The identification and functional characterisation of novel targets of Protein Kinase B (PKB/c-akt) action

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CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

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Proefschrift Universiteit Utrecht – met een samenvatting in het Nederlands
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Cover Art & Design:	Sue van Soest
Lay-out & technical support:	Ingeborg de Buijzer
Print:	Gildeprint, Enschede
ISBN:	978-90-393-4537-5

This research described in this thesis was performed at the Department of Immunology and the Department of Pulmonary Diseases, University Medical Centre Utrecht and was financially supported by a VIDI innovational research grant from ZonMW (NWO).

The identification and functional characterisation of novel targets of Protein Kinase B (PKB/c-akt) action

De identificatie en functionele karakterisatie van nieuwe targets van de werking van Proteïne Kinase B (PKB/c-akt)

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. W.H. Gispen,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
donderdag 7 juni 2007 des middags te 12.45 uur

door

Anna Gerardina Maria van Gorp

geboren op 8 februari 1979 te Breda

Promotor: Prof. dr. P.J. Coffer

But it's all in the game and the way you play it,
and you've got to play the game, you know.

I was born free, I've had the time of my life
and for all we know I'm going to live forever.

The Accidental
Ali Smith

Aan mama en papa

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The printing of this thesis was aided financially by contributions from Edward Jenner Stichting, Divisie Laboratoria en Apotheek (Abstract Prize), J.E. Jurriaanse stichting, Universiteit Utrecht, Dr. Ir. van de Laar Stichting, tebu-bio, Beun de Ronde BV and BD Biosciences.

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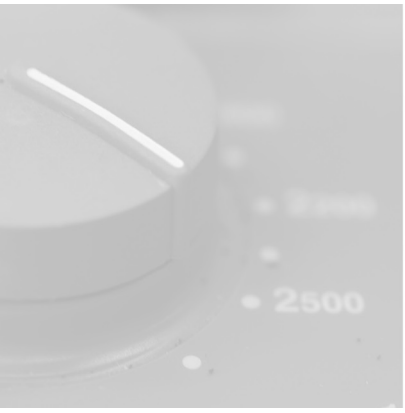
CHAPTER 1

General Introduction



Cancer is the result of a disturbed balance between cell division and growth on one hand, and programmed cell death on the other. Most cancers arise from a single cancer-initiating cell, a so-called cancer stem cell that has undergone malignant transformation driven by accumulative genetic alterations including tumour suppressor gene loss, oncogene amplification and chromosomal derangements (62). The term 'cancer stem cell' relates to a broad group of cells that share some common properties, such as self-renewal, ability to proliferate in defiance of normal growth-regulating mechanisms and by their ability to invade and destroy normal tissue (65). It is not yet clear if the cancer stem cells are derived from true tissue-derived stem cells or mature cells that have undergone a de-differentiation or transdifferentiation process (128,135).

Cellular behaviour is regulated by a complex network of signal transduction pathways. These signal transduction pathways regulate crucial processes such as cell motility, cell proliferation, and apoptosis. In many ways cancer is a disease of deregulated signal transduction: cancer cells grow when they should not and do not die (i.e. undergo apoptosis) when they should. Elucidation of disturbed signal transduction pathways involved in the carcinogenic process, both at the protein and the genomic levels, is critical for the identification of new targets for cancer treatment.

PI3K-PKB signalling in cancer

The phosphoinositol-3 kinase (PI3K) signalling pathway is crucial to many aspects of cell growth and survival. Dysregulation of the PI3K signalling pathway occurs by a variety of mechanisms in a large percentage of human tumours. For both PI3K and its downstream effector Protein kinase B (PKB/c-akt), the first evidence of a direct role in cancer was provided by the identification of homologous retroviral oncogene products which caused haemangiosarcomas in birds or lymphomas in mice respectively (14,30,159). Subsequently, it has become clear that the PI3K-PKB pathway is one of the most frequently targeted pathways in sporadic human tumours.

The most common mechanism of activation of the PI3K-PKB pathway is the loss of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a negative regulator of PI3K signalling. Sporadic mutations of PTEN are found in a high percentage of tumours, including breast, ovarian and colon cancers and glioblastoma (26). The loss of PTEN and subsequent activation of PI3K and PKB is also a critical event in human prostate cancer (109). Importantly, PTEN is now thought to be the second most commonly mutated tumour suppressor in humans, after p53.

A large-scale effort to sequence exons of PI3K genes in human tumours has also revealed clustered regions of point mutations in 20-30% of the breast, colon, brain and gastric tumours examined in this study (143). Most of these mutations enhance PI3K activity and have the potential to drive cellular transformation (144).

Human tumours also exhibit frequent dysregulation of PKB activity. These

alterations manifest as either gene amplifications that lead to overexpression of PKB or hyperactivation of PKB due to dysregulation of its upstream mediators. Amplification of PKB isoform encoding genes occurred, among others, in ovarian carcinomas, non-hodgkin lymphoma, pancreatic carcinomas, hepatocellular carcinomas and colon cancers (reviewed extensively in (6)).
Taken together, mutations in one or more components of PI3K-PKB pathway are estimated to count for up to 30% of all human cancers (104).

Phosphoinositide 3-kinases
Phosphatidylinositol (PI) is a minor component of eukaryotic cell membranes constituting approximately 8% of all phospholipids. The inositol headgroup of PI contains five free hydroxyl groups of which three (3', 4' and 5') can be phosphorylated *in vivo*. The 3' hydroxyl group can be phosphorylated by PI3Ks generating PI(3)P, PI(3,4)P_2 or PI(3,4,5)P_3. In a resting state, levels of 3-phosphoinositides are low but these levels are dramatically increased upon growth factor or cytokine stimulation. PI3K activity can be inhibited by well-known, and extensively used, small molecule inhibitors wortmannin and LY294002. *In vivo*, the actions of PI3Ks are negatively regulated by the phosphoinositol phosphatases PTEN and SH2 domain containing inositol 5'phosphatase (SHIP). SHIP dephosphorylates PI(3,4,5)P_3 at the 5' phosphate whereas PTEN dephosphorylates it at the 3' phosphate. Inactivating mutations of PTEN or SHIP therefore lead to increased levels of this phospholipid (105).
There are three classes of PI3Ks of which class I can be subdivided in IA and IB. *In vivo*, class I of PI3Ks is the main source of PI(3,4,5)P_3, the phosphoinositide necessary for activation of a variety of intracellular signalling molecules including PKB. Class IA PI3Ks are activated by tyrosine kinase coupled transmembrane receptors such as cytokine receptors and growth factor receptors (Fig. 1). Class IA PI3Ks exist as a heterodimeric complex consisting of an 85 and an 110 kDa subunit (112). The 110 kDa subunit contains the catalytic activity of the enzyme, whereas the p85 subunit is an adaptor protein with no enzymatic activity recruiting the catalytic subunit to the plasma membrane upon cellular stimulation. The adaptor subunit is derived from three distinct genes p85α, p85β and p55γ giving rise to eight isoforms of which p85α is expressed at the highest levels and in the most tissues (120). p85α contains two phosphotyrosine-binding Src homology 2 (SH2) domains, two proline rich motifs, a region of homology to the breakpoint cluster region (BCR) and an N-terminal SH3 domain. The SH2 domains are responsible for interaction of the p85 subunit with phosphorylated tyrosines in growth factor and cytokine receptors whereas the space between the two SH2, the inter-SH2 (iSH2) domain binds p110 (47). Translocation to the plasma membrane leads to both conformational changes in the PI3K heterodimer and access to the lipid substrates. The catalytic class IA p110 subunit exists in three isoforms p110α (68), p110β (71) and p110δ (172), all encoded by separate genes. The first two have a wide tissue distribution whereas

p110δ is mostly restricted to cell of the haematopoietic system. They all contain an N-terminal binding site for the iSH2 domain of p85 (47).
The class IB PI3K exists solely of p110γ which also exists as a heterodimer binding to a regulatory p101 subunit (161). The class IB PI3K is activated by G-protein-coupled receptors (GPCRs), a large family of membrane proteins that include the chemokine receptors (164). The Gβγ subunits of these receptors recruit p110γ from the cytosol to the membrane by interaction with its p101 subunit (19). This p101 regulatory subunit that tightly binds p110γ is critical for Gβγ-activated PtdIns(3,4,5)P_3 production (96,106,161).

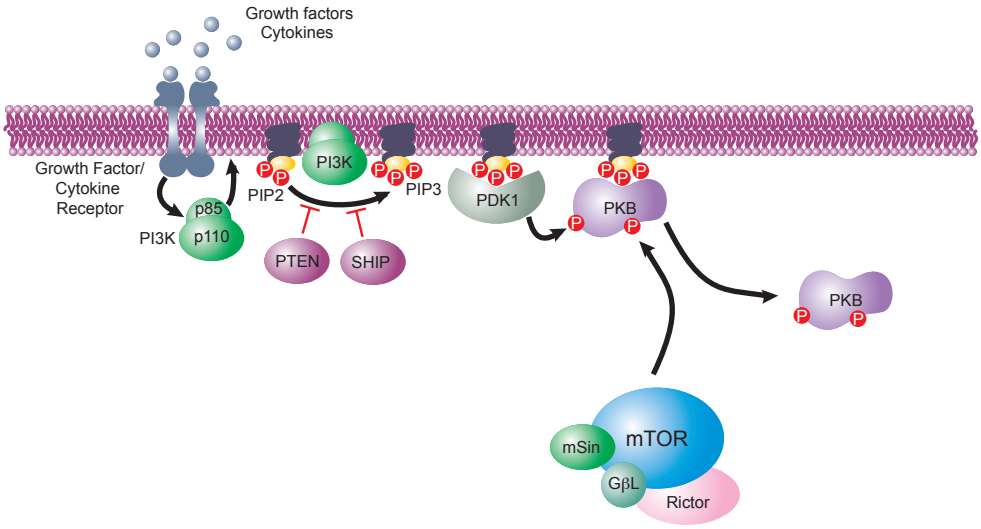


Figure 1: Activation of PI3K and PKB
Receptor-mediated generation of PI(3,4,5)P_3 by PI3K results in translocation of PKB to the plasma membrane and association with PI(3,4,5)P_3 through its PH domain. Binding of the PH domain to the plasma membrane leads to a conformational change in which the PH domain 'folds away' from the protein thereby exposing two critical phosphorylation sites. The recruitment of PKB to the plasma membrane leads to phosphorylation of both threonine (Thr) 308 and serine (Ser) 473 in PKBα. PDK-1 phosphorylates PKB at Thr308 whereas mTORC2 phosphorylates PKB at Ser473 resulting in maximum PKB activity.

All class I PI3Ks also bind the small GTPase $p21^{ras}$ (Ras) in a GTP-dependent manner with a domain that is similar to Ras binding domains (RBDs) present in other Ras regulated proteins (45,137,138,172). Ras binding induces a change in PI3K conformation stabilizing it at the plasma membrane thereby increasing its lipid kinase activity (121).
The PI(3,4,5)P_3 lipids generated by PI3Ks have high affinity for a variety of cytoplasmic proteins resulting in their translocation to the plasma membrane. Phospholipid products generated by PI3Ks associate with proteins that contain one

of a number of protein motifs that include the pleckstrin homology (PH) domain, SH2 domain, Phox homology (PX) domain, C2 domain and phosphotyrosine binding (PTB) domain. Translocation of these proteins to the membrane results in the formation of so-called 'signalosomes', where proteins associate and their activity is regulated.

How does PI3K induce PKB activation?

15 years ago Protein Kinase B (PKB/c-akt), a serine/threonine kinase was discovered independently by three research groups (14,37,77,159). In one report, PKB was described as the cellular homologue of the viral oncoprotein v-akt, the gene product of AKT-8, an acute transforming retrovirus that was isolated from a mouse lymphoma (14,159). The other two groups reported identification of a protein kinase whose kinase domain has significant homology with both protein kinase A (PKA) (65% homologous) and protein kinase C (PKC) (75% homologous), and it was subsequently named PKB (37,77).

PKB is a member of the AGC protein kinase family, which also includes serum- and glucocorticoid-induced protein kinase (SGK), mitogen- and stress-activated protein kinase (MSK), p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK), protein kinase C (PKC) isoforms, PKC-related kinase (PRK) and cyclic AMP (cAMP)-dependent protein kinase (PKA). This kinase family is defined by a high homology within their catalytic domains (13,171).

In mammalian cells there are three isoforms of PKB: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (Fig. 2). These isoforms are encoded by three separate genes on

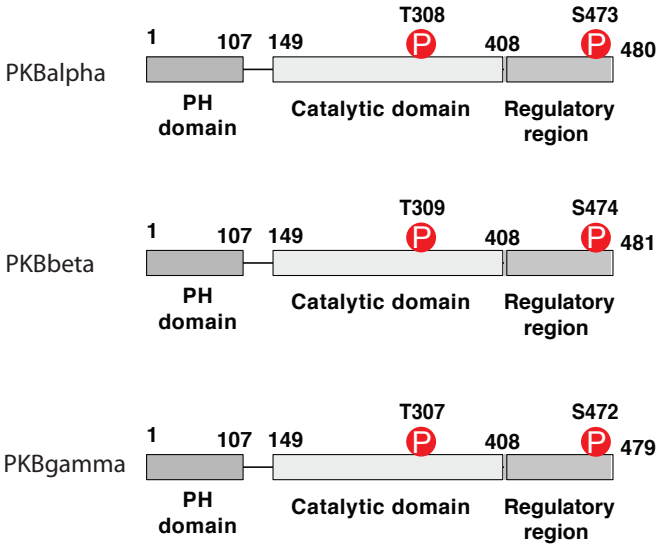


Figure 2: Schematic representation of the three human PKB isoforms

Depicted are the N-terminal PH domain, the catalytic domain which possesses specific kinase activity and the C-terminal regulatory domain. This figure also shows the two conserved regulatory phosphorylation sites.

chromosomes 14q32, 19q13 and 1q44 (14,33,37,77,91). PKBβ and PKBγ have 81% and 83% sequence similarity with PKBα (91). All three PKB isoforms are widely expressed however PKBα is expressed with highest levels in brain, heart and lung (92). The expression of PKBβ is highest in insulin-responsive tissues (4,5) while PKBγ is most abundantly expressed in the brain (111). All PKB isoforms contain an N-terminal PH domain, a central catalytic domain and a C-terminal regulatory region. Receptor-mediated generation of $PI(3,4,5)P_3$ by PI3K results in translocation of PKB to the plasma membrane and association with $PI(3,4,5)P_3$ through its PH domain which is essential for activation of the kinase (57,88). Binding of the PH domain to the plasma membrane leads to a conformational change in which the PH domain 'folds away' from the protein thereby exposing two critical phosphorylation sites. The recruitment of PKB to the plasma membrane leads to phosphorylation of both threonine (Thr) 308 and serine (Ser) 473 in PKBα. In PKBβ the equivalent phosphorylation sites are Thr309 and Ser474, and in PKBγ these are Thr305 and Ser472. The threonine is located in the catalytic domain, whereas serine is situated in the regulatory domain and phosphorylation of both residues is essential for maximum PKB activity (2). Phosphorylation on Thr308 primes PKB for phosphorylation on Ser473, which generates a conformational change that permits access to substrates, thereby fully activating PKB (3,149,160). Forced localisation of PKB at the plasma membrane results in its constitutive phosphorylation and activation (90). The kinase that phosphorylates PKB at Thr308 was identified as phosphoinositide-dependent kinase 1 (PDK-1) (3,163). Unsurprisingly, several other AGC kinases have also been found to be phosphorylated in their catalytic domain by PDK-1 resulting in their activation (34,76,97,127,132).

The identity of the Ser473 kinase, often referred to as PDK-2, has long remained elusive. Several candidates have been proposed including PDK-1 (8), integrin-linked kinase (ILK) (130), PKB itself (167) and DNA-PK (55). Recently, however, the "PDK-2-mystery" has been solved by Sarbassov and co-workers (148). These researchers convincingly demonstrated that mammalian target of rapamycin (mTOR) is the long sought after PDK2. mTOR exists in two distinct complexes within cells: mTORC1 that contains mTOR, GβL and Raptor (regulatory-associated protein of mTOR) (66,84,85,103) and another more recently described complex called mTORC2 containing mTOR, GβL and Rictor (rapamycin-insensitive companion of mTOR) (Fig. 3) (23,146). Only mTORC1 is sensitive to rapamycin and regulates translation and cell growth through ribosomal p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein (4E-BP). mTORC2 is also known to play a role in regulating the actin skeleton through Rho and PKCα (73,146). Since mTORC2 is insensitive to acute rapamycin treatment, mTOR functions discovered through this treatment are unlikely to be mediated by this complex.

Paradoxically, mTOR is also considered to be an important downstream mediator of PKB function, regulating protein translation. PKB can either activate mTOR through direct phosphorylation or via an indirect pathway. mTOR kinase activity is

activated by the GTPase Rheb, which in turn is controlled by the tuberous sclerosis complex (TSC1-TSC2). TSC2, which carries a GAP domain that regulates Rheb activity, is inactivated by PKB phosphorylation, which destabilizes TSC2 and disrupts its interaction with TSC1. Inactivation of TSC2 leads to activation of GTPase Rheb and subsequently mTORC1. Being both an upstream and downstream mediator of PKB signalling, it has been become almost impossible to evaluate the consequences of alterations of mTOR and PKB separately.

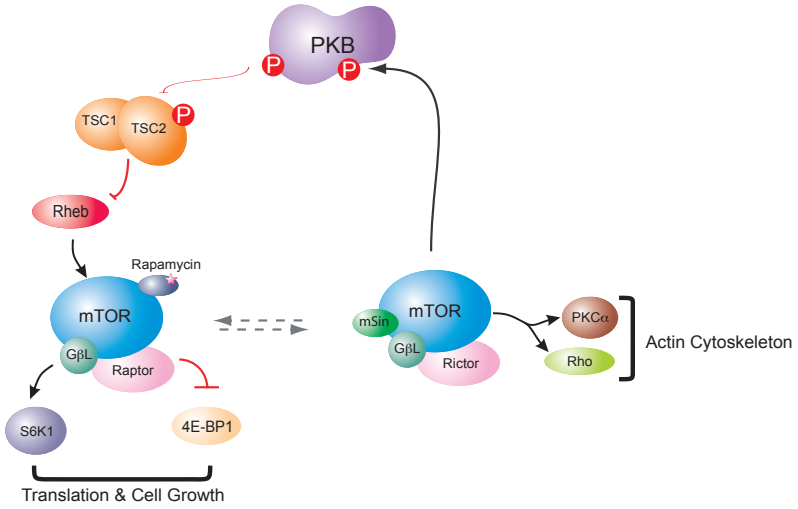


Figure 3: mTORC1 vs mTORC2
Activation of PKB leads to the phosphorylation and inhibition of TSC2, resulting in activation of Rheb. Active Rheb signals to the mTORC1 complex which when active phosphorylates p70S6K and 4E-BP, two important mediators of translation and cell growth. The regulation of mTORC2 activity remains unclear. mTORC2 is however responsible for activation of PKB and regulation of the actin cytoskeleton through regulation of PKCα and Rho.

mTOR and regulation of PKB activity

In recently published studies Raptor, Rictor and GβL deficient mice were generated to determine the role of mTORC1 and mTORC2 *in vivo*. Mice lacking Rictor or GβL survive until embryonic stage E10.5 whereas those lacking mTOR or Raptor die early in embryonic development (64,113). Furthermore, the Rictor null mice emphasized the role of mTORC2 as the primary kinase for Ser473 of PKB since Rictor null fibroblasts exhibit low proliferation rates, impaired PKB phosphorylation activity and diminished metabolic activity (156).

Recently, mSin1 was discovered as a novel component of mTORC2 but not mTORC1 (180). Alternative splicing generates at least five isoforms of the mSin1 protein (151), three of which assemble into mTORC2 to generate three distinct mTORC2s. All complexes can phosphorylate PKB *in vitro*, however the activity of

only two of them is regulated by insulin. It still remains to be identified which stimuli signal to PKB through this complex (180).
Genetic ablation of mSin1, similar to Rictor, results in embryonic lethality. It also disrupts the interaction between Rictor and mTOR, decreases PKB phosphorylation in both Drosophila and mammalian cells and diminishes PKB function *in vivo*. *In vitro*, mSin1 is required for TORC2 kinase activity, demonstrating that mSin1 together with Rictor are key components of mTORC2 and play an essential role in PKB phosphorylation and activation. Interestingly, the diminished PKB Ser473 phosphorylation observed in mSin1 knockouts affected only a subset of PKB substrates *in vivo* including Foxo1 and Foxo3a whereas other PKB targets as TSC and GSK3 as well as the mTORC1 effecters S6K and 4EBP remained unaffected (72). It could be that mSin1 through some yet to be identified mechanism regulates PKB substrates specificity however it could also be that there is some redundancy between AGC kinase family members in substrate phosphorylation. Indeed, there have already been reports of both RSK and p70S6K phosphorylating GSK3 (165,183).
Initially, mTORC2 was thought to be rapamycin insensitive since the complex did not respond to acute rapamycin treatment. However, subsequent studies have demonstrated that mTORC2 is sensitive to prolonged rapamycin treatment in a cell type specific manner (147). In these rapamycin-sensitive mTORC2 cells the assembly and thus activity of the complex is suppressed. Rapamycin blocks the interaction of Rictor to newly synthesized mTOR, whereas the interaction between raptor and mTOR is weakened immediately (147). However, for reasons yet unknown the level of this mTORC2 inhibition is cell type specific. It has been suggested that in some cell lines an unidentified protein or a post-translational modification blocks of the inhibitory site on mTOR. Interestingly, rapamycin-sensitive mTORC2 is often observed in haematopoietic cell lines.

