Gendron,C.M., and Longo,V.D. (2001). Regulation of longevity and stress resistance by Sch9 in yeast. Science *292*, 288-290.

Farr,A. and Roman,A. (1992). A pitfall of using a second plasmid to determine transfection efficiency. Nucleic Acids Res. *20*, 920.

Feng,J., Park,J., Cron,P., Hess,D., and Hemmings,B.A. (2004). Identification of a PKB/Akt hydrophobic motif Ser-473 kinase as DNA-dependent protein kinase. J. Biol. Chem. *279*, 41189-41196.

Figueroa,C., Tarras,S., Taylor,J., and Vojtek,A.B. (2003). Akt2 negatively regulates assembly of the POSH-MLK-JNK signaling complex. J Biol. Chem. *278*, 47922-47927.

Frauwirth,K.A., Riley,J.L., Harris,M.H., Parry,R.V., Rathmell,J.C., Plas,D.R., Elstrom,R.L., June,C.H., and Thompson,C.B. (2002). The CD28 signaling pathway regulates glucose metabolism. Immunity. *16*, 769-777.

Frech,M., Andjelkovic,M., Ingley,E., Reddy,K.K., Falck,J.R., and Hemmings,B.A. (1997). High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. J Biol. Chem. *272*, 8474-8481.

Freeburn,R.W., Wright,K.L., Burgess,S.J., Astoul,E., Cantrell,D.A., and Ward,S.G. (2002). Evidence that SHIP-1 contributes to phosphatidylinositol 3,4,5-trisphosphate metabolism in T lymphocytes and can regulate novel phosphoinositide 3-kinase effectors. J. Immunol. *169*, 5441-5450.

Freedman,D.A. and Levine,A.J. (1998). Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6. Mol. Cell Biol. *18*, 7288-7293.

Fu,Y., Maianu,L., Melbert,B.R., and Garvey,W.T. (2004). Facilitative glucose transporter gene expression in human lymphocytes, monocytes, and macrophages: a role for GLUT isoforms 1, 3, and 5 in the immune response and foam cell formation. Blood Cells Mol. Dis. *32*, 182-190.

Gao,T., Furnari,F., and Newton,A.C. (2005). PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol. Cell *18*, 13-24.

Garami,A., Zwartkruis,F.J., Nobukuni,T., Joaquin,M., Roccio,M., Stocker,H., Kozma,S.C., Hafen,E., Bos,J.L., and Thomas,G. (2003). Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol. Cell *11*, 1457-1466.

Garofalo,R.S., Orena,S.J., Rafidi,K., Torchia,A.J., Stock,J.L., Hildebrandt,A.L., Coskran,T., Black,S.C., Brees,D.J., Wicks,J.R., McNeish,J.D., and Coleman,K.G. (2003). Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. J Clin. Invest *112*, 197-208.

Gietz,R.D., Schiestl,R.H., Willems,A.R., and Woods,R.A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast *11*, 355-360.

Gold,M.R. and Aebersold,R. (1994). Both phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase products are increased by antigen receptor signaling in B cells. J. Immunol. *152*, 42-50.

Gold,M.R., Ingham,R.J., McLeod,S.J., Christian,S.L., Scheid,M.P., Duronio,V., Santos,L., and Matsuuchi,L. (2000). Targets of B-cell antigen receptor signaling: the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase-3 signaling pathway and the Rap1 GTPase. Immunol. Rev. *176*, 47-68.

Hanada,M., Feng,J., and Hemmings,B.A. (2004). Structure, regulation and function of PKB/AKT--a major therapeutic target. Biochim. Biophys. Acta *1697*, 3-16.

He,L., Simmen,F.A., Mehendale,H.M., Ronis,M.J., and Badger,T.M. (2006). Chronic ethanol intake impairs insulin signaling in rats by disrupting Akt association with the cell membrane. Role of TRB3 in inhibition of Akt/protein kinase B activation. J. Biol. Chem. *281*, 11126-11134.

Hill,M.M., Andjelkovic,M., Brazil,D.P., Ferrari,S., Fabbro,D., and Hemmings,B.A. (2001). Insulin-stimulated protein kinase B phosphorylation on Ser-473 is independent of its activity and occurs through a staurosporine-insensitive kinase. J Biol. Chem. *276*, 25643-25646.

Holdorf,A.D., Green,J.M., Levin,S.D., Denny,M.F., Straus,D.B., Link,V., Changelian,P.S., Allen,P.M., and Shaw,A.S. (1999). Proline residues in CD28 and the Src homology (SH)3 domain of Lck are required for T cell costimulation. J. Exp. Med. *190*, 375-384.