PKB function

Targeted deletion of specific isoforms of PKB in mice has proved to be a powerful tool for elucidating the physiological roles of PKB proteins, as well as the redundancy and specific functions of the different isoforms.
Single knockouts of all PKB isoforms are viable but exhibit distinct phenotypes. PKB$\alpha^{-/-}$ mice showed increased neonatal mortality and defects in both fetal as well as postnatal growth that persisted into adulthood (36). PKBα was also found to be the major isoform in placenta and that placenta lacking this protein cannot form a proper vascular labyrinth; this may restrict nutrient supply to the fetus and impair growth as observed (182). PKB$\beta^{-/-}$ mice were studied by two independent research groups and they described slightly different phenotypes probably due to the different mice strains used. However, both studies confirmed an important role for PKBβ in the regulation of glucose metabolism. Cho and co-workers described that PKB$\beta^{-/-}$ mice developed to adulthood without apparent growth defects but these

animals did exhibit insulin resistance and mild diabetes (35). However, Garofalo *et al.* observed mild growth retardation of the PKBβ null mutants coupled with severe lipoatrophy or decrease in adipose tissue (59). Lipoatrophy is also associated with severe insulin resistance and diabetes (133). PKBγ null mutant mice display a distinct phenotype without increased pertinatal mortality, growth retardation or altered glucose metabolism. However, the brains of adult PKBγ mutant mice show a dramatic reduction in size and weight caused by a significant reduction of both cell size and number at this location, suggesting a crucial and specific role for this PKB isoform in brain development (169).

Table 1. PKB gene-knockout mice and their phenotypes

Targeted PKB isoform	**Survival**	**Phenotype**	**Ref(s)**
PKBα$^{-/-}$	Viable but increased neonatal lethality	Growth retardation Altered placental development	(36,182)
PKBβ$^{-/-}$	Viable	Mild growth retardation Insulin resistance Mild diabetes Severe lipoatrophy Decrease in adipose tissue	(35,59)
PKBγ$^{-/-}$	Viable	Reduced brain size and weight	(169)
PKBα$^{-/-}$PKBβ$^{-/-}$	Death shortly after birth	Impaired development of skin and bone Skeletal muscle atrophy Defect in adipogenesis	(129)
PKBα$^{-/-}$PKBγ$^{-/-}$	Death at embryonic stage	Severe developmental effects in cardiovascular and nervous system Increased apoptosis in the brain	(181)
PKBα$^{+/-}$PKBγ$^{-/-}$	Viable	Similar to PKBγ$^{-/-}$	(181)
PKBα$^{-/-}$PKBγ$^{+/-}$	Death shortly after birth	Similar to PKBα$^{-/-}$	(181)
PKBα$^{+/-}$PKBβ$^{-/-}$PKBγ$^{-/-}$	Viable	Combined phenotype of PKBβ$^{-/-}$ and PKBγ$^{-/-}$	(50)

The most drastic phenotypes have been observed in mice that lacked more than one PKB isoform. PKBα and PKBβ double-knockout mice exhibit severe growth deficiency and die shortly after birth (129). These mice display impaired skin development, severe skeletal muscle atrophy and delayed bone development. Interestingly, these effects are very similar to the phenotypes of insulin growth factor 1 (IGF-1) receptor deficient mice, suggesting that PKB may indeed be the most crucial downstream effector of this receptor (101). Furthermore, these double-knockout mice display impeded adipogenesis due to impaired induction of peroxisome proliferating-activated receptor-γ (PPARγ) expression during adipocyte differentiation (129).
Recently, Yang and co-workers crossed PKBα with PKBγ knockout mice to generate compound (combined mutation of four alleles of PKBα and PKBγ) and double knockout mice (181). Similar to PKBα and PKBβ double-knockout mice, the PKBα and PKBγ double-knockout mice were not viable, dying at embryonic days 11 and 12 due to severe developmental defects in the cardiovascular and nervous systems. Increased apoptosis was also found in the developing brain of the double mutant embryos. $PKB\alpha^{-/-}PKB\gamma^{+/-}$ animals were also not viable and died within several days after birth, however, interestingly, the $PKB\alpha^{+/-}PKB\gamma^{-/-}$ mice survived normally (181). This is in accordance with a more recent study from the same group in which they generated $PKB\alpha^{+/-}PKB\beta^{-/-}PKB\gamma^{-/-}$ mice and were able to demonstrate that a single functional allele of PKBα is sufficient for successful embryonic development and postnatal survival (50).
In conclusion, these studies indicate that the three PKB isoforms have both redundant and non-redundant physiological functions. The relatively normal development and distinct physiological functions exhibited by single knockouts can be explained by differences in the tissue distribution and expression levels of these isoforms. These knockout studies provide evidence for a dominant role for PKBα in embryonic development and postnatal survival, as well as the redundant and non-redundant roles of PKBβ and PKBγ in growth, glucose metabolism and brain and testis development. To further understand the causes of the different phenotypes, it is important to define the specific PKB substrates involved in the development and function of the organs affected in these knockout mice.

PKB substrates

Aberrant PKB signalling not only plays a role in cancer onset and progression but it has also been suggested to play a role in other pathological conditions such as obesity, diabetes and premature aging. In normal cellular physiology, PKB is probably best known for its role in cellular survival. A study performed in PKBα and PKBβ double-knockout mouse embryonic fibroblasts in which PKBγ protein expression was inhibited by PKBγ siRNA showed that there is a dose-dependent effect of PKB expression on cell survival (102). The previously discussed mice knockout studies also support a role of PKB in metabolism, glycolysis, insulin signalling, growth,

vascularisation and lipid metabolism. Some of the PKB substrates that mediate these processes have been identified and these will be discussed below.

PKB: life and death decisions

Apoptosis is characterized by morphologically distinct features including plasma membrane blebbing, shrinking of the nucleus and the cytoplasm, loss of mitochondrial transmembrane potential, cleavage of intracellular proteins and degradation of chromosomal DNA (82,162,184). DNA is cleaved into fragments of distinct size, called "DNA laddering", a hallmark often used to characterize apoptotic cells (82). Furthermore, the distribution of lipids in the plasma membrane bilayer changes, resulting in an increase in external phosphatidylserine, a so-called 'eat me' signal which can be recognized by macrophages, which phagocytose dying cells.

The specific cleavage of proteins and induction of DNA laddering in cells undergoing apoptosis is carried out by a family of proteases, called caspases that cleave substrates after aspartate residues (42). Caspases are synthesized as inactive pro-enzymes that must themselves be cleaved at key aspartate residues to be activated. Initial caspase activity in apoptosis is induced through the recruitment of caspase pro-enzymes to activator molecules, promoting pro-caspase oligomerization and auto-activation (53,116). Caspase activity also results in the activation of caspase-activated deoxyribonuclease (CAD) which is responsible for chromosomal DNA degradation during apoptosis (52,142).

One of the caspases, **caspase 9**, is a direct target for PKB phosphorylation *in vitro* (Fig. 4) (27). This phosphorylation inhibits its protease activity however the phosphorylation site is not conserved in mouse or other rodents and the mechanism of action remains to be elucidated. Furthermore, PKB phosphorylation stabilizes X-linked inhibitor of apoptosis (**XIAP**) by preventing its ubiquitination and subsequent degradation (43). XIAP is able to directly bind and inhibit caspase activity.

Another important family of proteins involved in the control of apoptosis is the Bcl-2 family. This protein family can be divided into anti-apoptotic members such as Bcl-2 and Bcl-xl and pro-apoptotic members such as Bad and Bim (31,170). The Bcl-2 related proteins exert their apoptosis-regulating effects by modulating mitochondrial alterations that proceed caspase activation such as the loss of membrane potential and cytochrome c release (95). Anti-apoptotic Bcl-2 family members prevent this release whereas pro-apoptotic Bax can form pores to permeabilize the mitochondria (7,79). Upon release, cytochrome c directly activates caspases by binding to a cytoplasmic protein Apaf-1, resulting in a complex, which recruits caspase 9, in turn inducing the self-cleavage/activation of caspase 9 thereby activating a proteolytic cascade which ultimately results in cell death (158). Maintaining an appropriate balance between pro- and anti-apoptotic Bcl-2 family members is crucial in maintaining tissue homeostasis. Growth factors and cytokines have been demonstrated to be able to promote survival through transcriptional upregulation of anti-apoptotic Bcl-

2 family members or inactivation of pro-apoptotic Bcl-2 proteins.

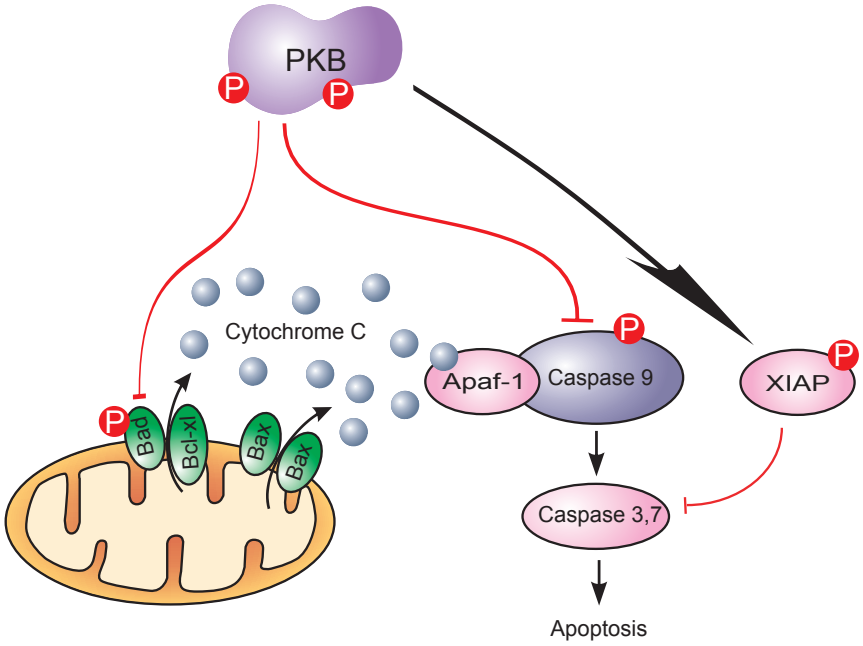


Figure 4: PKB: life and death decisions
PKB can regulate apoptosis by direct phosphorylation and inactivation of pro-apoptotic Bcl-2 family member Bad. Cytochrome c release from mitochondria, together with Apaf-1, promotes caspase activation. Caspase activity results in apoptosis through the cleavage of intracellular proteins and the degradation of chromosomal DNA. PKB can inhibit caspase-induced apoptosis by direct phosphorylation and inactivation of caspase 9 or by direct phosphorylation and stabilization of XIAP.

In vitro and *in vivo* PKB can directly phosphorylate and inactivate pro-apoptotic Bcl-2 family member, **Bad** (44). Bad promotes apoptosis by interacting with Bcl-xl in the mitochondrial membrane leading to the release of cytochrome c from the mitochondria into the cytoplasm (18,40,67). Like many PKB substrates, phosphorylation of Bad sequesters it from its targets by interaction with 14-3-3 proteins. 14-3-3 proteins are a family of dimeric proteins that can modulate interaction between proteins (1). Target proteins that interact with 14-3-3 proteins are involved in trafficking between cytoplasm and nucleus, between cytoplasm and plasma membrane and between cytoplasm and mitochondria as well as in regulation of diverse enzyme activities and protection against proteolysis. Phosphorylation by PKB results in the formation of a 14-3-3 binding site (1). Interestingly, PKB has also been shown to be able to phosphorylate at least one of the 14-3-3 isoforms, **14-3-3ζ** and regulate its homodimerization and subsequently also substrate binding (32).

Another Bcl-2 family member regulated by PKB activity is Bax. The expression of pro-apoptotic Bax is induced by p73 upon DNA damage and yes-associated protein (**YAP**) is a necessary coactivator for p73 in this function. PKB phosphorylation of YAP induces its binding to 14-3-3 proteins thereby promoting its localization to the cytoplasm, resulting in loss from the nucleus (11). Thus in this manner PKB suppresses the induction of the expression of Bax following DNA damage.

Another mechanism by which PKB inhibits apoptosis is the promotion of redistribution of hexokinase to mitochondria (61). Hexokinase binds to the outer mitochondrial membrane at sites where the voltage-dependent anion channel (VDAC) is located (177). VDAC plays an important role in regulating mitochondrial permeability transition and release of apoptotic factors such as cytochrome c. Forced overexpression of hexokinase antagonizes apoptosis whereas displacing it from the mitochondria increases cellular sensitivity to pro-apoptotic stimuli (61,107). Hexokinase carries out its antiapoptotic function in a glucose-dependent manner by antagonizing mitochondrial release of cytochrome c. The exact mechanism underlying this is still unclear but it has been suggested that mitochondrion-associated hexokinase antagonizes the proapoptotic function of tBid, an activator of pro-apoptotic Bcl-2 family members Bax and Bak thereby maintaining mitochondrial integrity and preventing the release of apoptogenic factors (108).

The most important regulator of DNA damage induced apoptosis is the tumour suppressor p53. p53 is crucial in regulating cell growth, survival and senescence, and is mutated or deleted in many types of tumours. p53 prevents the completion of the cell cycle if the DNA in the cell or the cell itself has been damaged. When the damage is minor, p53 induces cell cycle arrest until the damage is repaired, however if the damage is major and cannot be repaired, p53 triggers the cell to enter an apoptotic programme through both transcription-dependent and -independent mechanisms. p53 protein levels are regulated by E3 ubiquitin ligase **MDM2**-mediated ubiquitination and subsequent degradation. Phosphorylation of MDM2 by PKB enhances its nuclear localization and its ubiquitin ligase activity thus increasing p53 degradation (187). By increasing p53 degradation PKB is able to desensitize cells for DNA damage induced apoptosis.

The family of stress activated protein (SAP) kinases to which both c-Jun N-terminal kinase (JNK) and p38 kinase belongs is involved in the induction of apoptosis upon cellular stress through the regulation of AP-1 transcription factors. JNK and p38 kinase efficiently phosphorylate c-Jun and ATF-2, which are responsible for transcriptional activation of *c-jun* expression. Apoptosis signal-regulating kinase 1 (**ASK1**), a mitogen-activated protein kinase kinase kinase (MAPKKK) activates c-Jun N-terminal kinase (JNK) and p38 MAP kinase through the activation of MAPKKs. Phosphorylation of ASK1 by PKB inhibits its activity and thereby its apoptosis promoting potential (83). Direct phosphorylation by PKB also leads to inhibition of **SEK1** another upstream activator of JNK (126). In neurons, PKB inhibits apoptosis via phosphorylation and inhibition of mixed lineage kinase 3 (**MLK3**), a mitogen-

activated protein kinase kinase kinase (MAPKKK) that activates JNK (10).
The substrates discussed here demonstrate an important role for PKB in regulating cell survival. The fact that PKB inhibits apoptosis induced by DNA damage and cellular stress also contributes to its oncogenic potential. If PKB becomes constitutively active this can result in survival and proliferation of 'abnormal' cells contributing to cellular transformation.

Cellular metabolism control by PKB

Increased cellular metabolism is an important hallmark of cellular transformation. Transformed cells generally exhibit greatly increased levels of nutrient utilization compared with their non-transformed counterparts; in particular, glucose metabolism is increased (38,51,131,136). Cancer cells exhibit altered cellular glucose metabolism that relies primarily on glycolysis, rather than mitochondrial respiration is called the 'Warburg effect' (174). The fact that PKBβ$^{-/-}$ mice exhibit a diabetic phenotype shows that PKB function and cellular metabolism are intimately connected (Fig. 5). One of the best characterized mechanisms by which PKB can regulate metabolism is by direct phosphorylation of glycogen synthase kinase-3 (**GSK3**) in its N-terminal domain (41). This phosphorylated N-terminus inhibits GSK3 by acting as a pseudosubstrate, competing with primed substrates for the phosphate-binding and catalytic sites on GSK3. When active, GSK3 phosphorylates and inactivates glycogen synthase leading to decreased glycogen synthesis therefore PKB phosphorylation of GSK3 enhances glycogen synthesis. PKB not only affects glycogen synthesis but at the same time glycolysis is upregulated. PKB directly controls and enhances glycolysis by phosphorylation and activation of 6-phosphofructo-2-kinase (**PFK-2**), a regulator of glycolytic rate. Activation of PFK-2 results in increased production of fructose 2,6 biphosphate, an allosteric activator of phosphofructokinase 1 (PFK-1), a key regulator of the overall rate of glycolysis. An increase of kinetics of the reactions catalyzed by these enzymes results in an increase in the overall rate of glycolysis (46). PFK-2 has, like several other PKB substrates, been shown to be phosphorylated by several AGC protein kinase family members including p70S6K, mitogen-activated protein kinase-activated protein kinase-1 (MAPKAP-1), and PKB. PKB phosphorylation also activates **ATP citrate lyase**, a cytosolic enzyme that is critical for the conversion of glucose to cytosolic actyl CoA (12). Acetyl CoA is the requisite building block for all endogenous synthesis of acyl groups and sterols, therefore ATP citrate lyase regulates glucose-dependent lipogenesis (16). Enhanced glycolysis coincides with increased glucose uptake. Insulin-stimulated glucose uptake involves the recruitment of the cytosolic glucose transporter 4 isoform (GLUT4) to the plasma membrane of fat and muscle cells and PKB has been indicated to participate in this translocation (39,173). GLUT4-dependent glucose transport is reduced in PKBβ- deficient cells because of a failure to stimulate GLUT4 translocation from intracellular vesicles to the plasma membrane (35,59). Furthermore, PKBβ has been shown to be directly associated with GLUT4-

containing protein complexes (25).

A PKB substrate that has been suggested to play a direct role in this recruitment of GLUT4, the 160-kDa substrate for Akt (**AS160**) which functions as a Rab-GAP for RabGTPases, small G-proteins required for membrane trafficking (80). AS160 phosphorylation by PKB leads to its inhibition subsequently allowing GLUT4 vesicle recruitment to the plasma membrane (145). Another PKB substrate involved in GLUT4 transport is the FYVE domain-containing PI(3)P 5-kinase (**PIKfyve**) (15). PIKfyve is the mammalian homologue of yeast Fab1p that has been shown to be important for cargo sorting from late endosomes to lysosome/multivesicular body (157). Phosphorylation by PKB enhances PIKfyve kinase activity and its co-localization with a highly motile subpopulation of insulin-regulated aminopeptidase (IRAP)/GLUT4 vesicles, suggesting a role for PKB in insulin-regulated IRAP/GLUT4 trafficking. PKB modulates liver metabolism by phosphorylation and subsequent nuclear exclusion and inhibition of Hepatocyte nuclear factors 3 beta (**HNF3β/FOXa2**) transcriptional activity (178). The Foxa family of transcription factors is an important regulator of the gluconeogenic program in the liver particularly in response to fasting. In the nucleus, Foxa2 activates multiple genes driving increased glucose utilization, fatty acid oxidation and ketogenesis and these important metabolic genes in the liver are suppressed by the actions of insulin.

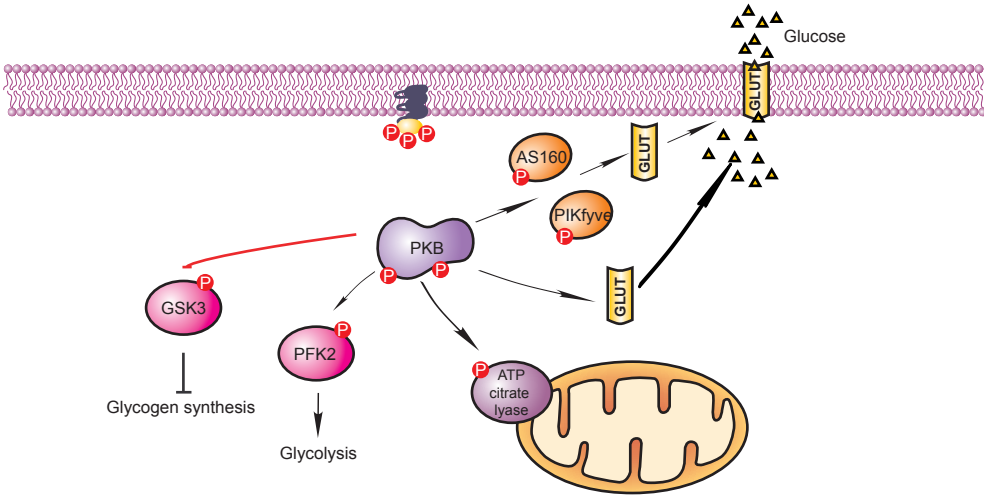


Figure 5: Regulation of metabolism by PKB

One of the best characterized mechanisms by which PKB can regulate metabolism is by direct phosphorylation and inhibition of GSK3 thereby enhancing glycogen synthesis. Furthermore, PKB directly controls and enhances glycolysis by phosphorylation and activation PFK-2, a regulator of glycolytic rate. PKB phosphorylation also activates ATP citrate lyase, a cytosolic enzyme that is critical for the conversion of glucose to cytosolic actyl CoA. PKB is able to increase glucose uptake through the recruitment of the cytosolic glucose transporter 4 isoform (GLUT4) to the plasma membrane of fat and muscle cells via its substrates AS160 and PIKfyve.

Roles of PKB in cellular proliferation

Cell division requires several co-ordinated distinct processes that can be subdivided into four stages (22,153). In G1 phase a cell is provided with an external growth signal that initiates cellular growth and the cell prepares for DNA replication. During S phase the cell duplicates its entire genome, after which it prepares for cellular division during G2 phase. Finally, nuclear division (mitosis) and cytoplasmic division (cytokinesis) is achieved in the M phase. At various stages of the cell-cycle, checkpoints control its progression and arrest the ongoing cell division whenever an abnormality is sensed. Growth factor signalling can regulate the activity of checkpoints at multiple levels ensuring initiation of DNA synthesis and progression through the cell-cycle. This is one of the reasons why changes in growth factor-controlled signal transduction pathways that lead to their constitutive activation are oncogenic: continuous activation of such pathways consistently inactivates the G1 checkpoint leading to uncontrolled cellular proliferation (110,115).

The cell cycle checkpoints are regulated by a variety of proteins. In G2 and M phases, the DNA damage checkpoint, which includes p53, ensures that the cell-cycle does not continue when DNA is damaged (117). Furthermore, in mitosis the spindle assembly checkpoint halts cell cycle progression in case certain chromosomes are misaligned or not properly attached to spindle microtubules (140). The G1-checkpoint, also referred to as the restriction point, cannot be inactivated unless the cell receives the correct external growth signals. At this checkpoint, the E2F-1 transcription factor plays a crucial role. Its activity is required for progression of the cell-cycle into S phase but is inhibited by pRb (60,176). In early G1, pRb is hypophosphorylated and bound to E2F-1. This complex actively represses transcription of genes required for G1/S progression. Activation of cyclinD/cdk4/6 and cyclinE/cdk2 complexes by growth factors causes pRb to be hyperphosphorylated (152). E2F-1 is then released from the inhibitory constraints imposed by Rb and actively transcribes genes required for G1/S transition (125). The cyclin/cdk complexes in turn can be inhibited by the $p27^{kip1}$, $p16^{INK4A}$ and $p21^{waf1/cip1}$ families of cdk-inhibitors (CKIs) that directly bind and inactivate the cyclin/cdk complex thus keep pRb in its hypophosphorylated form (154). Also growth factors can increase the activity or expression of the cyclin subunits of cyclin/cdk complexes, can relocalize cdk proteins from the cytoplasm to the nucleus and can decrease the activity or expression of CKIs.

Controlled progression through the cell cycle is thus essential for normal cellular proliferation and PKB regulates this process through a variety of mechanism (Fig. 6). PKB phosphorylates **p21Cip1**, which when in the nucleus plays a role in cell cycle inhibition and a block in proliferation by inhibition of cyclin-CDK complexes (186). Phosphorylation results in its cytoplasmic re-localization thereby inhibiting its function. PKB can also phosphorylate **p27Kip1** which inhibits cyclin E/CDK2 activity in the nucleus thereby blocking cell proliferation (155). However, similar to caspase 9, the PKB phosphorylation motif is not conserved between human and mouse. PKB controls cell cycle arrest after DNA damage by phosphorylation

of DNA damage effector kinase **Chk1** (87). This phosphorylation correlates with impairment of Chk1 activation by DNA damage. Active Chk1 blocks the activation of the Cdc2-cyclin B kinase complex, and hence entry into mitosis, by disrupting the translocation of the phosphatase Cdc25 from the cytoplasm to the nucleus. However, the Chk1 protein phosphorylated by PKB does not enter into protein complexes after replication arrest. Moreover, phosphorylated Chk1 fails to undergo activating phosphorylation by other kinases like ATM/ATR. This phosphorylation and association with other checkpoint proteins are essential steps in activation of Chk1.

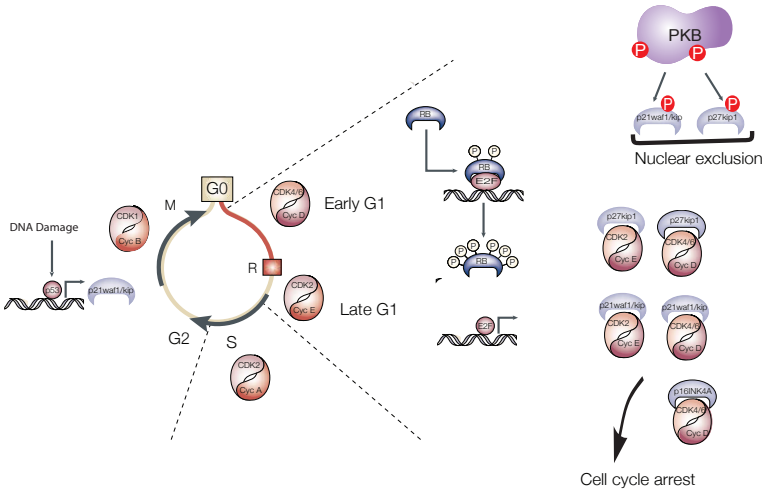


Figure 6: Roles of PKB in cellular proliferation

CDK/cyclin complexes promote progression through the cell cycle. The initial onset of the cell cycle requires phosphorylation of Rb, thus allowing transcription factor E2F to promote transcription of components of the cell cycle machinery. Active CDK/cyclin complexes can be inhibited by CKIs such as $p27^{kip1}$, $p16^{INK4A}$ and $p21^{waf1/cip1}$. PKB promotes cell cycle progression by phosphorylation and subsequent nuclear exclusion of $p27^{kip1}$ and $p21^{waf1/cip1}$.

FOXO transcription factors

FOXO transcription factors are the mammalian homologues of the nematode worm *Caenorhabditis elegans (C. elegans)* DAF-16. Normally, *C. elegans* lives for only a few weeks however when environmental conditions are unfavourable such in case of a lack of food or high population density, these worms can enter a so-called Dauer stage in which they can survive for months. This stage represents a phenotype that is characterized by low metabolic activity and increased resistance to stress (166). DAF-16 is one of the proteins responsible for the induction and maintenance of Dauer (69,98,118). Interestingly, the signals regulating DAF-16 are evolutionary conserved from *c. elegans* to humans. In *C. elegans*, DAF-16 is negatively regulated by DAF-2, an insulin receptor-like protein (86), via AGE-1, a homologue of the catalytic subunit of mammalian PI3K (124). The pathway also contains a

PKB-like (AKT1/AKT2), a PDK1-like (PDK1) and a PTEN-like (DAF-18) protein (119,123). The high evolutionary homology in this signalling pathway is a token of its importance.

In mammalians, it has been shown that PKB directly phosphorylates and inhibits members of the Foxo subfamily of forkhead transcription factors Foxo1, Foxo3a, Foxo4 and Foxo6 (20,75,94,134). PKB Phosphorylation inhibits Foxo1, Foxo3a and Foxo4 activity either by direct inhibition of its DNA binding (24,185) or through their cytoplasmic retention by interaction with 14-3-3 proteins, thereby sequestering them from their target genes (Fig. 7) (17). Foxo transcription factors regulate a variety of genes that influence cellular proliferation (p27kip1 and cyclin D) (49,150), survival (FasL and Bim) (21,48), metabolism (PEPCK and G6Pase) (114), and responses to stress (catalase and manganese superoxide dismutase (MnSOD) (93).

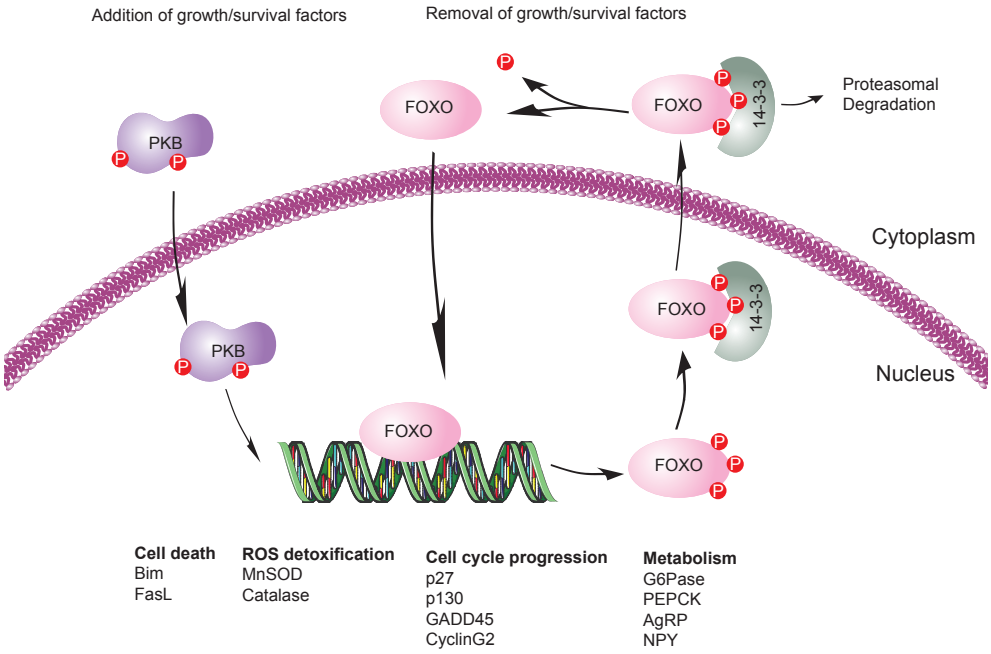


Figure 7: Regulation of FOXO transcriptional activity

Addition of growth/survival factors results in activation of PKB, which then translocates into the nucleus. Phosphorylation of FOXO by PKB results in release from DNA, and binding to 14-3-3 proteins. This complex is then transported out of the nucleus where it remains inactive in the cytoplasm or is degraded by the proteasome. Upon removal of growth/survival factors, FOXO is dephosphorylated, 14-3-3 is released, and FOXO is transported back into the nucleus where it is transcriptionally active.

In the past few years, the generation of Foxo null-mutant mice have provided greater insight in the importance of Foxo transcription factors and their functionally

diverse roles in mammals. Foxo1 knockout mice show lethality in the embryonic stage due to effects in angiogenesis (58,70). Foxo3a null mice are infertile due to abnormal ovarian follicular development (28), and also develop lymphoproliferation and inflammation (78,99). Foxo4 deficient mice are viable and have no distinct phenotype (70). Recently, Tothova and co-workers conditionally deleted Foxo1, Foxo3a and Foxo4 in the adult hematopoietic system and showed that these transcription factors play an essential role in the resistance to physiologic oxidative stress thereby maintaining the short- and long-term hematopoietic stem cell (HSC) population (168). Interestingly, broad somatic deletion of Foxo1, Foxo3a and Foxo4 resulted in a progressive cancer-prone condition characterized by thymic lymphomas and hemangiomas, indicating that Foxo transcription factors function as tumour suppressors (122). Regulation of the activity of Foxo transcription factors is thus a critical means through which PKB modulates cellular homeostasis.

Targeting the PI3K-PKB pathway in human cancer

The prevalence of a hyperactivated PI3K-PKB pathway in human cancers suggests that cancer cells might be more sensitive to inhibitors of this pathway than normal cells. The continued elucidation of the role of this pathway in cell growth, survival, and proliferation has shed light on why regulation of this pathway is often altered within tumours. The best known inhibitors upstream of this pathway are those inhibiting receptor tyrosine kinases such as Herceptin which blocks the Erb2/HER2 receptor in breast cancers and Gleevec which inhibits the BCR-Abl, c-KIT, and PDGF-R tyrosine kinase in e.g. chronic myeloid leukaemia (CML).

The most direct method to inhibit this pathway is to target PI3K itself. *In vitro*, PI3K activity inhibitors wortmannin and LY294002 have been extensively used as research tools. Conjugates of these inhibitors are being produced that have more suitable pharmalogical characteristics. Also a number of isoform-specific PI3K inhibitors have been developed (74,89,175,179). Selective inhibition of just one PI3K isoform could possibly minimize drug side effects. For PKB a number of isoform-selective PKB inhibitors, as well as inhibitors of the PH domain are under development (9,56,100). A number of other targets in the PI3K pathway for example PDK1 inhibitors and FOXO activators are also under evaluation in preclinical models (81).

The recent finding that mTOR is an upstream and downstream mediator of PKB signalling makes it an interesting target for drug development. Analogues of rapamycin such as CCI-779, RAD001 and AP23573 are likely to be the first mTOR perturbing molecules to be approved for anticancer use in humans (63). These molecules inhibit mTORC1 through the same mechanism of action as rapamycin, but have different pharmacokinetic and solubility properties that increase their desirability for clinical use. Rapamycin and its analogues have several anti-tumour properties in pre-clinical cancer models have a significant ability to reduce cell proliferation, cell survival and angiogenesis (29,54,139,141). Since the inhibition of

mTORC2 by rapamycin is time and dose dependent, PKB activity in tumours will also vary with the length of rapamycin treatment and the dosing regimen. The cell-type specific capacity to inhibit mTORC2 might help explain why the cellular effects of rapamycin vary among cancer cell lines. Rapamycin-mediated inhibition of PKB may also explain the side effects of the drug. Hyperlipidemia, high amounts of lipids in the bloodstream, which can speed hardening of the arteries, is commonly seen in patients treated with rapamycin analogues. Since rapamycin has been shown to strongly inhibit PKB phosphorylation in adipose tissue, a tissue type in which insulin-stimulated PKB activity plays an important role in suppressing lipolysis, PKB inhibition may allow high lipolysis, resulting in the accumulation of free fatty acids that can be used by the liver to generate triglycerides (147).

The continuing efforts to develop specific, high-affinity inhibitors against the PI3K-PKB pathway will surely yield new therapeutics to treat human cancer. To be able to achieve this the identification and characterisation of the complete network of PI3K- and PKB-mediated signalling events is of the utmost importance.

Outline of this thesis

The focus of this thesis is to identify and characterize novel signalling components downstream of PKB that can contribute to its transforming potential.

In **chapter two** we demonstrate that chronic PKB activation is in itself insufficient for cell survival. Higher levels of oxidative stress caused by prolonged PKB activation resulted in increased expression of Foxo3a leading to expression of its transcriptional targets Bim and p27kip1. Up-regulation of these proteins ultimately results in cell cycle arrest and apoptosis.

Chapter three describes a phosphoproteomic approach to identify novel PKB substrates. The potentially novel PKB substrates that were identified through this method are discussed here.

In **chapter four** we identify eIF4B as a novel substrate of PKB revealing an important mechanism by which PKB can regulate translation, potentially critical for its transforming capacity. PKB was found to phosphorylate eIF4B on Ser422 *in vitro* and *in vivo* after mitogen-stimulation. Furthermore, we identified Ser406 as a novel mitogen-regulated phosphorylation site. Phosphorylation of Ser406 was found to be regulated by p90 ribosomal S6 kinase (RSK) *in vivo*. Utilising a translational control reporter system (TCRS), phosphorylation of both residues was found to be physiologically relevant in regulating the translational activity of eIF4B.

In **chapter five**, we show that whereas PKB activation alone is insufficient for long-term cell survival, PI3K activation is. To discriminate between PI3K and PKB regulated signal transduction pathways, we performed microarray analysis on two cell lines, one containing an inducible active PKB and the other containing an inducible active PI3K. We compared gene expression following activation of these kinases. By doing this we have been able to model their distinct and overlapping functions in cellular physiology.

The consequences of these findings are discussed in **chapter six**. The identification and characterization of novel PKB-mediated signalling events provides novel targets for anti-cancer therapeutic targeting.

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CHAPTER 2

Chronic Protein Kinase B (PKB/c-akt) activation leads to apoptosis induced by oxidative stress-mediated Foxo3a transcriptional up-regulation

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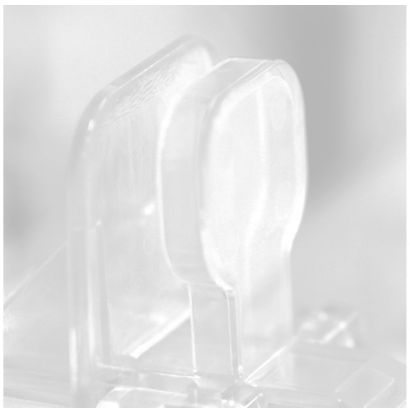
Cancer Res 2006 Nov 15;66(22):10760-9


ABSTRACT

Increased Protein Kinase B (PKB/c-akt) activation is a hallmark of diverse neoplasias providing both proliferative and anti-apoptotic survival signals. In this study, we investigated the effect of chronic PKB activation on cellular survival and proliferation using cytokine-dependent bone marrow-derived Ba/F3 cells in which PKBα activation can be directly, and specifically, induced by addition of 4-hydroxytamoxifen (4-OHT). Direct activation of PKB rescued Ba/F3 cells from cytokine-withdrawal-induced apoptosis, however, surprisingly, these anti-apoptotic effects were short-lived, cells only being protected for up to 48 hours. We observed that activation of PKB in survival factor deprived cells led to a dramatic increase of Foxo3a on both the transcriptional and protein level leading to expression of its transcriptional targets Bim and $p27^{kip1}$. High levels of PKB activity result in increased aerobic glycolysis and mitochondrial activity resulting in overproduction of reactive oxygen species (ROS). To determine whether oxidative stress might itself be responsible for Foxo3a upregulation we utilised hydrogenperoxide (H_2O_2) as an artificial inducer of oxidative stress and N-acetylcysteine (NAC), a thiol-containing radical oxygen scavenger. Addition of NAC to the culture medium prolonged the life-span of cells treated with 4-OHT and prevented the upregulation of Foxo3a protein levels caused by PKB activation. Conversely, treatment of Ba/F3 cells with H_2O_2 gave rise to an increase of Foxo3a on both transcriptional and protein levels suggesting that deregulated PKB activation leads to oxidative stress resulting in Foxo3a upregulation and subsequently cell death. Taken together our data provide novel insights into the molecular consequences of uncontrolled PKB activation.

INTRODUCTION

Cytokines of the interleukin(IL)-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) family play an important role as hematopoietic differentiation and survival factors (1). Critical anti-apoptotic signals induced by these cytokines include activation of phosphatidylinositol 3-kinase (PI3K), which in turn promotes activation of Protein Kinase B (PKB/c-akt) (2). In mammals there are three PKB isoforms, PKBα, PKBβ and PKBγ, which share a high degree of sequence homology. All three isoforms contain an N-terminal pleckstrin homology domain (PH), a catalytic domain and a C-terminal regulatory domain (3). PKBα is the most ubiquitously expressed and studies in PKBα-deficient mice have shown it to be indispensable for normal cell growth (4). The expression of PKBβ has its highest expression levels in insulin-responsive tissues. It is thus not surprising that PKBβ-deficient mice suffer from diabetes (5). PKBγ is expressed at the lowest levels except for the brain and testes. PKBγ-deficient mice show impaired brain development (6). PKBα and PKBβ double knock-out mice die shortly after birth (7).

PKB has been reported to inhibit apoptosis through a variety of molecular mechanisms, including direct phosphorylation and inhibition of the pro-apoptotic Bcl-2 family member Bad (8), glycogen synthase kinase-3 (GSK-3) (9) and caspase-9 (10). More recently, it also has been demonstrated that PKB directly phosphorylates and inhibits members of the FOXO subfamily of Forkhead transcription factors Foxo1, Foxo3a and Foxo4 (11). Phosphorylation of the FOXO proteins by PKB results in their cytoplasmic retention by interaction with 14-3-3 proteins, thereby sequestering them from their target genes (12). FOXO transcription factors regulate a variety of genes that influence cellular proliferation ($p27^{kip1}$ and cyclin D) (13, 14), survival (FasL and Bim) (15, 16), metabolism (PEPCK and G6Pase) (17) and responses to stress (MnSOD and catalase) (18). Therefore, regulation of FOXO activity is a critical means through which PKB modulates cellular homeostasis.

In a variety of human neoplasias PKB activity has been shown to be upregulated. This upregulation can be due to a variety of causes including mutation of its intracellular activators such as Ras or PI3K (19, 20), mutation or deletion of the tumour suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (21, 22) or enhanced expression of one or more of the PKB isoforms (23). Besides inhibition of apoptosis and uncontrolled cell growth, enhanced PKB activity can also give rise to another feature commonly seen in malignancies namely increased aerobic glycolysis (24). PKB directly regulates aerobic glycolysis by increasing surface expression of glucose transporters (25), stimulating the mitochondrial association of hexokinase (26), and by phosphorylation of PFK2 (27). The fact that tumour cells enhance their aerobic glycolytic metabolism is seen as one of the primary hallmarks of cancer, a phenomenon termed the Warburg effect (28). It has been demonstrated that after withdrawal of growth factors, cells rapidly lose surface expression of nutrient transporters such as Glut-1 and Glut-4 (29), leading to a rapid decline in

glycolysis and a decrease in mitochondrial potential, resulting ultimately in the release of cytochrome c and the initiation of apoptosis (30). The heightened levels of glycolysis and mitochondrial activity enforced by PKB activation could therefore be an important mediator of the enhanced resistance to apoptosis. However, glycolysis also results in the production of reactive oxygen species (ROS) in cells. Whereas ROS levels fluctuate during the cell cycle and are suggested to be necessary as second messengers, in excess they can result in oxidative stress causing damage to lipids, proteins and DNA (31). To counteract the effects of oxidative stress, cells have developed defense mechanisms in the form of intracellular antioxidants molecules, such as glutathione (GSH), catalase, superoxide dismutase (SOD), and thioredoxin (TRX), which protect cells from oxidative damage.

In this study, we have investigated the effect of chronic PKB activation on cellular survival and proliferation using cytokine-dependent bone marrow-derived Ba/F3 cells. For this purpose, we developed a system where PKBα can be inducibly activated. Surprisingly, we found that chronic PKB activation was unable to maintain long-term cell survival. Higher levels of oxidative stress caused by prolonged PKB activation resulted in increased expression of Foxo3a leading to expression of its transcriptional targets Bim and $p27^{kip1}$. Upregulation of these proteins ultimately results in cell cycle arrest and apoptosis. Taken together, our data suggest that chronic PKB activation is in itself insufficient for hematopoietic cell survival. These findings have important consequences for our understanding of the processes leading to cellular transformation.

MATERIALS AND METHODS

Cell culture

Ba/F3 cells were cultured in RPMI 1640 medium with 8% Hyclone serum (Gibco, Paisley, UK) and recombinant mouse IL-3 produced in COS cells (32). For the generation of clonal Ba/F3 cells stably expressing myrPKB:ER*, the SRα-myrPKB:ER* construct was electroporated into Ba/F3 cells together with pSG5 conferring neomycin resistance and maintained in the presence of 1mg/ml G418 (Gibco, Paisley, UK) and IL-3. Clonal cell lines were generated by limited dilution. For cytokine withdrawal experiments, cells were washed twice with PBS and resuspended in AimV medium (Gibco, Paisley, UK)

Antibodies and reagents

Polyclonal antibodies against total and phospho-PKB (Ser473) were from Cell Signaling Technologies (Hitchin, UK). Anti-$p27^{kip1}$ was from Transduction Laboratories (Lexington, Kentucky, USA). Anti-Bim was from Affinity BioReagents (Golden, CO, USA). Foxo3a, phospho-JNK(Thr183/Tyr185), actin and ER were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The phospho-Foxo3a(Thr32) was from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Phospho-GSK3α/β (Ser21/9), phospho-STAT5(Tyr694), phospho-P38(Thr180/Tyr182) and phospho-

MAPK42/44(Thr202/Tyr204) were from New England Biolabs (Hitchin, UK). CFSE was from Molecular Probes (Carlsbad, CA, USA). Buthionine sulfoximine (BSO) and dithiothreitol (DTT) were from Sigma (Seelze, Germany). Thiolyte® Monochlorobimane Reagent was from Calbiochem (Darmstadt, Germany). N-acetylcysteine was from Zambon (Amersfoort, The Netherlands).