Holz,M.K., Ballif,B.A., Gygi,S.P., and Blenis,J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. Cell *123*, 569-580.

Horn,S., Endl,E., Fehse,B., Weck,M.M., Mayr,G.W., and Jucker,M. (2004). Restoration of SHIP activity in a human leukemia cell line downregulates constitutively activated phosphatidylinositol 3-kinase/Akt/GSK-3beta signaling and leads to an increased transit time through the G1 phase of the cell cycle. Leukemia *18*, 1839-1849.

Huang,X., Begley,M., Morgenstern,K.A., Gu,Y., Rose,P., Zhao,H., and Zhu,X. (2003). Crystal structure of an inactive Akt2 kinase domain. Structure. *11*, 21-30.

Ichim,T.E., Li,M., Qian,H., Popov,I.A., Rycerz,K., Zheng,X., White,D., Zhong,R., and Min,W.P. (2004). RNA interference: a potent tool for gene-specific therapeutics. Am. J. Transplant. *4*, 1227-1236.

Imai,T., Sudo,K., and Miwa,T. (1994). Assignment of the human ribosomal protein S25 gene (RPS25) to chromosome 11q23.3 by sequence analysis of the marker D11S456. Genomics *20*, 142-143.

Ingley,E. and Hemmings,B.A. (2000). PKB/Akt interacts with inosine-5' monophosphate dehydrogenase through its pleckstrin homology domain. FEBS Lett. *478*, 253-259.

Inoki,K., Li,Y., Zhu,T., Wu,J., and Guan,K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol. *4*, 648-657.

Irie,H.Y., Pearline,R.V., Grueneberg,D., Hsia,M., Ravichandran,P., Kothari,N., Natesan,S.,

and Brugge,J.S. (2005). Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. J. Cell Biol. *171*, 1023-1034.

Iynedjian,P.B. (2005). Lack of evidence for a role of TRB3/NIPK as an inhibitor of PKB-mediated insulin signalling in primary hepatocytes. Biochem. J. *386*, 113-118.

Jakel,S. and Gorlich,D. (1998). Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. EMBO J. *17*, 4491-4502.

Jho,E., Lomvardas,S., and Costantini,F. (1999). A GSK3beta phosphorylation site in axin modulates interaction with beta-catenin and Tcf-mediated gene expression. Biochem. Biophys. Res. Commun. *266*, 28-35.

Johnson,G.L. and Lapadat,R. (2002). Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science *298*, 1911-1912.

Jones,P.F., Jakubowicz,T., Pitossi,F.J., Maurer,F., and Hemmings,B.A. (1991). Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. Proc. Natl. Acad. Sci. U. S. A *88*, 4171-4175.

Jones,R.G., Parsons,M., Bonnard,M., Chan,V.S., Yeh,W.C., Woodgett,J.R., and Ohashi,P.S. (2000). Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo. J. Exp. Med. *191*, 1721-1734.

Kane,L.P., Mollenauer,M.N., Xu,Z., Turck,C.W., and Weiss,A. (2002). Akt-dependent phosphorylation specifically regulates Cot induction of NF-kappa B-dependent transcription. Mol Cell Biol. *22*, 5962-5974.

Kanzaki,M. (2006). Insulin receptor signals regulating GLUT4 translocation and actin dynamics. Endocr. J. *53*, 267-293.

Kato,S., Ding,J., and Du,K. (2007). Differential activation of CREB by Akt1 and Akt2. Biochem. Biophys. Res. Commun. *354*, 1061-1066.

Kato,T., Muraski,J., Chen,Y., Tsujita,Y., Wall,J., Glembotski,C.C., Schaefer,E., Beckerle,M., and Sussman,M.A. (2005). Atrial natriuretic peptide promotes cardiomyocyte survival by cGMP-dependent nuclear accumulation of zyxin and Akt. J. Clin. Invest *115*, 2716-2730.

Kawakami,Y., Nishimoto,H., Kitaura,J., Maeda-Yamamoto,M., Kato,R.M., Littman,D.R., Leitges,M., Rawlings,D.J., and Kawakami,T. (2004). Protein kinase C betaII regulates Akt phosphorylation on Ser-473 in a cell type- and stimulus-specific fashion. J. Biol. Chem. *279*, 47720-47725.

Kim,A.H., Khursigara,G., Sun,X., Franke,T.F., and Chao,M.V. (2001). Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Mol. Cell Biol. *21*, 893-901.

Kim,A.H., Sasaki,T., and Chao,M.V. (2003). JNK-interacting protein 1 promotes Akt1 activation. J. Biol. Chem. *278*, 29830-29836.