Western blotting

Cells were lysed in laemmli buffer (0.12M Tris HCL pH 6.8, 4% SDS, 20% Glycerol, 0.05 μg/μl bromophenol blue, and 35mM β-mercaptoethanol), boiled for 5 minutes and the protein concentration was determined. Equal amounts of sample were analyzed by SDS PAGE, electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA) and probed with the respective antibodies. Immunocomplexes were detected using enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, UK).

Apoptosis assays

Cells were counted, washed twice with PBS, resuspended in AimV medium and seeded in 6-wells dishes (5x 10^4 cells per ml). Then the cells were cultured in the presence or absence of IL-3 and/or 4-hydroxytamoxifen (100nM), with the indicated amounts of N-Acetylcysteïne (250μM), buthionine sulfoximine (1.0 or 0.1mM) or hydrogenperoxide (H_2O_2) added to the medium. Cells were then harvested at the indicated time-points and washed with PBS. Samples were subsequently incubated for 15 minutes with Annexin-V-FITC (Bender MedSystems) in binding buffer (10mM Hepes NaOH pH 7.4, 150mM NaCL, 2.5mM $CaCl_2$). Cells were washed and resuspended in binding buffer containing 1μg/ml Propidium Iodide (Bender MedSystems, Vienna, Austria). FACS analysis was performed on a FACS Calibur at a wavelength of 550nm.

Proliferation assays

Cells were counted, washed with PBS, incubated in prewarmed PBS containing 2.5μM of carboxylfluorescein diacetate succinimidyl ester (CFSE) for 15 minutes. Cells were subsequently washed and resuspended in AimV medium with or without IL-3 and/or 4-hydroxytamoxifen (100nM) and seeded in 6-wells dishes (10^5 cells per ml). Proliferation was visualised as the decrease of fluorescent CFSE-probe per cell as measured by flow cytometry analysis in the FL-1 channel.

Measurement of GSH levels

Cells were cultured in the presence or absence of IL-3 and/or 4-hydroxytamoxifen (100nM), together with N-Acetylcysteïne (250μM), dithiothreitol (100μM), reduced glutathione (250μM) or buthionine sulfoximine (1.0 or 0.1mM) for the indicated time. To determine the total intracellular GSH content, samples (5 x 10^6 cells) were washed and resuspended in 400μl PBS containing 2mM monochlorobimane. Upon

incubation for 30 minutes at 37°C, cells were centrifuged and resuspended in 400μl of PBS. Aliquots of 100μl were taken to measure the fluorescence, either using the Fluoroskan Ascent from Thermolabsystems (Almere, The Netherlands) at excitation wavelength of 385nm and emission wavelength of 450nm or by FACS analysis on a FACS Calibur in the FL-1 channel.

Transient electroporations and luciferase assay

For transient transfections, Ba/F3 cells were electroporated by using a Bio-Rad Gene Pulser at 350V and 950μF with 10μg of the pGL3-Foxo3a promoter reporter construct plus 1μg of pRLTK Renilla plasmid (Promega) to normalize for transfection efficiency. The Foxo3a promoter construct contains 1146bp upstream of the mouse Foxo3a coding region cloned into the KpnI and XhoI sites in pGL3-basic. After transfection cells were cultured in RPMI medium containing 8% serum and IL-3 and left to recover for 16hr. Then cells were washed, transferred to cytokine-free medium and treated with 200nM tamoxifen. At the indicated time points the cells were washed twice with PBS and lysed in 100μL of Passive Lysis Buffer for 5 mins. After one freeze and thaw cycle, insoluble cell debris were spun down and the supernatant fraction was assayed for luciferase activity using Dual-Luciferase Reporter Assay System (Promega, Leiden, The Netherlands).

RNA isolation and Real-Time Quantitative PCR

Total RNA was isolated using the RNeasy Kit (QIAGEN), and the concentration and purity of each sample were assessed by absorbance at 260nm and by the 260nm/280 nm ratio, respectively. The integrity of the RNA was verified by observing the rRNA bands in ethidium bromide-stained gel under UV irradiation.

Equal amounts of total RNA (2μg) were reversed transcribed with Superscript™ III reverse transcriptase (Invitrogen). The resulting cDNA was amplified using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) with the following primer pairs:

mouse *FOXO3A*-sense (TCCCAGATCTACGAGTGGATGG);
mouse *FOXO3A*-antisense (CCTTCATTCTGAACGCGCAT);
mouse *L19*-sense (GGAAAAAGAAGGTCTGGTTGGA);
mouse *L-19*-antisense (TGATCTGCTGACGGGAGTTG)

L19, a nonregulated ribosomal housekeeping gene, was used as an internal control to normalize input RNA.

RESULTS

PKB activation is insufficient for long-term survival

To explore the mechanisms by which PKB regulates cytokine-mediated survival and proliferation in hematopoietic cells, we generated bone-marrow-derived Ba/F3 cells stably expressing an inducible active PKBα (myrPKB:ER). The activation of the myristoylated PKBα is, in the absence of 4-OHT, inhibited by heat-shock and

chaperone proteins that associate with the fused estrogen receptor (ER) hormone-binding domain. In the presence of 4-OHT these proteins dissociate allowing PKB to become phosphorylated and activated. Thus, in these cells PKB activation can be directly, and specifically, induced by addition of 4-OHT (Fig. 1a, upper panel). 4-OHT-mediated PKB activation was sufficient to rapidly induce the phosphorylation of Foxo3a and GSK-3 (Fig. 1a). In contrast to stimulation of the cells with IL-3, 4-OHT treatment was unable to induce phosphorylation of ERK1/2 MAP kinase, p38 MAP kinase, signal transducer and activator of transcription 5 (STAT5) or c-jun N-terminal kinase (JNK) (Fig. 1a, lower panels). The stability and activity of myrPKB:ER was followed over a longer period. myrPKB:ER cells were cytokine-starved and then treated with 4-OHT for up to 96 hours. As a control, cells were either cytokine-starved or stimulated with IL-3 for 24 hours. Stimulation of Ba/F3 myrPKB:ER cells with 4-OHT resulted in phosphorylation of the myrPKB:ER protein itself as well as Foxo3a and GSK3 for at least 96 hours (Fig. 1b). Prolonged activation of the myrPKB:ER protein did not result in its down regulation (Fig. 1b, upper panel).

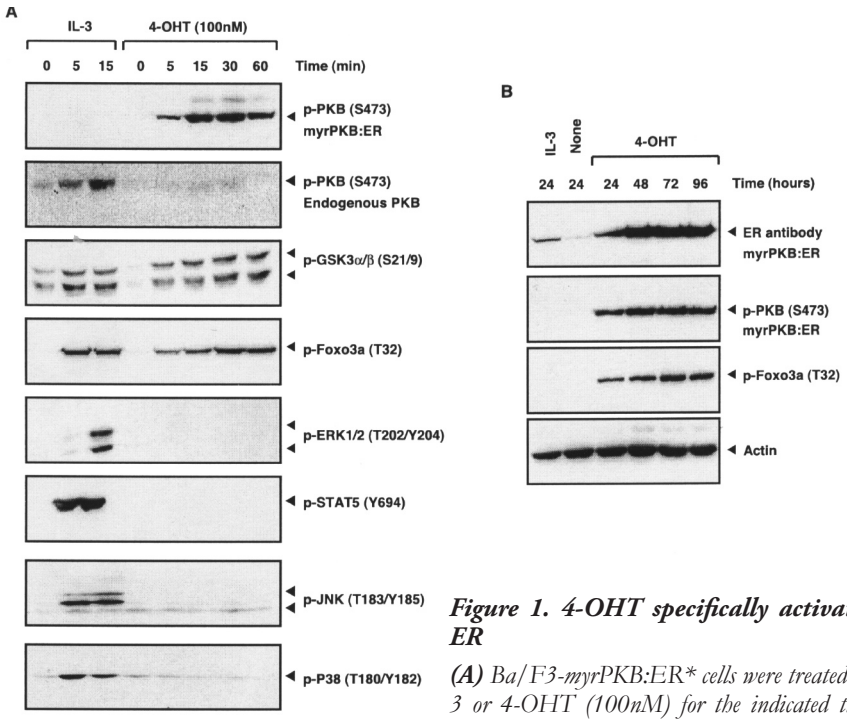


Figure 1. 4-OHT specifically activates myrPKB:ER

(A) *Ba/F3-myrPKB:ER* cells were treated with either IL-3 or 4-OHT (100nM) for the indicated times, lysed and equal amounts of protein were analyzed for levels of phospho-PKB (S473), phopho-Foxo3a (T32), phospho-GSK3α/β (S21/9), phospho-ERK1/2 (T202/Y204), phospho-STAT5 (Y694), phospho-JNK (T183/Y185) and phospho-p38 (T180/Y182).* ***(B)*** *Ba/F3-myrPKB:ER* cells were cytokine-starved or treated with either IL-3 or 4-OHT for the indicated times, lysed and equal amounts of protein were analyzed for levels of myrPKB:ER*, phospho-PKB (S473), phospho-Foxo3a (T32), phospho-GSK3α/β (S21/9).*

Subsequently, we investigated whether PKB activation alone was sufficient to induce proliferation. We cytokine-starved myrPKB:ER cells and left them untreated or treated with IL-3, 4-OHT or both for up to 96 hours. Using CFSE, we measured the decrease in fluorescence per cell every 24 hours, an indication of proliferation. In addition, proliferation was monitored by counting cells. Both methods showed that PKB activation alone was sufficient for cells to proliferate (Fig. 2a, 4-OHT) albeit not to the same extent as cells stimulated with IL-3 (Fig. 2a, IL-3). Cytokine-induced proliferation capacity was not perturbed when cells were stimulated with 4-OHT and IL-3 simultaneously (Fig. 2a, IL-3/4-OHT). Since activation of PKB is thought to be an important factor contributing to cell survival, we wished to establish the effect of chronic PKB activation on survival. We again cytokine-starved the myrPKB:ER cell line and left them untreated or treated them with IL-3, 4-OHT or both for up to 96 hours and measured the percentage of living cells every 24 hours. 4-OHT-mediated PKB activation rescued Ba/F3 cells from cytokine-withdrawal-induced apoptosis (Fig. 2b). Surprisingly, these anti-apoptotic effects were relatively short-lived, cells initiated programmed cell death after 48 hours (Fig. 2b). These apoptotic events were not due to reduced expression of myrPKB:ER nor due to inactivation of PKB judging from the phosphorylation status of myrPKB:ER and its substrates GSK-3 and Foxo3a (Fig. 1b). Importantly, chronic PKB activation in combination with IL-3 did not give rise to apoptosis (Fig. 2b). Since previously observed effects of PKB activation on survival and metabolism have been attributed to mTOR activity, we were interested if the transient anti-apoptotic activity of PKB was indeed mTOR-dependent. In order to investigate this, we measured apoptosis after 4-OHT stimulation in the absence or presence of rapamycin, a specific mTOR inhibitor. In contrast to IL-3, we observed that mTOR inhibition was able to almost completely block the rescue of cytokine-starved cells by PKB activation (Fig. 2c). These data demonstrate that the transient anti-apoptotic activity of PKB is dependent on mTOR activity, however PKB activation alone is insufficient to maintain longterm cell survival in the absence of additional factors.

Foxo3a expression is induced by prolonged activation of PKB

Since chronic PKB activation was insufficient for long-term survival, we sought to establish the mechanism underlying cell death. The myrPKB:ER cell line was cytokine starved and subsequently left untreated or treated with either IL-3 or 4-OHT for up to 96 hours. During prolonged 4-OHT treatment, we observed that Foxo3a remains phosphorylated (Fig. 1b), however Foxo3a protein levels increase dramatically (Fig. 3a). This increase in Foxo3a protein levels is already clearly visible within 24 hours of PKB activation (Fig. 3b). Quantitive analysis of the amounts of phosphorylated and unphosphorylated Foxo3a protein showed that the ratio between the two rapidly decreased after 48 hours of PKB activation (data not shown). Since chronic PKB activation in combination with cytokine treatment did not give rise to apoptosis or a delay in proliferation, we were interested if the presence of

cytokine would have an effect on Foxo3a upregulation. Cells were cytokine-starved and left untreated or treated with IL-3, 4-OHT or both for 24 hours. Foxo3a protein was only upregulated when PKB was activated in the absence of cytokine (Fig. 3c). These experiments indicate that PKB activation in the absence of additional survival factors results in upregulation of Foxo3a expression levels.

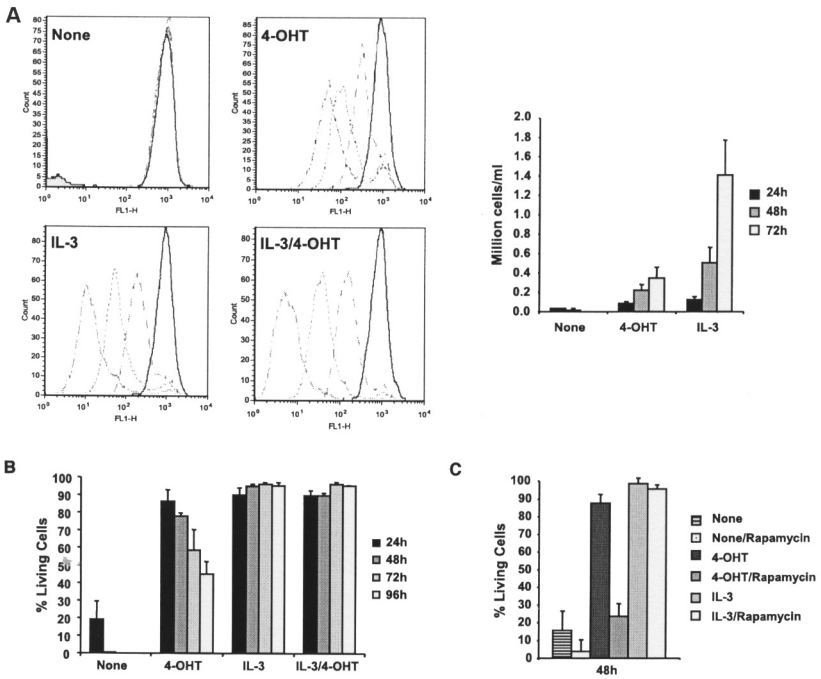


Figure 2. Chronic PKB activation induces apoptosis in cytokine starved Ba/F3 cells ***(A)*** *Left Ba/F3 myrPKB:ER* cells were cytokine-starved or treated with either 4-OHT, IL-3 or both IL-3 and 4-OHT for the indicated times; 24h (solid line -), 48h (Dashed line ---), 72h (Dotted line ⋯) and 96h (Dash-dotted line - ·). Proliferation was visualised as the decrease of fluorescent CFSE-probe per cell as measured by FACS analysis. Right Ba/F3 myrPKB:ER* cells were cytokine-starved or treated with either 4-OHT or IL-3 for the indicated times. Proliferation was monitored by counting cells at the indicated time points. Data is representative of at least three independent experiments and these results were observed in at least two independent clonal cell lines.* ***(B)*** *Ba/F3-myrPKB:ER* cells were cytokine-starved or treated with either IL-3, 4-OHT or both for the indicated times. The percentage of living cells was determined by FACS analysis of the percentage of Annexin-V negative cells. Results were presented as an average of three independent experiments* ± *S.E.M.* ***(C)*** *Ba/F3-myrPKB:ER* cells were cytokine-starved or treated with either IL-3 or 4-OHT for 48 hours in the presence or absence of rapamycin (20 ng/ml). The percentage of living cells was determined by FACS analysis of the percentage of Annexin-V negative cells. Results were presented as an average of three independent experiments* ± *S.E.M.*

Expression of Foxo3a transcriptional targets induced by long-term PKB activation

Since the upregulation of Foxo3a protein levels observed during chronic PKB activation leads to a decrease in the ratio of phosphorylated compared to total Foxo3a protein, we sought to establish if this leads to increased transcriptional activity.

Indeed, we found that together with the upregulation of Foxo3a, transcriptional targets Bim and $p27^{kip1}$ were also upregulated (Fig. 4). Upregulation of Foxo3a protein already occurs within the first 24 hours of PKB activation (Fig. 3b), while the upregulation of $p27^{kip1}$ and Bim expression occurs somewhat later. During prolonged PKB activation downregulation of Bcl-xl was also observed (Fig. 4). The upregulation of Bim and $p27^{kip1}$ and the downregulation of Bcl-xl are indicative for the fact that Foxo3a activity is no longer inhibited and provide an explanation as to why these cells are apoptotic after chronic PKB activation.

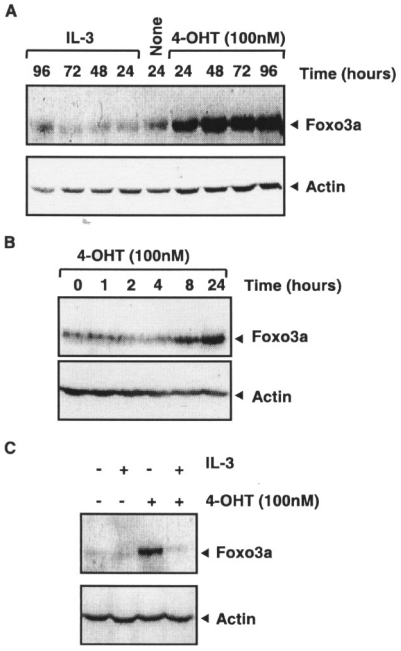


Figure 3. Chronic PKB activation leads to upregulation of Foxo3a protein

(A) *Ba/F3-myrPKB:ER* cells were cytokine-starved or treated with either IL-3 or 4-OHT for the indicated times, lysed and equal amounts of protein were analyzed for levels of Foxo3a and Actin.* ***(B)*** *Ba/F3-myrPKB:ER* cells were treated with 4-OHT for the indicated times, lysed and equal amounts of protein were analyzed for levels Foxo3a and Actin.* ***(C)*** *Ba/F3-myrPKB:ER* cells were cytokine-starved or treated with either IL-3, 4-OHT or both for 24 hours, lysed and equal amounts of protein were analyzed for levels of Foxo3a and Actin. Data is representative of at least three independent experiments and these results were observed in at least two independent clonal cell lines.*

Oxidative stress leads to an upregulation of Foxo3a protein

We have established that during prolonged PKB activation Foxo3a expression is upregulated resulting in transcription of pro-apoptotic target genes. However, we were interested in determining the molecular mechanism underlying this upregulation. It has been observed in various tumours in which PKB activity is enhanced that there is a concomitant increase in aerobic glycolysis and mitochondrial activity. This increase in metabolic activity in the cell can lead to increased levels of reactive oxygen species (ROS) resulting in oxidative stress. Cells have specific mechanisms to protect themselves against ROS, including the upregulation of MnSOD. However, while MnSOD has been reported to be a transcriptional target of Foxo3a (18), surprisingly we observed that after long-term PKB activation MnSOD levels actually decrease (Fig. 4a), indicating that at least one important protective mechanism against oxidative stress is impaired.

To determine whether an increase in intracellular oxidative stress might be responsible for increased Foxo3a expression we utilized hydrogen peroxide (H_2O_2) and N-acetylcysteine (NAC), a widely used thiol-containing oxygen radical scavenger. Addition of H_2O_2 to the culture medium induced apoptosis in Ba/F3 cells which was prevented by the addition of NAC (Fig. 4b). To determine if an increase in ROS can indeed lead to increased levels of Foxo3a protein, Ba/F3 cells were treated with H_2O_2 and subsequently cell lysates were prepared and analysed for Foxo3a expression. Similar to chronic PKB activation, H_2O_2 treatment was also able to elevate levels of Foxo3a protein (Fig. 4c).

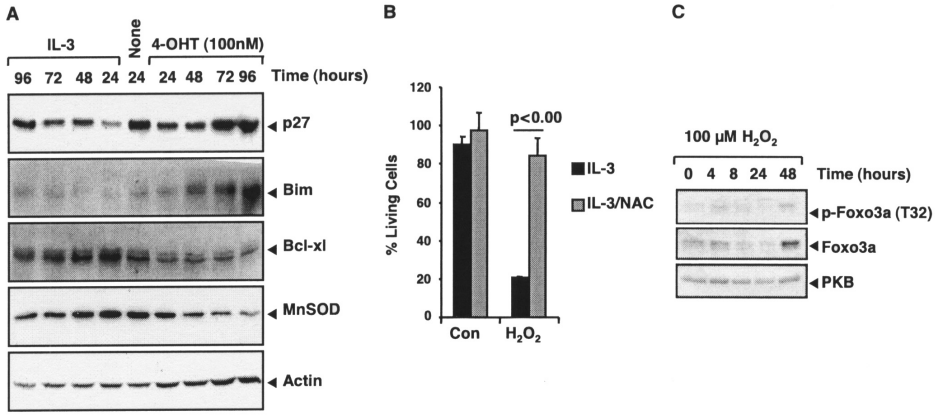


Figure 4. Chronic PKB activation and H_2O_2 lead to changes in expression of Foxo3a transcriptional targets

(A) *Ba/F3-myrPKB:ER* cells were cytokine-starved or treated with either IL-3 or 4-OHT for the indicated times, lysed and equal amounts of protein were analyzed for levels of $p27^{kip1}$, Bim, Bcl-xl, MnSOD and Actin. Data is representative of at least three independent experiments and these results were observed in at least two independent clonal cell lines.* ***(B)*** *Ba/F3-myrPKB:ER* cells were cultured for 24h in the presence of IL-3 with or without N-acetyl-cysteine (250 μM) and with or without H_2O_2 (200 μM) added to the medium. The cells treated with NAC were pretreated for 24h in normal culture medium supplemented with this radical oxygen scavanger. The percentage of living cells was determined by FACS analysis of the percentage of Annexin-V negative cells. Results were presented as an average of three independent experiments ± S.E.M. Data were analysed using one-way ANOVA corrected with Bonferroni's post test for multiple comparisons.* ***(C)*** *Ba/F3 wildtype cells were cultured in the presence of IL-3 and 100 μM H_2O_2 for the indicated times, lysed and equal amounts of protein were analyzed for levels of Foxo3a, phospho-Foxo3a (Thr 32), phosphor-PKB (Ser473) and PKB. Data is representative of at least three independent experiments.*

In order to investigate whether increased oxidation results in the death of Ba/F3 myrPKB:ER cells after prolonged PKB activation, we measured apoptosis after 4-OHT stimulation in the absence or presence of NAC. We observed that addition of NAC was able to significally decrease the amount of apoptosis observed after PKB activation (Fig. 5a). Although NAC is a free radical scavenger, it can also act as an aminothiol and a precursor of intracellular cysteine and reduced glutathione (GSH). GSH, the most abundant thiol antioxidant in mammalian cells, directly reacts with

ROS, functions as a cofactor for antioxidant enzymes like gluthatione preroxidases (GPxs) and maintains thiol redox potential in cells. Under normal circumstances, biological environments and reducing thiol buffers such as GSH within cells are predominantly in a reduced state. However, under conditions of oxidative stress, the endogenous pool of reduced thiols can become depleted. To test if thiol buffers are reduced after prolonged PKB activation, we measured GSH levels using the GSH-sensitive probe monochlorobimane. Buthionine sulphoximine (BSO), a selective GSH biosynthesis inhibitor was used as a negative control (Fig. 5b, Left). GSH levels are significantly decreased in both cytokine-starved cells and in cells which have PKB chronically activated. We also observed that GSH depletion through BSO treatment (Fig. 5b, Middle) results in apoptosis in the Ba/F3 cells either in the presence of cytokine or together with chronic PKB activation (Fig. 5b, Right). Addition of NAC to the medium is able to significally replenish the depleted pool of GSH (Fig. 5b, Left). To investigate if NAC may function by replenishing a depleted pool of reducing thiols, we measured apoptosis after 4-OHT stimulation in the absence or presence of GSH or dithiothreitol (DTT), a general thiol reductant. We observed that addition of either GSH or DTT significally decreased the amount of apoptosis observed after PKB activation (Fig. 5c), revealing the key role of oxidation in the apoptotic effect of chronic PKB activation.

Furthermore, we investigated if glutathione depletion by BSO has an effect on Foxo3a protein levels. As shown in Figure 5d (top panel), glutathione depletion gave rise to similar upregulation of Foxo3a as was observed with chronic PKB activation. Moreover, addition of NAC to the culture medium was able to prevent the upregulation of Foxo3a protein compared to cells treated with 4-OHT alone (Fig. 5d, Bottom panel). Thus, Ba/F3 cells are sensitive to increased levels of oxidative stress leading to upregulation of Foxo3a and apoptosis. Relieving the levels of oxidative stress by addition of NAC can delay Foxo3a upregulation and the onset of apoptosis.

Increased ROS levels induce transcriptional activity of the Foxo3a promotor

Although we have demonstrated regulation of Foxo3a protein levels by chronic PKB activation, we wished to determine whether this could occur at the transcriptional level. Ba/F3 cells were treated with 4-OHT in the presence or absence of transcription inhibitor actinomycin D. Actinomycin D completely blocked the upregulation of Foxo3a protein by 4-OHT (Fig. 6a). Therefore, we examined if the effect of PKB activation on FOXO3a protein levels was also reflected on the level of FOXO3a mRNA. Realtime quantative PCR showed an increase in FOXO3a mRNA after 4 hours of PKB activation (Fig. 6b). Furthermore, H_2O_2 treatment also resulted in an increase of Foxo3a mRNA (Fig. 6c).

In addition to analyzing Foxo3a mRNA, we also examined Foxo3a promoter regulation by PKB activation. To this end, Ba/F3 cells were transiently transfected with a luciferase reporter construct under the control of the Foxo3a promoter.

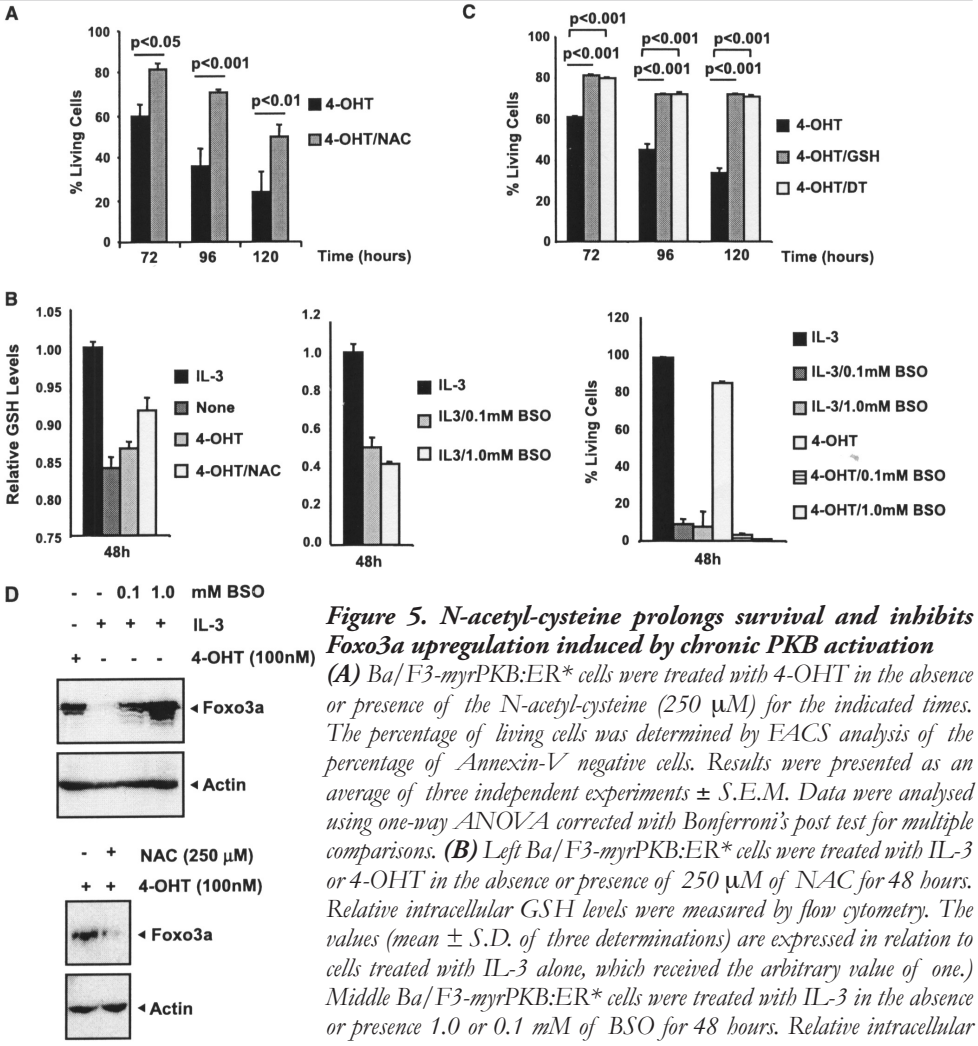


Figure 5. N-acetyl-cysteine prolongs survival and inhibits Foxo3a upregulation induced by chronic PKB activation

(A) *Ba/F3-myrPKB:ER* cells were treated with 4-OHT in the absence or presence of the N-acetyl-cysteine (250 μM) for the indicated times. The percentage of living cells was determined by FACS analysis of the percentage of Annexin-V negative cells. Results were presented as an average of three independent experiments ± S.E.M. Data were analysed using one-way ANOVA corrected with Bonferroni's post test for multiple comparisons.* ***(B)*** *Left Ba/F3-myrPKB:ER* cells were treated with IL-3 or 4-OHT in the absence or presence of 250 μM of NAC for 48 hours. Relative intracellular GSH levels were measured by flow cytometry. The values (mean ± S.D. of three determinations) are expressed in relation to cells treated with IL-3 alone, which received the arbitrary value of one.) Middle Ba/F3-myrPKB:ER* cells were treated with IL-3 in the absence or presence 1.0 or 0.1 mM of BSO for 48 hours. Relative intracellular GSH levels were measured by a Fluoroskan Ascent from Thermo Labsystems (Almere, The Netherlands). The values (mean ± S.D. of three determinations) are expressed in relation to cells treated with IL-3 alone, which received the arbitrary value of one.) Right Ba/F3-myrPKB:ER* cells were treated with IL-3 in the absence or presence 1.0 or 0.1 mM of BSO for 48 hours. The percentage of living cells was determined by FACS analysis of the percentage of Annexin-V negative cells. Results were presented as an average of three independent experiments ± S.E.M.* ***(C)*** *Ba/F3-myrPKB:ER* cells were treated with 4-OHT in the absence or presence of GSH (250 μM) or DTT (100 μM)for the indicated times. The percentage of living cells was determined by FACS analysis of the percentage of Annexin-V negative cells. Results were presented as an average of three independent experiments ± S.E.M. Data were analysed using one-way ANOVA corrected with Bonferroni's post test for multiple comparisons.* ***(D)*** *Top Ba/F3-myrPKB:ER* cells were treated with 4-OHT, IL-3 alone or IL-3 in the presence of 0.1 mM or 1.0 mM BSO for 48 hours, lysed and equal amounts of protein were analyzed for levels of Foxo3a and actin. Data is representative of at least three independent experiments and these results were observed in at least two independent clonal cell lines. Bottom Ba/F3-myrPKB:ER* cells were treated with 4-OHT in the absence or presence of N-acetyl-cysteine (250 μM) for 24 hours, lysed and equal amounts of protein were analyzed for levels of Foxo3a and actin. Data is representative of at least three independent experiments and these results were observed in at least two independent clonal cell lines.*

Subsequently, luciferase activity was measured after 4-OHT mediated PKB activation. As shown in Figure 6d (upper panel), chronic PKB activation induced Foxo3a promoter activity after 24 hours. To investigate whether Foxo3a promoter activity was also oxidative stress-dependent, luciferase activity was measured after addition of H_2O_2 to the culture medium. Figure 6d (lower panel) demonstrates that upon addition of H_2O_2, luciferase reporter activity increases. Taken together, these data suggest that chronic PKB activation leads to a state of oxidative stress which subsequently induces Foxo3a transcription, leading to increased levels of Foxo3a protein. This increased Foxo3a expression leads to transcriptional upregulation of pro-apoptotic target genes eventually resulting in programmed cell death.

DISCUSSION

A tumour develops when the balance between generation and growth of new cells, and death and removal of excess cells is perturbed. Aberrant PKB activation has now been implicated in the pathogenesis of a variety of human cancers. Mouse models have suggested that unregulated PKB signaling can contribute to malignancy either alone or in cooperation with additional genetic alterations (33). However, to properly interpret studies investigating the role of this kinase in oncogenesis it is important to take into account that diverse genetic events and cellular changes occur in the development of a tumor. We have utilized a cellular model in which PKB activity can be rapidly and inducibly activated providing a simplified model for understanding the molecular mechanisms underlying transforming capacity of this protein. Interestingly, our data suggest that chronic PKB activation can lead to cellular oxidative stress, eventually resulting in apoptosis through increased Foxo3a expression. Importantly, this demonstrates that PKB activity alone is insufficient for cell survival and additional signals induced by cytokine stimulation are required. Indeed it has been demonstrated that expression of either activated Ras or PKB alone is insufficient to cause glioblastoma in mice, while their co-expression leads to aggressive disease (34).

The fact that dysregulation of PKB activation can lead to oxidative stress in cells can be explained by the mechanism by which PKB inhibits apoptotic events. PKB-mediated survival depends at least in part on the maintenance of glucose metabolism. PKB activation has been shown to maintain mitochondrial membrane potential and hexokinase activity in situations that would otherwise lead to apoptosis such as serum withdrawal. PKB activation leads to an increase in nutrient uptake and enhanced cellular metabolism through an mTOR-dependent mechanism (35). mTOR can be activated by PKB either by direct phosphorylation (36) or by phosphorylation and inhibition of TSC2 (37). In our system, PKB-mediated survival is also dependent on mTOR activity and thus the key role mTOR plays in coordinating the cellular response to extracellular nutrient levels is of great importance to survival induced by chronic PKB activation. However, metabolic activity also results in the production of reactive oxygen species (ROS) and, in absence of sufficient cellular mechanisms

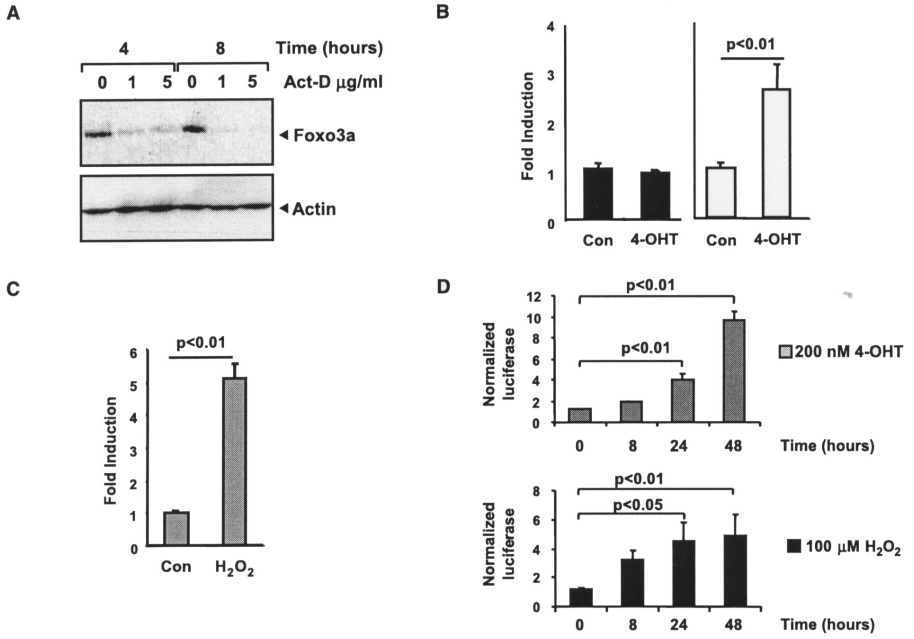


Figure 6. PKB activation and H_2O_2 upregulate Foxo3a mRNA levels and promotor activity ***(A)*** *Ba/F3-myrPKB:ER* cells were cultured for the indicated times in the presence of 4-OHT with or without the indicated concentrations of actinomycin D added to the medium, lysed and equal amounts of protein were analyzed for levels of Foxo3a and Actin.* ***(B)*** *Ba/F3 myrPKB:ER* cells were cultured with or without 4OHT (Right panel) for 4 hours and as a control wild type Ba/F3 cells were incubated with 4OHT for 4 hours (left panel), RNA was isolated, cDNA synthesized and real time PCR was performed using specific primers for Foxo3a. Results were normalized for the housekeeping gene L19. The values (relative change of Foxo3a mRNA ± S.D.) are expressed in relation to untreated cells, which received the arbitrary value of one. Data is representative of at least three independent experiments. Data were analysed using one-way ANOVA corrected with Bonferroni's post test for multiple comparisons.* ***(C)*** *Ba/F3 myrPKB:ER* cells were cultured with or without H_2O_2 for 4 hours, RNA was isolated, cDNA synthesized and real time PCR was performed using specific primers for Foxo3a. Results were normalized for the housekeeping gene L19. The values (relative change of Foxo3a mRNA ± S.D.) are expressed in relation to untreated cells, which received the arbitrary value of one. Data is representative of at least three independent experiments. Data were analysed using one-way ANOVA corrected with Bonferroni's post test for multiple comparisons.* ***(D)*** *Ba/F3-myrPKB:ER* cells were transiently transfected with pGL3-Foxo3a promoter construct and subsequently treated with 4-OHT (upper panel) or H_2O_2 (lower panel). Cells were harvested at the indicated times and assayed for luciferase activity. Results are corrected for cotransfected renilla activity, and the relative luciferase activity is standardized to that observed with the empty vector. The values (relative change of Foxo3a promoter activity ± S.D.) are expressed in relation to untreated cells, which received the arbitrary value of one. Results were presented as an average of three independent experiments. Data in this graph were analysed using ANOVA statistics with Dunnets post test for multiple comparisons (all points vs. control).*

to compensate for this, a state of oxidative stress. Unfortunately, we were unable to detect increased levels of ROS due to lack of sensitivity of available methods. However, experiments utilizing both H_2O_2 and NAC support that chronic PKB activation can lead to oxidative stress and that the observed effects on Foxo3a regulation are oxidative stress dependent. In our system, the chronic activation of

PKB leads to a prolonged situation of cellular oxidation resulting in depletion of cellular protection mechanisms including MnSOD and GSH.

Excessive ROS and oxidative stress can result in cellular damage but also in altered signal transduction. Recently, there have been several reports describing the regulation of FOXO transcription factors by cellular oxidative stress. Essers et al. (38) showed that oxidative stress induces the activation of FOXO4. Oxidative stress leads to the activation of small GTPase Ral and this results in a JNK-dependent phosphorylation of FOXO4, leading to nuclear translocation and transcriptional activation. Another post-translational modification observed for FOXOs in response to cellular stress is changes in acetylation. In 2005, three independent groups reported that FOXOs are acetylated in response to cellular stress. While these studies show that regulation of acetylation of FOXOs leads to changes in transcriptional activation, the functional consequences remain unclear (39-41). Here, we are the first to demonstrate that Foxo3a can also be regulated by oxidative stress at the level of expression. This demonstrates that cellular oxidative stress can modulate FOXO transcriptional activity through multiple molecular mechanisms.

How could cellular oxidation lead to upregulation of Foxo3a protein levels? We have clearly shown that Foxo3a expression is regulated at the level of transcription (Figs. 6a,d). Studies have demonstrated that changes in redox status can regulate phosphorylation and activation of a variety of intracellular signaling molecules (42). This can subsequently lead to changes in the activity of redox sensitive transcription factors including components of the NFκb and AP-1 complexes (43). Indeed, with homology to Foxo3a, regulation of AP-1 activity through oxidative stress can be achieved through changes in transcription of genes encoding AP-1 subunits, control of mRNA stability, post-translational processing and turnover of protein. It is likely that oxidation-mediated modulation of such transcription factors is responsible for the increased Foxo3a expression we observe after chronic PKB activation.

Here, we investigate the effect of chronic PKBα on survival and proliferation of Ba/F3 cells, recently Jin et al. (44) investigated the effect of chronic PKBβ activation in human kidney epithelial cells (HEK293). This chronic activation resulted in alterations in cellular growth, size and the appearance of aneuploid cells. After prolonged activation cells underwent extensive multinucleation caused by both endomitosis and cell fusion. In our system, we do not observe these phenomena, this is most likely do to the distinct nature of the two cell lines used in these studies. It is also possible that due to their factor-dependent nature, hematopoietic cell lines are less likely to accumulate major alterations since they are more prone to apoptosis than HEK293 cells. Transgenic mice expressing constitutively active PKB targeted to the lymphocyte population demonstrate lymphoproliferation (45, 46). In our study, we also observed increased proliferation when PKB was activated in the presence of cytokine (Fig 2a, left) but since survival was already optimal under these conditions there was no further advantage of PKB activation (Fig. 2b). This indicates that in our system the anti-apoptotic activity of PKB does not lead to apoptosis in

combination with other stimulating factors, but that chronic activation enhances proliferation and could increase resistance to apoptotic stimuli as was shown by Jones et al (47). PKB activation merely enhances existing survival and proliferation signals but its chronic activation appears to make these cells more sensitive to targeting or depletion of protective mechanisms such as GSH or MnSOD.

Insulin and IGF-1 have beneficial effects on cardiomyocyte function and survival including protection against ischemic injury. This is thought to be mediated in part due to PKB activation (48), and acute PKB activation is cardioprotective. However, recent studies have demonstrated that chronic PKB activation actually results in decreased functional recovery and increased injury after ischaemia/reperfusion injury (IRI) (49). In these studies, PKB-induced injury could be abrogated by constitutive PI3K activation. Interestingly, we found that in contrast to PKB, chronic PI3K activation was indeed sufficient for cytokine-independent Ba/F3 survival (data not shown). Our data suggests a possible molecular mechanism underlying the detrimental effects of chronic PKB activation in IRI, suggesting that prolonged PKB activation in cardiomyocytes might also lead to increased oxidative stress and cellular damage.

We demonstrate that increased expression of Foxo3a ultimately results in upregulation of $p27^{kip1}$ and Bim protein levels together with downregulation of Bcl-xl. This can be explained since Bcl-xL expression is inhibited by Bcl-6, which has been shown to be a transcriptional target of Foxo4 (50). Another known Foxo3a target MnSOD is downregulated during Foxo3a activation in this cytokine deprived situation. This indicates that, in contrast to other cell systems, Foxo3a transcriptional upregulation is insufficient to protect cells against oxidative stress. Bone marrow derived Ba/F3 cells apparently do not have an adequate mechanism to deal with the enhanced oxidative environment caused by chronic PKB activation.

A cell must successfully overcome a series of hurdles before it can be considered to be cancerous. Our data suggest that while chronic activation of PKB may indeed be a crucial factor in bypassing normal failsafe mechanisms, allowing factor independent growth and survival, additional mechanisms are required to enable PKB "transformed" cells to cope with the detrimental effects of uncontrolled glycolysis. Dysregulation of PKB activity is therefore in itself insufficient for hematopoietic cell survival, and cells are "culled" through upregulation of the pro-apoptotic Foxo3a transcription factor. These findings have important consequences for our understanding of the processes leading to cellular transformation.