Kim,A.H., Yano,H., Cho,H., Meyer,D., Monks,B., Margolis,B., Birnbaum,M.J., and Chao,M.V. (2002a). Akt1 regulates a JNK scaffold during excitotoxic apoptosis. Neuron *35*, 697-709.

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

Kim,H.H., Tharayil,M., and Rudd,C.E. (1998). Growth factor receptor-bound protein 2 SH2/SH3 domain binding to CD28 and its role in co-signaling. J. Biol. Chem. *273*, 296-301.

Knobbe,C.B. and Reifenberger,G. (2003). Genetic alterations and aberrant expression of genes related to the phosphatidyl-inositol-3'-kinase/protein kinase B (Akt) signal transduction pathway in glioblastomas. Brain Pathol. *13*, 507-518.

Kohn,A.D., Summers,S.A., Birnbaum,M.J., and Roth,R.A. (1996). Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol. Chem. *271*, 31372-31378.

Kubota,S., Copeland,T.D., and Pomerantz,R.J. (1999). Nuclear and nucleolar targeting of human ribosomal protein S25: common features shared with HIV-1 regulatory proteins. Oncogene *18*, 1503-1514.

Kumar,C.C. and Madison,V. (2005). AKT crystal structure and AKT-specific inhibitors. Oncogene *24*, 7493-7501.

Laine,J., Kunstle,G., Obata,T., and Noguchi,M. (2002). Differential regulation of Akt kinase isoforms by the members of the TCL1 oncogene family. J. Biol. Chem. *277*, 3743-3751.

Laine,J., Kunstle,G., Obata,T., Sha,M., and Noguchi,M. (2000). The protooncogene TCL1 is an Akt kinase coactivator. Mol Cell *6*, 395-407.

Lavery,W.L. and Goyns,M.H. (2002). Increased expression of the S25 ribosomal protein gene occurs during ageing of the rat liver. Gerontology *48*, 369-373.

Lee,J.Y., Kim,H., Ryu,C.H., Kim,J.Y., Choi,B.H., Lim,Y., Huh,P.W., Kim,Y.H., Lee,K.H., Jun,T.Y., Rha,H.K., Kang,J.K., and Choi,C.R. (2004). Merlin, a tumor suppressor, interacts with transactivation-responsive RNA-binding protein and inhibits its oncogenic activity. J. Biol. Chem. *279*, 30265-30273.

Li,M.L., Latoud,C., and Center,M.S. (1991). Cloning and sequencing a cDNA encoding human ribosomal protein S25. Gene *107*, 329-333.

Li,Y., Dowbenko,D., and Lasky,L.A. (2002). AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. J. Biol. Chem. *277*, 11352-11361.

Liang,J., Zubovitz,J., Petrocelli,T., Kotchetkov,R., Connor,M.K., Han,K., Lee,J.H., Ciarallo,S., Catzavelos,C., Beniston,R., Franssen,E., and Slingerland,J.M. (2002). PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. Nat. Med. *8*, 1153-1160.

Liu,A.X., Testa,J.R., Hamilton,T.C., Jove,R., Nicosia,S.V., and Cheng,J.Q. (1998a). AKT2, a member of the protein kinase B family, is activated by growth factors, v-Ha-ras, and v-src through phosphatidylinositol 3-kinase in human ovarian epithelial cancer cells.

Cancer Res. *58*, 2973-2977.

Liu,Q., Oliveira-Dos-Santos,A.J., Mariathasan,S., Bouchard,D., Jones,J., Sarao,R., Kozieradzki,I., Ohashi,P.S., Penninger,J.M., and Dumont,D.J. (1998b). The inositol polyphosphate 5-phosphatase ship is a crucial negative regulator of B cell antigen receptor signaling. J. Exp. Med. *188*, 1333-1342.

Lohrum,M.A., Ludwig,R.L., Kubbutat,M.H., Hanlon,M., and Vousden,K.H. (2003). Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell *3*, 577-587.

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

Luban,J. and Goff,S.P. (1995). The yeast two-hybrid system for studying protein-protein interactions. Curr. Opin. Biotechnol. *6*, 59-64.

Maira,S.M., Galetic,I., Brazil,D.P., Kaech,S., Ingley,E., Thelen,M., and Hemmings,B.A. (2001). Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. Science *294*, 374-380.

Mauro,V.P. and Edelman,G.M. (2002). The ribosome filter hypothesis. Proc. Natl. Acad. Sci. U. S. A *99*, 12031-12036.

Maxam,A.M. and Gilbert,W. (1977). A new method for sequencing DNA. Proc. Natl. Acad. Sci. U. S. A *74*, 560-564.

Mayo,L.D. and Donner,D.B. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc. Natl. Acad. Sci. U. S. A *98*, 11598-11603.