Acknowledgements

We would like to thank Ines Soeiro for technical assistance. We would like to thank Dr. A. Klippel for kindly providing us with the SRα-myrPKB:ER* construct. Ankie van Gorp and Kim Birkenkamp are supported by grants from ZonMW.

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CHAPTER 3

Phosphoproteomic identification of novel Protein Kinase B (PKB/c-akt) substrates

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ABSTRACT

To define the precise role protein kinases play in cellular physiology, it is necessary to have a clear picture of the *in vivo* substrates through which those kinases exert their actions. For Protein kinase B (PKB/c-akt) there have been many reports describing *in vitro* substrates, however many of these remain to be validated *in vivo*. Validation of "proper" PKB substrates is complicated by the lack of specific inhibitors for this kinase.

In this study we made use of a phosphoproteomic approach in order to identify novel PKB substrates. Cytokine-dependent bone marrow-derived Ba/F3 cells, in which PKBα activation can be directly, and specifically, induced by addition of 4-hydroxytamoxifen (4-OHT) were stimulated with 4-OHT in order to induce phosphorylation of PKB substrates. Phosphorylated proteins were separated by phospho-Ser/Thr affinity purification, analyzed by 2D SDS-PAGE and western blotting utilizing an antibody raised against the minimal PKB consensus phosphorylation site (RXRXXS/T). Hybridisation with this phospho-PKB substrate antibody resulted in the identification of several proteins whose phosphorylation was regulated upon specific PKB activation. These included eukaryotic translation initiation factor 4B (eIF4B), 5'-Phosphoribosyl N-formylglycinamide amidotransferase (FGARAT), Minichromosome Maintenance proteins (MCM) 3, MCM6, Lamin B1 and Moesin. Here we discuss the role of those identified proteins as novel *in vivo* PKB targets and the feasibility of this approach for phosphoprotein characterization.

INTRODUCTION

To define the precise role protein kinases play in cellular physiology, it is necessary to have a clear picture of the *in vivo* substrates through which those kinases exert their actions. While there have been many reports concerning *in vitro* substrates for Protein kinase B (PKB/c-akt), many of the substrates that mediate its actions *in vivo* remain to be validated. The lack of good specific PKB inhibitors has made it difficult to confirm potential substrates *in vivo*. Studies analysing PKB knock-out mice have revealed that it is indispensable for normal cellular functioning (18,19,86,99,111). In mammals there are three PKB isoforms, PKBα, PKBβ and PKBγ, which share a high degree of sequence homology (58). All three isoforms contain an N-terminal pleckstrin homology domain (PH), a catalytic domain and a C-terminal regulatory domain (23). PKBα is the most ubiquitously expressed and studies in PKBα-deficient mice have shown it to be indispensable for normal cell growth (19). The null mutant mice show defects in both fetal and postnatal growth, which persisted into adulthood. PKBβ is most abundantly expressed in insulin-responsive tissues and indeed PKBβ-deficient mice suffer from diabetes (18). PKBγ has a more restricted expression profile, being predominantly expressed in the brain and testes, with null mutant mice exhibiting impaired brain development (99). $PKB\alpha^{(-/-)}$ and $PKB\beta^{(-/-)}$ as well as $PKB\alpha^{(-/-)}$ and $PKB\gamma^{(-/-)}$ double knock-out mice die shortly after birth or during embryonic development, respectively (86,111). Interestingly, Dummler and co-workers found that a single functional allele of PKB ($PKB\alpha^{(-/+)}$/$PKB\beta^{(-/-)}$/$PKB\gamma^{(-/-)}$) is sufficient for successful embryonic development and postnatal survival; however these mice show glucose and insulin intolerance and their total body weight as well as that of brain and testis was significantly reduced (30).

Given the importance of PKB in a variety of cellular functions as highlighted by the phenotypes of null mutant mice; numerous laboratories have sought to identify PKB substrates that could explain its critical role in cellular physiology. While a variety of PKB substrates have been identified (Table 1), many have only been validated through *in vitro* kinase assays. It is thus important to re-evaluate the relevance of these studies through *in vivo* identification and validation of PKB substrate.

A confounding factor in the identification of "true" PKB substrates is that PKB is a member of the AGC protein kinase family, which also includes serum- and glucocorticoid-induced protein kinase (SGK), mitogen- and stress-activated protein kinase (MSK), p70 ribosomal S6 kinase (S6K), p90 ribosomal S6 kinase (RSK), protein kinase C (PKC) isoforms, PKC-related kinase (PRK) and cyclic AMP (cAMP)-dependent protein kinase (PKA) (7,102). This kinase family is defined by a high homology within their catalytic domains, resulting in similar substrate consensus sequences. Most AGC kinase family members phosphorylate serine and/or threonine residues that lie C-terminal to clusters of basic amino acid residues that are critical for their specificity (1). *In vitro*, AGC protein kinases are often promiscuous and can phosphorylate the same substrates. However, it is often unclear *in vivo* which

kinase phosphorylates a certain substrate in response to a physiological stimulus. For example the well characterized PKB substrate GSK3 can also be phosphorylated by p70S6K under conditions of cellular insulin resistance where PKB is inactive (112).

Table 1: Known substrates of PKB

Depicted are the described substrates of PKB, species, position of phosphorylation by PKB, their PKB phosphorylation consensus sequence(s), Scansite 2.0 percentile and stringency, and the primary reference(s).

Protein	Species	Position	Sequence	Scansite 2.0 Percentile/ Stringency	Reference(s)
14-3-3zeta	Human Mouse Drosophila	58 58 61	GARRSS GARRSS GARRSS	3.907/Low 3.907/Low 4.135/Low	(87,108)
AHNAK	Human Mouse	5782 5555	RHRSNS RHRSNS	0.018/High 0.018/High	(94)
Androgen receptor	Human Mouse Human Mouse	213 208 791 771	RAREAS RAREAT RMRHLS RMRHLS	0.729/Med 0.729/Med 1.770/Low 1.770/Low	(70,107)
APS	Human Mouse Rat	598 588 588	RSRSNS HSRSNS RSRSNS	0.071/High 2.692/Low 0.222/Med	(52)
ARK5	Human	600	RQRIRS	0.837/Med	(96)
AS160	Human Mouse Human Mouse Human Mouse Human Mouse	588 594 318 324 341 348 42 648	RGRLGS RGRLGS RSRCSS RSRCSS RRRHAS RRRHAS RRRAHT RRRAHT	0.011/High 0.018/High 0.067/High 0.049/High 0.105/High 0.105/High 0.005/High 0.005/High	(50)
ASK1	Human Mouse	83 90	RGRGSS RGRGNS	0.075/High 0.764/Med	(54)

Ataxin-1	Human Mouse	775 752	RKRRWS KRRRWS	0.047/High 0.181/High	(16)
ATP-citrate lyase	Human Mouse	455 455	PSRTAS PSRTAS	0.107/High 0.107/High	(10)
Bad	Human Mouse Human Mouse	75 112 99 136	RSRHSS RSRHSS RGRSRS RGRSRS	0.046/High 0.080/High 0.010/High 0.010/High	(24)
BRCA1	Human Mouse	509 504	RKRRPT RKRSTS	0.206/Med 0.009/High	(2)
Caspase 9	Human Human	183 196	RTRTGS RRRFSS	0.113/High 2.044/Low	(14)
CHK1	Human Mouse	280 280	RPRVTS RPRATS	0.131/High 0.137/High	(55)
Cot1/TPL2	Human Mouse	400 400	QPRCQS QPRCQS	1.824/Low 1.824/Low	(49)
CREB	Human Mouse	133 133	LSRRPS LSRRPS	Not Found Not Found	(29)
CRHSP24	Human Mouse	52 53	RTRTFS RTRTFS	0.020/High 0.020/High	(3)
EDG1	Human Mouse	236 196	RSRRLT RSRRLT	0.148/High 0.148/High	(68)
eNOS	Human Mouse	1177 1176	RIRTQS RIRTQS	0.022/High 0.022/High	(28)
ER alpha	Human Mouse	167 171	RERLAS RERLSS	0.241/Med 0.268/Med	(103)
Ezrin	Human Mouse	567 567	RDKYKT RDKYKT	4.333/Low 4.333/Low	(93)
FANCA	Human	1149	RSRDPS	0.477/Med	(80)
Filamin C	Human Mouse	2233 2234	RERLGS RERLGS	0.023/High 0.023/High	(73)

FOXA2	Human Mouse	156 156	YRRSYT YRRSYT	2.102/Low 2.102/Low	(27)
FOXO1	Human Mouse Elegans Human Mouse Elegans Human Mouse Elegans	24 24 54 256 253 242 319 316 314	RPRSCT RQRSCT RDRCNT RRRAAS RRRAAS RERSNT RPRTSS RPRTSS RPRTQS	0.003/High 0.008/High 0.053/High 0.049/High 0.049/High 0.017/High 0.102/High 0.102/High 0.168/High	(89)
FOXO3a	Human Mouse Elegans Human Mouse Elegans Human Mouse Elegans	32 32 54 253 252 240 315 314 314	RPRSCT RPRSCT RDRCNT RRRAVS RRRAVS RERSNT RSRTNS RSRTNS RPRTQS	0.001/High 0.001/High 0.053/High 0.112/High 0.112/High 0.017/High 0.283/Med 0.283/Med 0.168/High	(12)
FOXO4	Human Mouse Elegans Human Mouse Elegans Human Mouse Elegans	32 32 54 197 197 240 262 262 314	RPRSCT RPRSCT RDRCNT RRRAAS RRRAAS RERSNT RPRSSS RPRSSS RPRTQS	0.000/High 0.000/High 0.053/High 0.085/High 0.085/High 0.017/High 0.108/High 0.108/High 0.168/High	(59)
FOXO6	Mouse Mouse	26 184	RPRSCT RRRAVS	0.001/High 0.052/High	(47)
FRAP/ mTOR	Human Mouse Human Mouse	2446 2446 2173 2173	RSRTRT RSRTRS RPRKLT RPRKLT	0.048/High 0.048/High 0.166/High 0.166/High	(75,91)

	Human Mouse	2448 2448	RTRTDS RTRTDS	0.030/High 0.030/High	
Gab2	Human Mouse	159 160	RERKSS RERKSS	0.105/High 0.105/High	(72)
GABA(A) receptor β2	Human Mouse	410 410	LRRRAS LRRRAS	Not Found Not Found	(106)
Girdin/ APE	Human	1416	RERQKS	0.178/High	(31)
GSK3α	Human Mouse Drosophila	21 21 9	RARTSS RARTSS RPRTSS	0.015/High 0.015/High 0.013/High	(21)
GSK3β	Human Mouse Drosophila	9 9 9	RPRTTS RPRTTS RPRTSS	0.008/High 0.008/High 0.013/High	(21)
hTERT	Human Human	227 824	RRRGGS RIRGKS	0.071/High 2.949/Low	(51)
Huntington	Human Mouse Human Mouse Human Mouse	421 399 1024 1003 2068 2046	RSRSGS RGRSGS TTRALT TTRALT RFRLST RFRLST	0.030/High 0.024/High 0.180/High 0.180/High 0.104/High 0.085/High	(40)
IKKα	Human Mouse	23 23	RERLGT RERLGT	0.176/High 0.156/High	(81)
IRS1	Human Mouse Human Mouse Human Mouse Human Mouse	307 302 527 522 330 325 270 265	RSRTES RSRTES RKRTHS RKRTHS RVRASS RVRASS RPRSKS RPRSKS	0.006/High 0.006/High 0.010/High 0.010/High 0.133/High 0.133/High 0.175/High 0.141/High	(84)

METTL1	Human Mouse	27 21	RQRAHS RQRAHS	0.235/Med 0.235/Med	(15)
MDM2	Human Mouse Zebrafish Human Mouse Zebrafish	166 163 147 186 183 169	RRRAIS RRRSIS RRRRSS RKRHKS RKRRRS RKRHKS	0.116/High 0.055/High 0.073/High 0.464/Med 0.006/High 0.675/Med	(114)
MLK3	Human Mouse	674 675	RERGES RERGES	0.225/Med 0.893/Med	(5)
Nur77	Human Mouse	351 354	RGRLPS RGRLPS	0.321/Med 0.321/Med	(85)
	Human Rat	329 330	RTRSLS RTRSLS	0.524/Med 1.233/Low	(37)
p21CIP1	Human Mouse	145 140	RKRRQT RKRRQT	0.634/Med 0.634/Med	(113)
p27kip	Human Cat	157 157	RKEPAT RKRPAT	0.296/Med 0.280/Med	(92)
p47Phox	Human Mouse Human Mouse	304 305 328 329	PPRRSS PPRRST AYRRNS TYRRNS	1.757/Low 1.984/Low 2.724/Low 2.353/Low	(17,39)
PDE3A	Human Mouse	294 292	RRRSSS RRRSSS	0.085/High 0.097/High	(35)
PDE3	Human Mouse	295 273	RPRRRS RPRRRS	0.779/Med 0.779/Med	(56)
PED/PEA-15	Human Mouse	116 116	IIRQPS IIRQPS	Not Found Not Found	(98)
PFK2	Human Mouse Human Mouse	465 469 482 486	RMRRNS RMRRNS RPRNYS RPRNYS	0.024/High 0.024/High 0.045/High 0.045/High	(25)
PIKfyve	Human Mouse	307 318	RNRSAS RNRSAS	0.030/High 0.030/High	(9)

PRAS40	Human Mouse	246 247	RPRLNT RPRLNT	0.868/Med 0.868/Med	(61)
PTP1B	Human Mouse	50 50	RYRDVS RYRDVS	1.104/High 1.104/High	(88)
Rac1	Human Mouse Drosophila Elegans	71 71 71 71	RLRPLS RLRPLS RLRPLS RLRPLS	0.250/Med 0.250/Med 0.250/Med 0.250/Med	(63)
Raf1	Human Mouse Elegans Drosophila	259 259 312 312	RQRSTS RQRSTS RDRSSS RSRRCS	0.135/High 0.135/High 0.071/High 0.109/High	(115)
SEK1	Human Mouse	80 78	RLRTHS RLRTHS	0.117/High 0.118/High	(82)
Tau	Human	214	KERPGS	2.337/Low	(62)
TopBP1	Human Mouse	1159 1161	RARLAS RARLAS	1.496/Low 1.357/Low	(71)
TSC2	Human Mouse Human Mouse	981 981 1462 1465	RCRSIS RCRSIS RPRGYT RPRGYT	0.031/High 0.031/High 0.005/High 0.005/High	(42)
WNK1	Human Mouse	60 58	RRRRHT RRRRHT	0.064/High 0.064/High	(48,104)
XIAP	Human Mouse	87 87	RHRKVS RHRRIS	1.205/Low 0.796/Med	(22)
Yap1	Human Mouse	328 347	ELRTMT ELRTMT	0.873/Med 0.873/Med	(6)
YB-1	Human Mouse	102 100	RKYLRS RKYLRS	1.071/Low 1.071/Low	(95)

All three PKB isoforms possess indistinguishable substrate specificity towards synthetic peptides (105). Originally the minimum sequence motif required for efficient phosphorylation of small peptide substrates by PKB is RxRxxS/Tz, where x is any amino acid, and z is a bulky hydrophobic residue [phenylalanine (F) or leucine (L) (1). However, many of the previously identified PKB substrates (for

example the Forkhead transcription factors, Foxo1, Foxo3a and Foxo4) do not contain this hydrophobic residue and therefore it can be considered non-essential. It has also been shown that variations in the amino acids surrounding the essential -3 and -5 argenine residue can influence the ability of specific AGC kinases to phosphorylate a certain residue *in vitro* (1,74,76). Knowledge of the optimal motif of a protein kinase can accelerate discovery of targets by allowing prediction of sites from a global search of genome sequences or by a restricted search of candidate proteins. However, ultimately, such predictions must be verified by both *in vitro* and *in vivo* experiments. Also as previously highlighted, care must be taken in drawing conclusions from *in vitro* assays where, it is likely that various members of the AGC kinase family may act promiscuously in phosphorylating substrates. In contrast however, *in vivo* phosphorylation of substrates is likely to be a highly regulated process.

Using a phosphoproteomic approach, we have analysed *in vivo* PKB substrate phosphorylation in bone marrow-derived Ba/F3 cells. Our studies have revealed a number of potentially novel PKB substrates, including eukaryotic translation initiation factor 4B (eIF4B), 5'-Phosphoribosyl N-formylglycinamide amidotransferase (FGARAT), Minichromosome Maintenance proteins (MCM) 3, MCM6, Lamin B1 and Moesin. The identification of eIF4B as a valid PKB substrate will be discussed in chapter 4. In this chapter we discuss the potential validity of the other proteins as novel PKB phosphorylation targets.

MATERIAL AND METHODS

Cell culture

Ba/F3 cells were cultured in RPMI 1640 medium with 8% Hyclone serum (Gibco, Paisley, UK) and recombinant mouse IL-3 produced in COS cells(26). For the generation of clonal Ba/F3 cells stably expressing myrPKB:ER*, the SRα-myrPKB:ER* construct was electroporated into Ba/F3 cells together with pSG5 conferring neomycin resistance and maintained in the presence of 1mg/ml G418 (Gibco, Paisley, UK) and IL-3. Clonal cell lines were generated by limited dilution. For cytokine withdrawal experiments, cells were washed twice with PBS and resuspended in AimV medium (Gibco, Paisley, UK).

Antibodies and reagents

Monoclonal antibodies against phospho-PKB (Ser473) and the polyclonal antibodies against Phospho (Ser/Thr) PKB substrate and phospho-Ezrin(Thr567)/Radixin(Thr564)/Moesin(Thr558) were from Cell Signaling Technologies (Hitchin, UK). Actin was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The phospho-Foxo3a (Thr32) antibody was from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Phospho-MAPK42/44(Thr202/Tyr204) and phosphorylated p38 (Thr180/Tyr182) were from New England Biolabs (Hitchin, UK). 4-hydroxytamoxifen (4-OHT) was purchased from Sigma (Seelze, Germany).

2D SDS-PAGE

Thr/Ser phosphorylated proteins were isolated with the PhosphoProtein Purification Kit from Qiagen (Hilden, Germany). Eluted proteins were desalted using PD-10 desalting columns (Amersham, Buckinghamshire, UK) and freeze dried and subsequently dissolved in electric focusing (IEF) buffer (8 M urea, 2 M Thiourea, 4% CHAPS, 20 mM DTT, 0.2% Biolythe, pH 3–10, and 0.2% bromophenolblue) and subjected to 2D electrophoresis by using Immobiline dry strips (pH 4–7; 11 cm) on an Ettan IPGphor system (GE Healthcare) for separation in the first dimension. Subsequently, separation in the second dimension was performed on 10% polyacrylamide gels by SDS-PAGE using a Sturdier Vertical Slab Gel Unit (Hoefer Scientific Instruments). The gels were transferred to PVDF membrane (Millipore, Bedford, MA) and probed with the respective antibodies. Immunocomplexes were detected using enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, UK) or subjected to silver staining by using standard techniques.

Mass spectrometry

The corresponding spot on the silver stained 2D gel was subjected to mass spectrometry. In-gel proteolytic digestion of the silver-stained spot using trypsin (Roche) was performed essentially as described previously (5). Samples were subjected to nanoflow liquid (LC) chromatography (Agilent 1100 series) and concentrated on a C18 precolumn (100 μM ID, 2 cm). Peptides were separated on an analytical column (100 μM ID, 20 cm) at a flow rate of 200 nl/min with a 60-min linear acetonitrile gradient from 0 to 80%. The LC system was directly coupled to a QTOF Micro tandem mass spectrometer (Micromass Waters). A survey scan was performed from 400–1,200 amu s–1, and precursor ions were sequenced in MS/MS mode at a threshold of 150 counts. Data were processed and subjected to database searches by using Proteinlynx Global Server version 2.1 (Micromass Waters) or MASCOT software (Matrixscience) against the SWISSPROT and the National Center for Biotechnology Information nonredundant database, with a 0.3 Da mass tolerance for both precursor ion and fragment ion.

RESULTS AND DISCUSSION

In order to identify novel PKB substrates, we made use of cytokine-dependent bone-marrow-derived Ba/F3 cells which are normally dependent on interleukin (IL-) 3 for their survival and proliferation. Stimulation of Ba/F3 cells with IL-3 results in the activation of multiple intracellular signal transduction pathways. To specifically study the role of PKB in phosphorylation events following cellular activation by IL-3, a Ba/F3 cell line stably expressing an inducible active PKBα (myrPKB:ER) was generated, as previously described in van Gorp et al (101). The activation of myrPKB:ER is, in the absence of 4-hydroxytamoxifen (4-OHT), inhibited by heat-shock and chaperone proteins that associate with the fused estrogen receptor (ER) hormone-binding domain. In the presence of 4-OHT these proteins dissociate

allowing PKB to become rapidly phosphorylated and activated (Fig. 1). myrPKB:ER* cells were cytokine starved and left unstimulated or stimulated with 4-OHT for 15 minutes. Phosphorylated proteins were separated by phospho-Ser/Thr affinity purification, analyzed by 2D SDS-PAGE and western blotting utilizing an antibody raised against the minimal PKB consensus phosphorylation site (RXRXXS/T) as described in Material and Methods (Fig. 2) (1). Hybridisation with this phospho-PKB substrate antibody resulted in the identification of several proteins whose phosphorylation was regulated upon specific PKB activation. Protein spots whose reactivity to the antibody was altered upon PKB activation were visually compared to the spots visible on a silver-stained duplicate of each gel. Proteins were eluted from the gels and their identity was established using tandem mass spectrometry (as described in Material and Methods). This phosphoproteomic approach to identify novel PKB substrates has been visualised in a schematic in figure 3.