Melese,T. and Xue,Z. (1995). The nucleolus: an organelle formed by the act of building a ribosome. Curr. Opin. Cell Biol. *7*, 319-324.

Mendell,J.T. and Dietz,H.C. (2001). When the message goes awry: disease-producing mutations that influence mRNA content and performance. Cell *107*, 411-414.

Milburn,C.C., Deak,M., Kelly,S.M., Price,N.C., Alessi,D.R., and van Aalten,D.M. (2003). Binding of phosphatidylinositol 3,4,5-trisphosphate to the pleckstrin homology domain of protein kinase B induces a conformational change. Biochem. J. *375*, 531-538.

Mitsuuchi,Y., Johnson,S.W., Sonoda,G., Tanno,S., Golemis,E.A., and Testa,J.R. (1999). Identification of a chromosome 3p14.3-21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2. Oncogene *18*, 4891-4898.

Miyake,K., Flygare,J., Kiefer,T., Utsugisawa,T., Richter,J., Ma,Z., Wiznerowicz,M., Trono,D., and Karlsson,S. (2005). Development of cellular models for ribosomal protein S19 (RPS19)-deficient diamond-blackfan anemia using inducible expression of siRNA against RPS19. Mol. Ther. *11*, 627-637.

Na,S.Y., Patra,A., Scheuring,Y., Marx,A., Tolaini,M., Kioussis,D., Hemmings,B.A., Hunig,T., and Bommhardt,U. (2003). Constitutively active protein kinase B enhances Lck and Erk activities and influences thymocyte selection and activation. J. Immunol. *171*,

1285-1296.

Nagel,J.E., Smith,R.J., Shaw,L., Bertak,D., Dixit,V.D., Schaffer,E.M., and Taub,D.D. (2004). Identification of genes differentially expressed in T cells following stimulation with the chemokines CXCL12 and CXCL10. BMC. Immunol. *5*, 17.

Nakatani,K., Thompson,D.A., Barthel,A., Sakaue,H., Liu,W., Weigel,R.J., and Roth,R.A. (1999). Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. J. Biol. Chem. *274*, 21528-21532.

Nishiyama,T., Yamamoto,H., Uchiumi,T., and Nakashima,N. (2007). Eukaryotic ribosomal protein RPS25 interacts with the conserved loop region in a dicistroviral intergenic internal ribosome entry site. Nucleic Acids Res.

Ogawara,Y., Kishishita,S., Obata,T., Isazawa,Y., Suzuki,T., Tanaka,K., Masuyama,N., and Gotoh,Y. (2002). Akt enhances Mdm2-mediated ubiquitination and degradation of p53. J. Biol. Chem. *277*, 21843-21850.

Osman,N., Turner,H., Lucas,S., Reif,K., and Cantrell,D.A. (1996). The protein interactions of the immunoglobulin receptor family tyrosine-based activation motifs present in the T cell receptor zeta subunits and the CD3 gamma, delta and epsilon chains. Eur. J. Immunol. *26*, 1063-1068.

Ozes,O.N., Mayo,L.D., Gustin,J.A., Pfeffer,S.R., Pfeffer,L.M., and Donner,D.B. (1999). NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature *401*, 82-85.

Park,H.S., Kim,M.S., Huh,S.H., Park,J., Chung,J., Kang,S.S., and Choi,E.J. (2002). Akt (protein kinase B) negatively regulates SEK1 by means of protein phosphorylation. J. Biol. Chem. *277*, 2573-2578.

Pasumarthi,K.B., Nakajima,H., Nakajima,H.O., Jing,S., and Field,L.J. (2000). Enhanced cardiomyocyte DNA synthesis during myocardial hypertrophy in mice expressing a modified TSC2 transgene. Circ. Res. *86*, 1069-1077.

Pekarsky,Y., Koval,A., Hallas,C., Bichi,R., Tresini,M., Malstrom,S., Russo,G., Tsichlis,P., and Croce,C.M. (2000). Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. Proc. Natl. Acad. Sci. U. S. A *97*, 3028-3033.

Peng,X.D., Xu,P.Z., Chen,M.L., Hahn-Windgassen,A., Skeen,J., Jacobs,J., Sundararajan,D., Chen,W.S., Crawford,S.E., Coleman,K.G., and Hay,N. (2003). Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev. *17*, 1352-1365.

Peterson,R.T. and Schreiber,S.L. (1999). Kinase phosphorylation: Keeping it all in the family. Curr. Biol. *9*, R521-R524.

Phan,L., Zhang,X., Asano,K., Anderson,J., Vornlocher,H.P., Greenberg,J.R., Qin,J., and Hinnebusch,A.G. (1998). Identification of a translation initiation factor 3 (eIF3) core complex, conserved in yeast and mammals, that interacts with eIF5. Mol. Cell Biol. *18*, 4935-4946.

Potter,C.J., Pedraza,L.G., and Xu,T. (2002). Akt regulates growth by directly phosphorylating Tsc2. Nat. Cell Biol. *4*, 658-665.

Prasad,K.V., Cai,Y.C., Raab,M., Duckworth,B., Cantley,L., Shoelson,S.E., and Rudd,C.E. (1994). T-cell antigen CD28 interacts with the lipid kinase phosphatidylinositol 3-kinase by a cytoplasmic Tyr(P)-Met-Xaa-Met motif. Proc. Natl. Acad. Sci. U. S. A *91*, 2834-2838.

Quaye,I.K., Toku,S., and Tanaka,T. (1996). Sequence requirement for nucleolar localization of rat ribosomal protein L31. Eur. J. Cell Biol. *69*, 151-155.

Rane,M.J., Coxon,P.Y., Powell,D.W., Webster,R., Klein,J.B., Pierce,W., Ping,P., and McLeish,K.R. (2001). p38 Kinase-dependent MAPKAPK-2 activation functions as 3-phosphoinositide-dependent kinase-2 for Akt in human neutrophils. J. Biol. Chem. *276*, 3517-3523.

Rathmell,J.C., Elstrom,R.L., Cinalli,R.M., and Thompson,C.B. (2003a). Activated Akt promotes increased resting T cell size, CD28-independent T cell growth, and development of autoimmunity and lymphoma. Eur. J. Immunol. *33*, 2223-2232.

Rathmell,J.C., Fox,C.J., Plas,D.R., Hammerman,P.S., Cinalli,R.M., and Thompson,C.B. (2003b). Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. Mol Cell Biol. *23*, 7315-7328.

Raught,B., Gingras,A.C., Gygi,S.P., Imataka,H., Morino,S., Gradi,A., Aebersold,R., and Sonenberg,N. (2000). Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4GI. EMBO J. *19*, 434-444.

Remy,I. and Michnick,S.W. (2004). Regulation of apoptosis by the Ft1 protein, a new modulator of protein kinase B/Akt. Mol. Cell Biol. *24*, 1493-1504.

Rodionova,E., Conzelmann,M., Maraskovsky,E., Hess,M., Kirsch,M., Giese,T., Ho,A.D., Zoller,M., Dreger,P., and Luft,T. (2006). GSK-3 mediates differentiation and activation of pro-inflammatory dendritic cells. Blood.

Rondinone,C.M., Carvalho,E., Wesslau,C., and Smith,U.P. (1999). Impaired glucose transport and protein kinase B activation by insulin, but not okadaic acid, in adipocytes from subjects with Type II diabetes mellitus. Diabetologia *42*, 819-825.

Rossig,L., Jadidi,A.S., Urbich,C., Badorff,C., Zeiher,A.M., and Dimmeler,S. (2001). Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells. Mol. Cell Biol. *21*, 5644-5657.

Rothe,M., Ko,Y., Albers,P., and Wernert,N. (2000). Eukaryotic initiation factor 3 p110 mRNA is overexpressed in testicular seminomas. Am. J. Pathol. *157*, 1597-1604.

Ruggero,D. and Sonenberg,N. (2005). The Akt of translational control. Oncogene *24*, 7426-7434.

Russo,G., Ricciardelli,G., and Pietropaolo,C. (1997). Different domains cooperate to target the human ribosomal L7a protein to the nucleus and to the nucleoli. J. Biol. Chem. *272*, 5229-5235.

Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B., and Erlich,H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science *239*, 487-491.

Samstag,Y. and Nebl,G. (2005). Ras initiates phosphatidyl-inositol-3-kinase (PI3K)/PKB mediated signalling pathways in untransformed human peripheral blood T lymphocytes. Adv. Enzyme Regul. *45*, 52-62.

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

Schlegel,J., Piontek,G., and Mennel,H.D. (2002). Activation of the anti-apoptotic Akt/protein kinase B pathway in human malignant gliomas in vivo. Anticancer Res. *22*, 2837-2840.

Schmidt,C., Lipsius,E., and Kruppa,J. (1995). Nuclear and nucleolar targeting of human ribosomal protein S6. Mol. Biol. Cell *6*, 1875-1885.

Scoles,D.R., Yong,W.H., Qin,Y., Wawrowsky,K., and Pulst,S.M. (2006). Schwannomin inhibits tumorigenesis through direct interaction with the eukaryotic initiation factor subunit c (eIF3c). Hum. Mol. Genet. *15*, 1059-1070.