The visual comparison of the spots with the silver-stained duplicate proved difficult since many proteins exhibited low expression levels and high reactivity with the antibody did not always correlate with expression level thus hindering identification of immunoreactive spots. Furthermore, the different treatment that the gels underwent during either the immuno blotting or silver-staining caused them to differ in size. Therefore, in many cases the identity of several adjacent protein spots was determined to increase the likelihood of identifying the correct immunoreactive protein. The identification of direct *in vivo* substrates of specific protein kinases is complicated by another issue: the existence of protein kinase cascades. Among the substrates of PKB are a variety of kinases including GSK-3, ASK1 and Raf1. Therefore, the changes seen in proteome phosphorylation upon PKB activation could be indirectly due to altered activity of other kinases. However, due to the relatively short stimulation with 4-OHT (15 minutes), we hoped to minimized these effects.

As mentioned previously, the minimum sequence motif required for efficient phosphorylation of small peptide substrates by PKB has been defined: PKB specifically phosphorylates Serine (Ser) or Threonine (Thr) residues in its substrates within the motif RxRxxS/T. To eliminate false positives, we analysed the proteins identified by mass spectrometry for the presence of this PKB phosphorylation site motif. Since throughout evolution the basic layout of signalling pathways appears conserved, especially the PI3K-PKB module, we therefore also investigated whether these motifs were evolutionary conserved. In this manner, we were able to identify six proteins with potential PKB phosphorylation sites (Table 2). These six proteins were analysed further by *in silico* analysis using Scansite 2.0 (77). Each sequence is represented as a position specific scoring matrix based on results from an oriented peptide library. Each identified motif is compared to the motif matrix of interest (in this case PKB) and scored with a percentile. Threshold values were assigned to decide which scores are likely to suggest real interactions. In a high stringency search only scores that fall within the top 0.2% when compared to all records are reported as a hit.

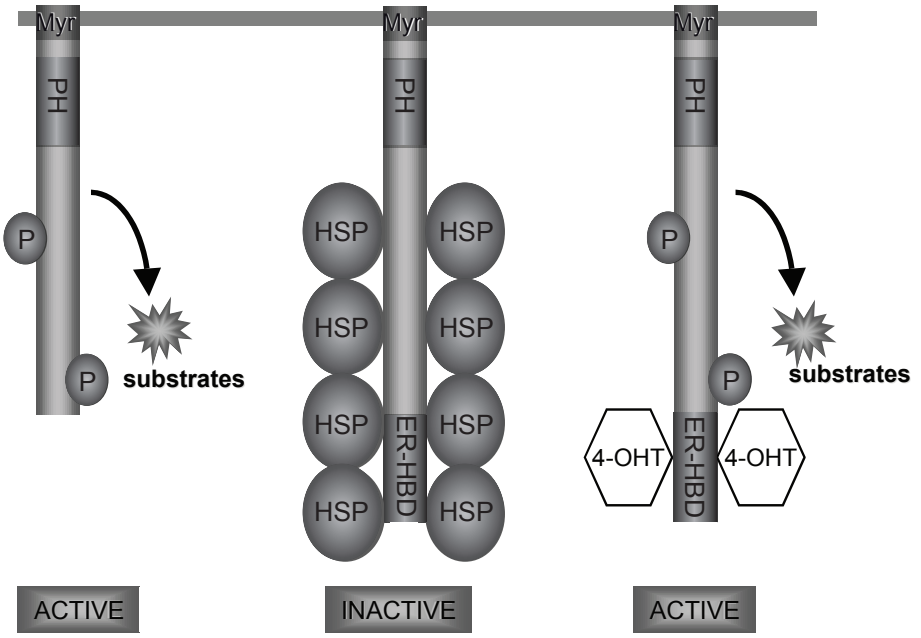

Figure 1: 4-OHT activates PKB in the myrPKB:ER* cells
The myristilation (myr) tail of myrPKB:ER heightens the affinity of the protein for the membrane. The activation of myrPKB:ER is, in the absence of 4-hydroxytamoxifen (4-OHT), inhibited by heat-shock and chaperone proteins that associate with the fused estrogen receptor (ER) hormone-binding domain (HBD). In the presence of 4-OHT these proteins dissociate allowing PKB to become rapidly phosphorylated and activated.*

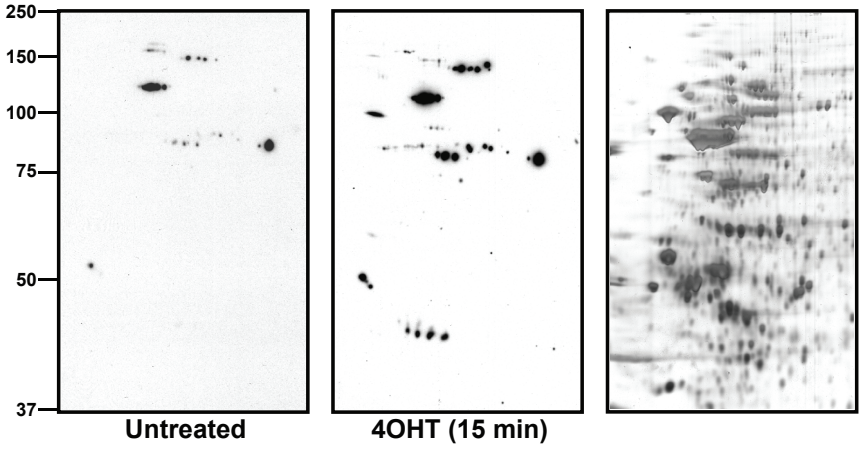

Figure 2: Detection of potentially novel PKB substrates
Ba/F3-myrPKB:ER cells were cytokine starved and left untreated (Left and Right Panel) or treated with 4-OHT (100nM) for 15 minutes (Middle Panel), lysed, Ser/Thr phosphorylated proteins were isolated and subjected to 2D electrophoresis. 2D gels were either blotted and analyzed with phospho (Ser/Thr) PKB substrate antibody (Left and Middle Panel) or silver stained for total protein (Right Panel).*

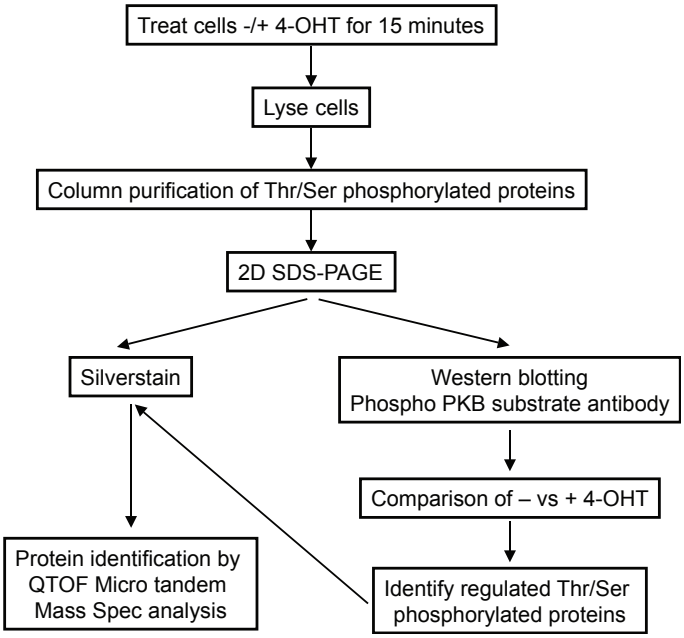

Figure 3: Schematic of phosphoproteomic identification of novel PKB substrates

These values were found to increase the reliability of prediction of true positive 'hits' while minimizing the number of false positives (77). As shown in Table 1 most confirmed *in vivo* PKB substrates fall in this high stringency category. Eukaryotic translation initiation factor 4B (eIF4B) and 5'-Phosphoribosyl N-formylglycinamide amidotransferase (FGARAT) have phosphorylation motifs identified through a high stringency search. Lamin B1 and MCM3/6 had potential PKB phosphorylation motifs however; these were based on a medium stringency screen.

eIF4B

Characterisation of eIF4B phosphorylation by PKB is described in Chapter 4.

FGARAT

FGARAT catalyzes the fourth reaction in the *de novo* synthesis of purines. Purines are biological essential molecules and defects in purine metabolism are associated with human diseases such as Lesch-Nyhan Syndrom, immune deficiencies, mental retardation and autism, sensorineural deafness, and gouty arthritis (4,83). Interestingly, *de novo* synthesis of purines is essential for DNA synthesis and thus proliferation, and purine synthesis has been shown to be deregulated in cancers (83) therefore altering the activity of an enzyme involved in purine synthesis by phosphorylation could contribute to the transforming potential of PKB.

Table 2: PKB substrates identified by phosphoproteomic analysis
Shown are the possible position(s) of PKB phosphorylation, conservation between human and mouse and Scansite 2.0 percentile and stringency.

Protein	Species	Position	Sequence	Scansite 2.0 Percentile/ Stringency
eIF4B	Human	406	RERHPS	0.163/High
	Mouse	406	RERHPS	0.163/High
	Human	420	RERSRT	0.053/High
	Mouse	420	RERSRT	0.053/High
	Human	422	RSRTGS	0.052/High
	Mouse	422	RSRTGS	0.059/High
FGARAT	Human	1151	RRRPDT	0.018/High
	Elegans	1159	RSRPDT	0.047/High
LaminB1	Human	302	RMRIES	0.214/Med
	Mouse	303	RMRIES	0.214/Med
Moesin	Human	558	RDKYKT	Not found
	Mouse	558	RDKYKT	Not found
	Human	384	RKRAQS	1.407/Low
	Mouse	384	RKRAQS	1.407/Low
MCM3	Human	694	RKRRKT	0.240/Med
	Mouse	694	RKRRKT	0.466/Med
MCM6	Human	568	IDRVYS	0.324/Med
	Mouse	568	IDRVYS	0.324/Med

MCM3 and MCM6

The DNA replication licensing system ensures that eukaryotic chromosomes replicate precisely once per cell cycle. Replication licensing requires the recruitment of the Minichromosome Maintenance proteins (MCM) at origins and takes place during late mitosis and the G1 phase, in preparation for the next round of chromosome duplication (8). MCMs are essential in all eukaryotes and are absolutely required for initiation of DNA replication (53,100). Depletion of each of these MCM proteins has demonstrated that they are essential for growth and specifically for the initiation and elongation of replication forks (64,110). MCM2-7 proteins can form a heterohexamer as well as other subcomplexes, they have conserved helicase motifs and exhibit DNA helicase and ATP hydrolysis activities *in vitro* (43,90). Importantly, none of the MCM proteins can substitute for the function of another. MCM proteins are thought to be activated during the G1 to S phase transition by conformational

changes coupled to chemical modifications. Genetic experiments indicate that phosphorylation of MCM2 by Cdc-Dbf4, the G1/S kinase, is a required step in this transition (33,69). The activated MCM complex appears to play a role in the DNA unwinding step, acting as a replicating helicase and moves along with the replication fork. Phosphorylation of the N-terminal domain of MCM4 by either Cdk1/cyclin B or Cdk2/cyclin A is associated with the inactivation of helicase activity of the MCM complex during the G2 and M phase of the cell cycle (44,45,57). Acetylation of MCM3 by MCM3 acetylase protein MCM3AP inhibits the initiation DNA replication (97). Interestingly, most studied MCM phosphorylation sites reside in the N-terminal domain of the proteins whereas the potential PKB phosphorylation site is located more C-terminal. Only ataxia-telangiectasia-mutated (ATM) protein kinase phosphorylates MCM3 at a more C-terminal location on Ser 535 in response to ionizing radiation unfortunately the function of this phosphorylation has yet to be elucidated. Since ATM functions as a replication checkpoint kinase, inhibition of DNA helicase activity upon DNA damaging ionizing radiation might be expected (20). Phosphorylation of MCM3 and MCM6 by PKB, however, would most likely be an activating signal, since PKB activation itself has been observed to have a positive effect on proliferation. MCM phosphorylation could therefore be a way for PKB to control normal growth and development.

Lamin B1

Lamins are intermediate filament proteins expressed in the nuclei of multicellular eukaryotes. Like other intermediate filament proteins, lamins consist of an N-terminal head domain, a central coiled-coil (rod) domain responsible for dimerization, and a large globular C-terminal tail. Lamins are important for nuclear architecture; they provide mechanical strength, determine nuclear shape, and anchor and space the nuclear pore complexes. Lamins are also found at sites of DNA replication and reprocessing, and in association with replication proteins and RNA polymerases (34). Mammalian cells express two types of lamins, the A and B types. Lamins A and C, the major A-type lamins, are alternatively spliced isoforms of a single gene, LMNA. B-type lamins (lamins B1 and B2) are encoded by separate genes, LMNB1 and LMNB2, respectively.

Currently there are no diseases associated with mutations or loss of LNMB1 or LMNB2 alleles. It is assumed that mutations in these genes are lethal, since loss of either lamin B1 or lamin B2 is lethal to dividing cells (36), which is consistent with fundamental roles in replication and transcription. A-type lamins are expressed mainly in differentiated tissues. In contrast >150 mutations in LMNA have been linked to various inherited diseases called the 'laminopathies' (41,109).

Lamin phosphorylation is a key event in the mitotic breakdown of the nuclear lamina (67). In cells undergoing mitosis, cyclin dependent kinases (CDKs) mediate phosphorylation of lamins A, B, and C. This event follows the interphase phosphorylation of lamins by protein kinase C (PKC) (13). Interphase

phosphorylation of lamins A and C has been reported following insulin treatment of quiescent fibroblasts (32). Although Ser302 of LaminB1 was identified in a medium stringency search, it is a highly conserved site among the lamins. Table 3 shows that in Lamin A, C and B2, a serine in this region is identified in a high stringency Scansite 2.0 search. This residue is not known as a substrate for PKC or CDKs and is located in the central coiled-coil domain. Phosphorylation of this domain has been found during mitosis and is thought to be involved in the regulation of lamin assembly and disassembly (38). Therefore, by phosphorylation of Lamin B1, PKB could play a role in regulating nuclear lamina structural dynamics during cell cycle.

Table 3: Proteins related to the selected possible novel PKB substrates in Table 2

Shown are the possible position(s) of PKB phosphorylation, conservation between human and mouse and Scansite 2.0 percentile and stringency.

Protein	Species	Position	Sequence	Scansite 2.0 Percentile/ Stringency
LaminA	Human	301	RIRIDS	0.174/High
	Mouse	301	RIRIDS	0.174/High
	Human	404	RGRASS	0.128/High
	Mouse	404	RGRASS	0.128/High
LaminC	Human	301	RIRIDS	0.174/High
	Mouse	301	RIRIDS	0.174/High
	Human	404	RGRASS	0.128/High
	Mouse	404	RGRASS	0.128/High
LaminB2	Human	294	RMRLES	0.063/High
	Mouse	296	RMRVES	0.082/High
Ezrin	Human	567	RDKYKT	4.333/Low
	Mouse	567	RDKYKT	4.333/Low
Radixin	Human	564	RDKYKT	Not found
	Mouse	564	RDKYKT	Not found

Moesin

Moesin is a member of the Ezrin-Radixin-Moesin (ERM) protein family. These proteins share a high sequence homology (70%) and a common structure. They contain binding sites for membrane adhesion molecules on the N terminus and actin binding sites on the C terminus and, thus, function as membrane-cytoskeletal linkers. Activity of these proteins is regulated by an intramolecular interaction between the 4.1 ERM domain and the C-terminal domain. Phosphorylation of a

conserved threonine (Ezrin Thr567, Radixin Thr564 and Moesin Thr558) in the actin-binding domain of ERM proteins has been demonstrated to be important for their activation by relieving the head to tail interaction (11,46).
The consensus PKB phosphorylation motif around Ser384 in moesin was only identified in a low stringency search. However since Thr567 in ezrin had been reported as a PKB phosphorylation site (Table 3), the homologous residue in moesin Thr558 was investigated further as a potentially novel PKB phosphorylation site. BaF3 myrPKB:ER cells were stimulated with 4-OHT for up to an hour resulting in activation of PKB and phosphorylated of Foxo3a (Fig 4). PKB activation resulted

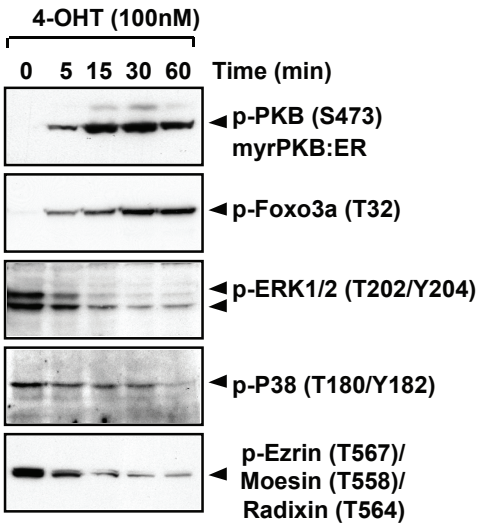

Figure 4: PKB activation decreases Moesin phosphorylation
Ba/F3-myrPKB:ER cells were cytokine starved and left untreated or treated with 4-OHT (100nM) for the indicated times, lysed and equal amounts of protein were analyzed for levels of phospho-PKB (S473), phospho-Foxo3a (T32), phospho-ERK1/2 (T202/Y204) , phospho-P38 (T180/Y182), phospho-Ezrin (T567)/Moesin (T558)/Radixin (T564).*

in a decrease of basal ERK1/2 and p38 phosphorylation, most likely caused by the direct phosphorylation and inactivation of MAP kinase kinases Raf1 and ASK1. Interestingly, phosphorylation of ERM family members was also decreased upon PKB activation. Taking together, these results indicate that in Ba/F3 cells the ERM family members appear not to be phosphorylated by PKB on this previously reported residue. The fact that dephosphorylation of the ERM family members coincides with the dephosphorylation of ERK1/2 and p38 MAPK suggests a potential role for MAP kinases regulating ERM phosphorylation. In contrast to our data, Lan et al. found ezrin phosphorylation at Thr567 increased upon p38 MAPK inhibition, whereas MEK inhibition had no effect (65). However, Koss and co-workers found decreased ERM phosphorylation upon TNFα stimulation after p38 MAPK inhibition (60). Taken together, it appears that the Thr558 residue in moesin and the homologues residues in ezrin and radixin are not true PKB substrates. However, the Ser384 residue in moesin was not investigated in this study and although its low stringency status makes it an unlikely PKB substrate, this residue is still a possible PKB substrate.

Recently, several interesting novel approaches have been described for analysis of the phosphoproteome. Olsen and co-workers described a global *in vivo* analysis of the phosphoproteome and its temporal dynamics upon growth factor stimulation (78). In this method different populations of the same cells were labelled by stable isotope labelling by amino acids in cell culture (SILAC) method (79) and stimulated for different times. Subsequently the cells were mixed, proteins were extracted and enzymatically digested. The phosphopeptides present in the resulting peptide pool were enriched on TiO_2-beads (66) and subsequently analyzed and quantified by mass spectrometry. This method has a couple of major advantages: mixing the cells ensures that all samples are treated the same. The isotope labelling makes it possible to distinguish between the differentially treated fractions in the mass spectra of the peptides. Also when short stimulation times are used, the total amount of protein is not expected to change and therefore non-phosphorylated peptides can be used to determine quantification errors. Such advances in analysis of the phosphoproteome will be crucial in increasing our understanding of intracellular signaling *in vivo* and may result in the re-evaluation of many previously described PKB substrates.

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CHAPTER 4

AGC kinases control phosphorylation and activation of eukaryotic translation initiation factor 4B

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Submitted for publication


ABSTRACT

Eukaryotic translation initiation factor 4B (eIF4B) plays a critical role in stimulating the helicase activity of eIF4A to unwind inhibitory secondary structures in mRNAs and recruit the 40S ribosomal subunit to those mRNAs. eIF4B is a multiphosphorylated protein and phosphorylation can control its functional activity. In a screen to identify novel Protein Kinase B (PKB/c-akt) substrates, we identified eIF4B as a potential target. PKB was found to phosphorylate eIF4B on Ser422 *in vitro* and *in vivo* after mitogen-stimulation. Furthermore, we identified Ser406 as a novel mitogen-regulated phosphorylation site. Phosphorylation of Ser406 was found to be regulated by p90 ribosomal S6 kinase (RSK) *in vivo*. Utilising a translational control reporter system (TCRS), phosphorylation of both residues was found to be physiologically relevant in regulating the translational activity of eIF4B. These data provide novel insight into complex multi-kinase regulation of eIF4B phosphorylation and reveal an important mechanism by which PKB can regulate translation, potentially critical for the transforming capacity of this AGC kinase family member.

INTRODUCTION

Regulation of protein translation is crucial for the specific expression of proteins important for development, differentiation, cell growth and apoptosis (13,21). The ability of cells to regulate this process allows a rapid response to external stimuli without the necessity of mRNA synthesis, processing and transport. In transformed cells, components of the translation machinery are often deregulated or misexpressed and changes in the nucleolus; the suborganelle of the nucleus which functions as the centre of ribosome biogenesis, have long been recognized as a reliable marker of cellular transformation (7,29).

Translational control mostly occurs at the level of initiation. The initiation phase of translation is regulated by a number of eukaryotic translation initiation factors (eIFs) (9,12). Initially, eIF4E binds to the cap structure at the 5' end of the mRNA. eIF4E is part of a trimeric complex, termed eIF4F, together with scaffolding protein eIF4G and ATPase/RNA helicase eIF4A. eIF4A unwinds the secondary structure of the 5'UTR allowing the 40S ribosomal subunit to bind to the mRNA. The helicase activity of eIF4A is significantly increased by the co-factor eIF4B (18,35,36). eIF4B itself has three functional domains, namely two mRNA binding domains (22,25) and a DRYG domain necessary for dimerization and binding to eIF3 (24). The two RNA binding domains have distinct affinities for RNA, the argenine rich motif (ARM) binds mRNA with higher affinity and is essential for RNA helicase activity (22). The RNA recognition motif (RRM) binds with high affinity to 18S rRNA (23). Therefore, besides being a co-factor for eIF4A, eIF4B is thought to exhibit a bridge function between mRNA and rRNA (22).