Shankar,D.B., Cheng,J.C., and Sakamoto,K.M. (2005). Role of cyclic AMP response element binding protein in human leukemias. Cancer *104*, 1819-1824.

Shiraishi,I., Melendez,J., Ahn,Y., Skavdahl,M., Murphy,E., Welch,S., Schaefer,E., Walsh,K., Rosenzweig,A., Torella,D., Nurzynska,D., Kajstura,J., Leri,A., Anversa,P., and Sussman,M.A. (2004). Nuclear targeting of Akt enhances kinase activity and survival of cardiomyocytes. Circ. Res. *94*, 884-891.

Siebenlist,U., Durand,D.B., Bressler,P., Holbrook,N.J., Norris,C.A., Kamoun,M., Kant,J.A., and Crabtree,G.R. (1986). Promoter region of interleukin-2 gene undergoes chromatin structure changes and confers inducibility on chloramphenicol acetyltransferase gene during activation of T cells. Mol. Cell Biol. *6*, 3042-3049.

Silver,P.A., Keegan,L.P., and Ptashine,M. (1984). Amino terminus of the yeast GAL4 gene product is sufficient for nuclear localization. Proc. Natl. Acad. Sci. U. S. A *81*, 5951-5955.

Smith,D.B. and Johnson,K.S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene *67*, 31-40.

Soucek,T., Yeung,R.S., and Hengstschlager,M. (1998). Inactivation of the cyclin-dependent kinase inhibitor p27 upon loss of the tuberous sclerosis complex gene-2. Proc. Natl. Acad. Sci. U. S. A *95*, 15653-15658.

Spahn,C.M., Beckmann,R., Eswar,N., Penczek,P.A., Sali,A., Blobel,G., and Frank,J. (2001). Structure of the 80S ribosome from Saccharomyces cerevisiae--tRNA-ribosome and subunit-subunit interactions. Cell *107*, 373-386.

Staal,S.P. (1987). Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc. Natl. Acad. Sci. U. S. A *84*, 5034-5037.

Stahl,J.M., Sharma,A., Cheung,M., Zimmerman,M., Cheng,J.Q., Bosenberg,M.W., Kester,M., Sandirasegarane,L., and Robertson,G.P. (2004). Deregulated Akt3 activity promotes development of malignant melanoma. Cancer Res. *64*, 7002-7010.

Stahl,M., Dijkers,P.F., Kops,G.J., Lens,S.M., Coffer,P.J., Burgering,B.M., and Medema,R.H. (2002). The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. J. Immunol. *168*, 5024-5031.

Stokoe,D., Stephens,L.R., Copeland,T., Gaffney,P.R., Reese,C.B., Painter,G.F., Holmes,A.B., McCormick,F., and Hawkins,P.T. (1997). Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science *277*, 567-570.

Sun,M., Paciga,J.E., Feldman,R.I., Yuan,Z., Coppola,D., Lu,Y.Y., Shelley,S.A., Nicosia,S.V., and Cheng,J.Q. (2001). Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. Cancer Res. *61*, 5985-5991.

Suzuki,H., Terauchi,Y., Fujiwara,M., Aizawa,S., Yazaki,Y., Kadowaki,T., and Koyasu,S. (1999). Xid-like immunodeficiency in mice with disruption of the p85alpha subunit of phosphoinositide 3-kinase. Science *283*, 390-392.

Takahashi,Y., Hirayama,S., and Odani,S. (2005). Ribosomal proteins cross-linked to the initiator AUG codon of a mRNA in the translation initiation complex by UV-irradiation. J. Biochem. (Tokyo) *138*, 41-46.

Tanaka,M., Konishi,H., Touhara,K., Sakane,F., Hirata,M., Ono,Y., and Kikkawa,U. (1999). Identification of myosin II as a binding protein to the PH domain of protein kinase B
2. Biochem. Biophys. Res. Commun. *255*, 169-174.

Tellam,J., Fogg,M.H., Rist,M., Connolly,G., Tscharke,D., Webb,N., Heslop,L., Wang,F., and Khanna,R. (2007). Influence of translation efficiency of homologous viral proteins on the endogenous presentation of CD8+ T cell epitopes. J. Exp. Med. *204*, 525-532.

Terada,N., Patel,H.R., Takase,K., Kohno,K., Nairn,A.C., and Gelfand,E.W. (1994). Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. Proc. Natl. Acad. Sci. U. S. A *91*, 11477-11481.

Thomas,C.C., Deak,M., Alessi,D.R., and van Aalten,D.M. (2002). High-resolution structure of the pleckstrin homology domain of protein kinase b/akt bound to phosphatidylinositol (3,4,5)-trisphosphate. Curr. Biol. *12*, 1256-1262.

Thornberry,N.A. (1998). Caspases: key mediators of apoptosis. Chem. Biol. *5*, R97-103.

Thut,C.J., Goodrich,J.A., and Tjian,R. (1997). Repression of p53-mediated transcription by MDM2: a dual mechanism. Genes Dev. *11*, 1974-1986.

Toker,A. and Newton,A.C. (2000). Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. J Biol. Chem. *275*, 8271-8274.

Tolan,D.R., Hershey,J.W., and Traut,R.T. (1983). Crosslinking of eukaryotic initiation factor eIF3 to the 40S ribosomal subunit from rabbit reticulocytes. Biochimie *65*, 427-436.

Tremblay,F., Lavigne,C., Jacques,H., and Marette,A. (2001). Defective insulin-induced GLUT4 translocation in skeletal muscle of high fat-fed rats is associated with alterations in both Akt/protein kinase B and atypical protein kinase C (zeta/lambda) activities. Diabetes *50*, 1901-1910.

Trencia,A., Perfetti,A., Cassese,A., Vigliotta,G., Miele,C., Oriente,F., Santopietro,S., Giacco,F., Condorelli,G., Formisano,P., and Beguinot,F. (2003). Protein kinase B/Akt binds and phosphorylates PED/PEA-15, stabilizing its antiapoptotic action. Mol Cell Biol. *23*, 4511-4521.

Tschopp,O., Yang,Z.Z., Brodbeck,D., Dummler,B.A., Hemmings-Mieszczak,M., Watanabe,T., Michaelis,T., Frahm,J., and Hemmings,B.A. (2005). Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis. Development *132*, 2943-2954.

Tsujita,Y., Muraski,J., Shiraishi,I., Kato,T., Kajstura,J., Anversa,P., and Sussman,M.A. (2006). Nuclear targeting of Akt antagonizes aspects of cardiomyocyte hypertrophy. Proc. Natl. Acad. Sci. U. S. A *103*, 11946-11951.

Uchiumi,T., Kikuchi,M., and Ogata,K. (1986). Cross-linking study on protein neighborhoods at the subunit interface of rat liver ribosomes with 2-iminothiolane. J. Biol. Chem. *261*, 9663-9667.

Valasek,L., Mathew,A.A., Shin,B.S., Nielsen,K.H., Szamecz,B., and Hinnebusch,A.G. (2003). The yeast eIF3 subunits TIF32/a, NIP1/c, and eIF5 make critical connections with the 40S ribosome in vivo. Genes Dev. *17*, 786-799.

Valasek,L., Nielsen,K.H., Zhang,F., Fekete,C.A., and Hinnebusch,A.G. (2004). Interactions of eukaryotic translation initiation factor 3 (eIF3) subunit NIP1/c with eIF1 and eIF5 promote preinitiation complex assembly and regulate start codon selection. Mol. Cell Biol. *24*, 9437-9455.

Van Der Heide,L.P., Hoekman,M.F., and Smidt,M.P. (2004). The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. Biochem. J. *380*, 297-309.

van Weeren,P.C., de Bruyn,K.M., Vries-Smits,A.M., van Lint,J., and Burgering,B.M. (1998). Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. J Biol. Chem. *273*, 13150-13156.

Viniegra,J.G., Martinez,N., Modirassari,P., Losa,J.H., Parada,C.C., Lobo,V.J., Luquero,C.I., varez-Vallina,L., Cajal,S., Rojas,J.M., and Sanchez-Prieto,R. (2005). Full activation of PKB/Akt in response to insulin or ionizing radiation is mediated through ATM. J. Biol. Chem. *280*, 4029-4036.

Virgilio,L., Narducci,M.G., Isobe,M., Billips,L.G., Cooper,M.D., Croce,C.M., and Russo,G. (1994). Identification of the TCL1 gene involved in T-cell malignancies. Proc. Natl. Acad. Sci. U. S. A *91*, 12530-12534.

Westermann,P., Gross,B., and Bielka,H. (1980). Neighbourhoods of proteins S2--S3, S3--S3a, S15--S15a, and S5--S25 within the small subunit of rat liver ribosomes. Acta Biol. Med. Ger *39*, 1147-1152.

Westermann,P. and Nygard,O. (1983). The spatial arrangement of the complex between eukaryotic initiation factor eIF-3 and 40 S ribosomal subunit. Cross-linking between factor and ribosomal proteins. Biochim. Biophys. Acta *741*, 103-108.

Westermann,P., Nygard,O., and Bielka,H. (1981). Cross-linking of Met-tRNAf to eIF-2 beta and to the ribosomal proteins S3a and S6 within the eukaryotic inhibition complex, eIF-2 .GMPPCP.Met-tRNAf.small ribosomal subunit. Nucleic Acids Res. *9*, 2387-2396.

Williams,M.R., Arthur,J.S., Balendran,A., van der,K.J., Poli,V., Cohen,P., and Alessi,D.R. (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. Curr. Biol. *10*, 439-448.

Williams,T.M., Burlein,J.E., Ogden,S., Kricka,L.J., and Kant,J.A. (1989). Advantages of firefly luciferase as a reporter gene: application to the interleukin-2 gene promoter. Anal. Biochem. *176*, 28-32.

Wilson,D.N. and Nierhaus,K.H. (2005). Ribosomal proteins in the spotlight. Crit Rev. Biochem. Mol. Biol. *40*, 243-267.

Wolfraim,L.A., Walz,T.M., James,Z., Fernandez,T., and Letterio,J.J. (2004). p21Cip1 and p27Kip1 act in synergy to alter the sensitivity of naive T cells to TGF-beta-mediated G1 arrest through modulation of IL-2 responsiveness. J. Immunol. *173*, 3093-3102.

Wool,I.G., Chan,Y.L., and Gluck,A. (1995). Structure and evolution of mammalian ribosomal proteins. Biochem. Cell Biol. *73*, 933-947.

Xiao,C., Yang,B.F., Asadi,N., Beguinot,F., and Hao,C. (2002). Tumor necrosis factor-related apoptosis-inducing ligand-induced death-inducing signaling complex and its modulation by c-FLIP and PED/PEA-15 in glioma cells. J. Biol. Chem. *277*, 25020-25025.

Xu,X., Sakon,M., Nagano,H., Hiraoka,N., Yamamoto,H., Hayashi,N., Dono,K., Nakamori,S., Umeshita,K., Ito,Y., Matsuura,N., and Monden,M. (2004). Akt2 expression correlates with prognosis of human hepatocellular carcinoma. Oncol. Rep. *11*, 25-32.

Yamada,E., Okada,S., Saito,T., Ohshima,K., Sato,M., Tsuchiya,T., Uehara,Y., Shimizu,H., and Mori,M. (2005). Akt2 phosphorylates Synip to regulate docking and fusion of GLUT4-containing vesicles. J Cell Biol. *168*, 921-928.

Yang,J., Cron,P., Good,V.M., Thompson,V., Hemmings,B.A., and Barford,D. (2002). Crystal structure of an activated Akt/protein kinase B ternary complex with GSK3-peptide and AMP-PNP. Nat. Struct. Biol. *9*, 940-944.

Yang,L., Lin,H.K., Altuwaijri,S., Xie,S., Wang,L., and Chang,C. (2003a). APPL suppresses androgen receptor transactivation via potentiating Akt activity. J. Biol. Chem. *278*, 16820-16827.

Yang,Z.Z., Tschopp,O., Baudry,A., Dummler,B., Hynx,D., and Hemmings,B.A. (2004). Physiological functions of protein kinase B/Akt. Biochem. Soc. Trans. *32*, 350-354.

Yang,Z.Z., Tschopp,O., Hemmings-Mieszczak,M., Feng,J., Brodbeck,D., Perentes,E., and Hemmings,B.A. (2003b). Protein kinase B alpha/Akt1 regulates placental development and fetal growth. J. Biol. Chem. *278*, 32124-32131.

Yewdell,J.W. and Nicchitta,C.V. (2006). The DRiP hypothesis decennial: support, controversy, refinement and extension. Trends Immunol. *27*, 368-373.

Yoeli-Lerner,M., Yiu,G.K., Rabinovitz,I., Erhardt,P., Jauliac,S., and Toker,A. (2005). Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol. Cell *20*, 539-550.

Yuan,Z.Q., Sun,M., Feldman,R.I., Wang,G., Ma,X., Jiang,C., Coppola,D., Nicosia,S.V., and Cheng,J.Q. (2000). Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. Oncogene *19*, 2324-2330.

Yusuf,I., Zhu,X., Kharas,M.G., Chen,J., and Fruman,D.A. (2004). Optimal B-cell proliferation requires phosphoinositide 3-kinase-dependent inactivation of FOXO transcription factors. Blood *104*, 784-787.

Zhao,S., Konopleva,M., Cabreira-Hansen,M., Xie,Z., Hu,W., Milella,M., Estrov,Z., Mills,G.B., and Andreeff,M. (2004). Inhibition of phosphatidylinositol 3-kinase dephosphorylates BAD and promotes apoptosis in myeloid leukemias. Leukemia *18*, 267-275.

Zhou,B.P., Liao,Y., Xia,W., Spohn,B., Lee,M.H., and Hung,M.C. (2001). Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat. Cell Biol. *3*, 245-252.

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