Translational control is intimately connected to the regulation of intracellular signal transduction pathways. Phosphorylation of initiation factors provides an important means to control the rate of mRNA binding (32). The phosphorylation state of eIF4E, eIF4G, eIF4B and eIF3 positively correlates with both translation and growth rates of the cell. Changes in phosphorylation, and thus translation, occur in response to a wide variety of extracellular signals including, viral infection, heat-shock and in response to cellular growth factors and cytokines (12,21). Global changes in protein synthesis after these events are relatively small but a subgroup of mRNAs exhibits a dramatic change in their rate of translation. Rajasekhar *et al.* recently demonstrated that upon Protein Kinase B (PKB/c-akt) and RAS signalling the profile of mRNA associated to polysomes was drastically altered, although the underlying mechanism remains unclear (31). Interestingly, these mRNAs mainly encoded proteins involved in the regulation of growth, transcription, cell-cell interactions and morphology. Thus, by controlling translation efficiency, general stimuli, such as growth factors and cytokines, can selectively induce or suppress the translation of specific set of genes and deregulation of these cellular mechanisms controlling translation can lead to cellular transformation (14).

Mammalian target of rapamycin (mTOR) plays a major role in the regulation of global and specific mRNA translation. mTOR is activated by phosphatidylinositol

3-kinase (PI3K) through PKB either by direct phosphorylation (26), or by phosphorylation of TSC2 which inactivates its GAP activity for the small G protein Rheb, a potent activator of mTOR (16). The best-studied downstream targets of mTOR activation are those involved in translation regulation, namely p70 S6 kinase (p70S6K) and eIF4E-binding proteins, (4E-BPs). p70S6K phosphorylates ribosomal protein S6, whose hyperphosphorylation status correlates with translation activity. The phosphorylation of the inhibitory 4E-BPs is required for their release of the proto-oncogene eIF4E resulting in increased cap-dependent translation (34,37). De-regulation of activation of the phosphatidylinositol 3-kinase (PI3K) pathway is found in a large variety of human cancers (20) and importantly, inhibition of translation by a specific mTOR inhibitor, rapamycin, can effectively block transformation initiated by perturbed PI3K signalling (10). This indicates that PI3K/PKB/mTOR mediated regulation of translational control is crucial for maintenance of neoplasia.

eIF4B has long been known as a hyperphosphorylated protein (5), and eIF4B phosphorylation is responsive to extracellular stimuli including serum, insulin and phorbol esters (6). It had however remained elusive which kinase(s) are responsible for the phosphorylation of eIF4B, however, recently, two reports have been published concerning the regulation of phosphorylation a specific serine residue (Ser422). Raught *et al.* (33) implicated p70S6K as the specific Ser422 kinase but subsequently Shahbazian and co-workers (41) proposed that p70S6K and p90 S6 kinase (RSK) were both able to phosphorylate this residue. Both p70S6K and RSK are members of the AGC protein kinase family, which also contains PKB (30). This kinase family is defined by the high homology within their catalytic domains, resulting in similar substrate consensus sequences. The activity of these kinases, however, is differentially regulated, whereas PKB and p70S6K are components of the PI3K-mTOR pathway, RSK is activated by signalling through the small GTPase RAS.

Recent evidence that long-term rapamycin treatment can inhibit PKB activity (39) made us re-examine the importance of mTOR signalling versus PKB signalling in the regulation of translation initiation. In this study, we show that PKB *in vitro* and *in vivo* can phosphorylate eIF4B within the RNA-binding domain at serine (Ser422). We demonstrate that PKB is the dominant AGC protein kinase family member phosphorylating Ser422 upon insulin stimulation *in vivo*. We also demonstrate regulation of a novel phosphorylation site (Ser406) and show that phosphorylation of this residue is regulated by RSK *in vivo*. Furthermore, we demonstrated that a non-phosphorylatable form of eIF4B enhances recognition of a uORF thus modulating translation. These data provide novel insight into the complex regulation of eIF4B phosphorylation *in vivo*. Furthermore, we demonstrate for the first time that eIF4B phosphorylation is a novel mechanism by which PKB can regulate protein translation and may be critical for the transforming potential of this AGC kinase family member.

MATERIAL AND METHODS

Cell culture

Ba/F3 cells were cultured in RPMI 1640 medium with 8% Hyclone serum (Gibco, Paisley, UK) and recombinant mouse IL-3 produced in COS cells(4). For the generation of clonal Ba/F3 cells stably expressing myrPKB:ER*, the SRα-myrPKB:ER* construct was electroporated into Ba/F3 cells together with pSG5 conferring neomycin resistance and maintained in the presence of 1mg/ml G418 (Gibco, Paisley, UK) and IL-3. Clonal cell lines were generated by limited dilution. For cytokine withdrawal experiments, cells were washed twice with PBS and resuspended in AimV medium (Gibco, Paisley, UK). A14 cells and COS cells were cultured in Dulbecco's modified Eagles Medium (Gibco, Paisley, UK) with 8% FCS (Gibco, Paisley, UK). A14 cells are NIH 3T3 derived cells that overexpress the insulin receptor (1). A14 cells were serum starved in DMEM supplemented with 0.1% FCS.

Antibodies and reagents

Monoclonal antibodies against phospho-PKB (Ser473) and the polyclonal antibodies against Phospho-eIF4B (Ser422) and Phospho (Ser/Thr) PKB substrate antibodies were from Cell Signaling Technologies (Hitchin, UK). Actin was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The phospho-Foxo3a (Thr32) and Phospho-Foxo3a (Ser253) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Phospho-MAPK42/44(Thr202/Tyr204) and Phospho S6 (S235/S236) were from New England Biolabs (Hitchin, UK). The Anti-FLAG M2 monoclonal antibody peroxidase conjugate was purchased from Sigma (Seelze, Germany). 4-hydroxytamoxifen (4-OHT) and insulin were purchased from Sigma (Seelze, Germany). LY294002, U0126 and rapamycin were obtained from Biomol International LP (Hamburg, Germany)

Western blotting

A14 cells were lysed in 1x sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue) and boiled for 5 minutes. BaF3 cells were lysed in laemmli buffer (0.12M Tris HCL pH 6.8, 4% SDS, 20% Glycerol, 0.05 μg/μl bromophenol blue, and 35mM β-mercaptoethanol), boiled for 5 minutes and the protein concentration was determined. Equal amounts of sample were analyzed by SDS PAGE, electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA) and probed with the respective antibodies. Immunocomplexes were detected using enhanced chemiluminescence (ECL, Amersham, Buckinghamshire, UK).

Immunoprecipitation

For the immunoprecipitation assays either COS or A14 cells (9 cm dishes) were transfected with a total of 10 μg of plasmid DNA by the calcium phosphate or

Polyethylene imine (PEI) precipitation method. The following morning the cells were washed with PBS and fresh medium was added to the cells. For serum starvation cells were again washed with PBS at the end of the day and DMEM containing 0.1% FCS was added to the cells. After another 24 hours of growth cells were stimulated as indicated and lysed in RIPA lysis buffer (20 mM Tris pH 7.8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodiumdeoxycholin, 5mM EDTA, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). Lysates were centrifuged at maximum speed for 10 minutes to remove DNA and cellular debris. A part of the lysate was taken as a control for stimulations, 5x sample buffer was added to a final concentration of 1x (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue) and boiled for 5 minutes. The rest of the lysate was incubated for at least 2 hours with FLAG M2 agarose beads from Sigma (Seelze, Germany) at 4°C, subsequently beads were washed four times with RIPA lysis buffer and boiled in 1x sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue).

Kinase assay

After immunoprecipitation and washing, kinase buffer (20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP) and 200 ng of active PKBα (Upstate Biotechnology Inc., Lake Placid, NY, USA) was added to the FLAG M2 agarose beads and incubated at 37°C for 30 minutes. After incubation, 5x sample buffer was added to a final concentration of 1x (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue) and boiled for 5 minutes.

Tandem Mass spectrometry

Immunoprecipitated eIF4B was digested with Trypsin (Roche) and enriched for phosphorylated peptides using a ~5mm length TiO_2 microcolumn, packed in GE-Loader tip with a 3M Empore C8 plug from an extraction disc, essentially as described (17). Peptides were loaded onto this column in buffer A (80% acetonitrile, 0.1% trifluoric acid)/ 200g/l DHB(2,5-dihydroxibenzoic acid). Columns were washed once in bufferA/DHB followed by a wash in buffer A. The bound peptides were eluted with 20μl 1% ammonia in 5μl 10% Formic acid. Samples were directly subjected to nanoflow liquid (LC) chromatography (Agilent 1100 series) and concentrated on a C18 precolumn (100um ID, 2cm). Peptides were separated on an aqua™ C18 reversed phase column (kind gift of Prof. A. Heck, dimensions; 75μM ID, 20 cm) at a flow rate of 200nl/min with a 60 min. linear acetonitrile gradient from 0 to 90%. The LC system was directly coupled to a QTOF Micro tandem mass spectrometer (Micromass Waters, UK). A survey scan was performed from 400-1200 amu s^{-1} and precursor ions were sequenced in MS/MS mode at a threshold of 150 counts. Data were processed and subjected to database searches using MASCOT software (Matrixscience) against SWISSPROT and the NCBI non-redundant database, allowing for the detection of phosphorylation

residues, with a 0.25 Da mass tolerance for both precursor ion and fragment ion. The identified peptides were confirmed by manual interpretation of the spectra.

Translational control reporter system

A14 cells were transfected with 5μg of FLAG-tagged wild type or mutant eIF4B together with 5μg of TCRS vector (43). The following morning the cells were washed with PBS and fresh medium was added to the cells. At the end of the day the cells were again washed with PBS and DMEM containing 0.1% FCS and insulin were added to the cells. After 24 hours cells were lysed in 1x sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue). Equal amounts of sample were analyzed by SDS PAGE, electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA) and probed with the respective antibodies.

RESULTS

Identification of eIF4B as a PKB substrate

To identify novel PKB substrates, we made use of cytokine-dependent bone-marrow-derived Ba/F3 cells which are normally dependent on interleukin (IL-) 3 for their survival and proliferation. To specifically study the role of PKB in phosphorylation events following cellular activation by IL-3, a Ba/F3 cell line stably expressing an inducible active PKBα (myrPKB:ER) was made, as previously described in van Gorp *et al.* (42). The activation of myrPKB:ER is, in the absence of 4-hydroxytamoxifen (4-OHT), inhibited by heat-shock and chaperone proteins that associate with the fused estrogen receptor (ER) hormone-binding domain. In the presence of 4-OHT these proteins dissociate allowing PKB to become rapidly phosphorylated and activated.

myrPKB:ER cells were cytokine starved and the phosphorylation patterns of unstimulated cells were compared to those stimulated with 4-OHT for 15 minutes. Phosphorylated proteins were separated by phospho-Ser/Thr affinity purification, analyzed by 2D gel electrophoreses and western blotting utilizing an antibody raised against the minimal PKB consensus phosphorylation site (RXRXXS/T) (27,44). We observed several proteins whose phosphorylation was upregulated upon PKB activation. Tandem mass spectrometry identified one of these proteins as eIF4B (data not shown).

To identify potential PKB phosphorylation sites in eIF4B, we performed *in silico* analysis using Scansite 2.0 (28). A high stringency analysis of eIF4B identified two serines likely to be phosphorylated by PKB, serines 406 (Ser406) and 422 (Ser422) in the ARM region (Fig. 1A).

Due to the availability of a specific antibody raised against the phosphorylated Ser422 on eIF4B, we analyzed whether PKB activation resulted in phosphorylation of this residue. COS cells were transfected with FLAG-tagged eIF4B, or eIF4B in which Ser422 had been mutated to alanine. eIF4B was immunoprecipitated and incubated

with active PKB. PKB was indeed able to directly phosphorylate eIF4B at Ser422 (Fig. 1B, compare lane 1 to 3) and mutation of this site abolished phosphorylation (Fig. 1B, lanes 2 and 4). Importantly, analysis of phosphorylated residues on eIF4B after incubation with PKB by mass spectrometry clearly demonstrated Ser422 as the primary phosphorylated site (Fig. 1C). Taken together, these data indicate that phosphorylation of Ser422 is the primary phospho-acceptor site in eIF4B that can be regulated by PKB.

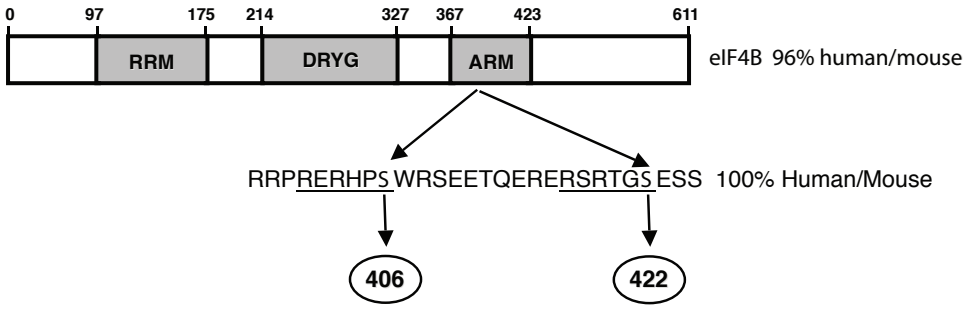
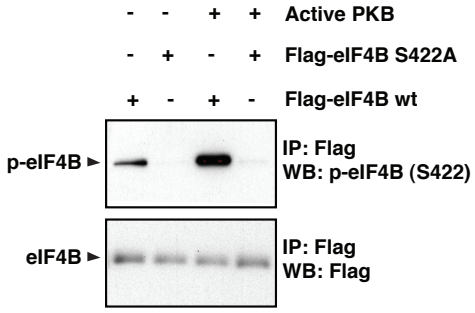
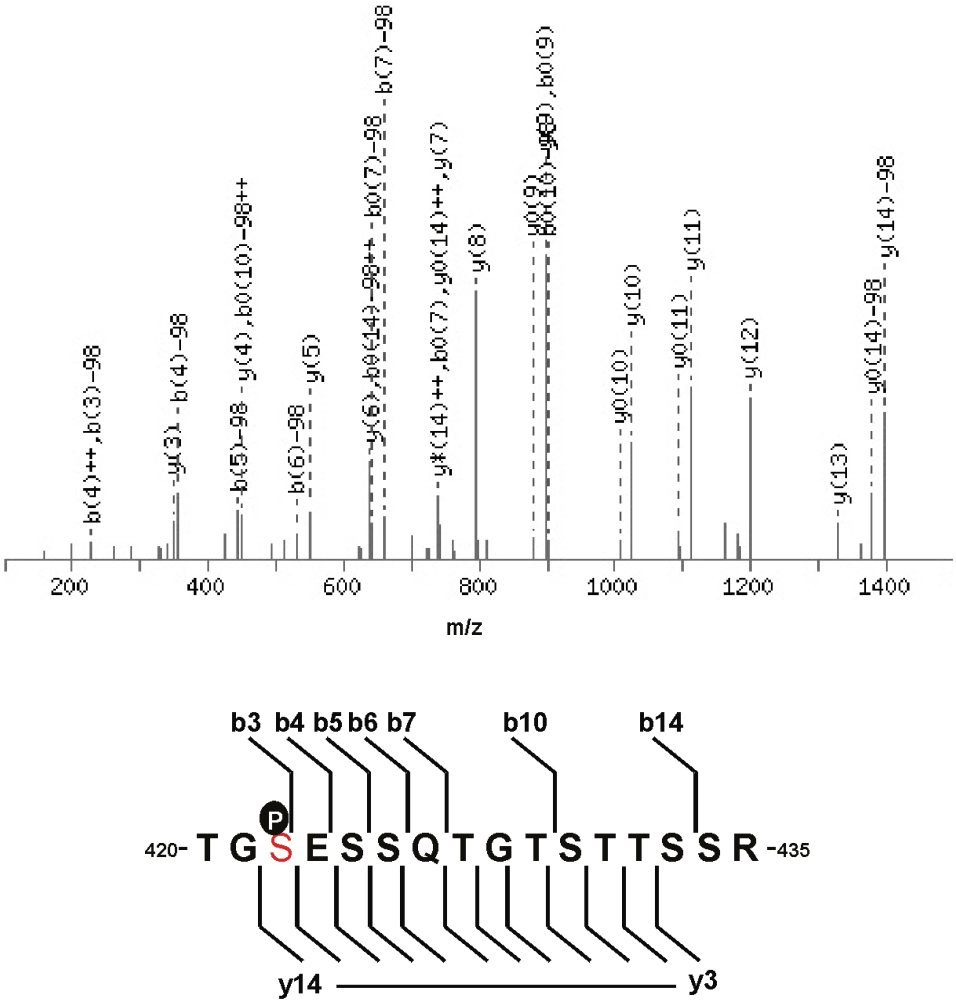


Figure 1. PKB phosphorylates eIF4B on Ser422 in vitro

(A) *Schematic representation of the eIF4B protein. The three functional domains of eIF4B, the RRM, DRYG domain and the ARM are shown. Also shown are the two serines (Ser406 and Ser422, bold and italic) within their PKB phosphorylation consensus sequence (underlined) that were identified by in silico analysis by Scansite 2.0. Human and mouse eIF4B are 96% homologous. The region in which the two serines are localized is 100% conserved between these two species.*



(1 B) *FLAG-tagged eIF4B or FLAG-tagged eIF4B in which Ser422 was mutated to alanine was phosphorylated by PKB in an in vitro kinase assay. Proteins were incubated without active PKB present as a control. Samples were analyzed for levels of phospho-eIF4B (S422) and FLAG.*



(1 C) *Identification of the PKB phosphorylated Ser422 on eIF4B by mass spectrometry. Flag-tagged eIF4B protein was expressed in COS cells and immunoprecipitated protein was phosphorylated in vitro by PKB, separated on SDS-PAGE and trypsin digested. The resulting peptides were seperated utilising TiO_2 phosphopeptide-enrichment columns and subjected to tandem mass spectrometry (LC-MS/MS). MS/MS spectrum (top) and sequence (bottom) of the Ser422 phosphorylated peptide of eIF4B (AA 420-435) as identified by MASCOT software (See experimental procedures). Identified b and y ions are indicated. The phosphorylated serine (Ser422) is indicated in grey.*

Role of PKB in the phosphorylation of eIF4B on Ser422 in vivo

To investigate the *in vivo* phosphorylation status of eIF4B on Ser422, we again made use of the BaF3 myrPKB:ER cell line. BaF3 myrPKB:ER cells were cytokine starved overnight and stimulated with either IL-3 or 4-OHT for the times indicated. Both stimulation with IL-3 and 4-OHT induced phosphorylation of eIF4B on Ser422

as well as that of the PKB substrate forkhead transcription factor Foxo3a (3) and the p70S6K substrate ribosomal protein S6 (Fig 2A). Since it has been previously

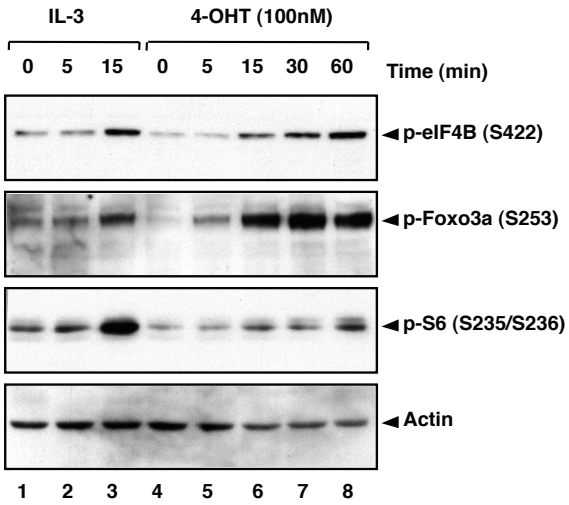
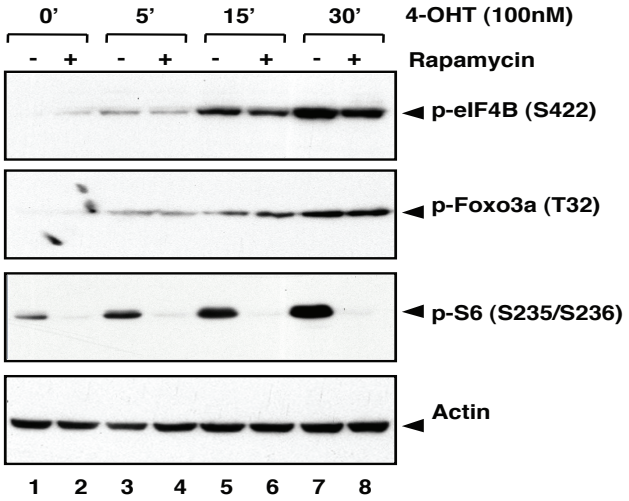


Figure 2. PKB activation sufficient and necessary for eIF4B phosphorylation of Ser422 in Ba/F3 myrPKB:ER cells

(A) *Ba/F3-myrPKB:ER* cells were cytokine starved and left untreated or treated with either IL-3 or 4-OHT (100nM) for the indicated times, lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phopho-Foxo3a (S253), phospho-S6 (S235/S236) and actin.*

demonstrated by Raught *et al.* that phosphorylation of eIF4B on Ser422 can be mediated by p70S6K activity (33), we investigated whether inhibition of its upstream activator, the mTOR/Raptor complex by pre-incubation of the cells with rapamycin, could abolish phosphorylation on this site. Inhibition of the mTOR/Raptor-p70S6K pathway by rapamycin completely inhibited phosphorylation of p70S6K target S6 but not the phosphorylation of Foxo3a (Fig. 2B). However, eIF4B phosphorylation was only very modestly reduced by pre-treating cells with rapamycin indicating that p70S6K is not responsible for PKB-mediated eIF4B Ser422 phosphorylation (Fig. 2B). Previously, Shahbazian and co-workers proposed that p70S6K and RSK can synergistically regulate eIF4B Ser422 phosphorylation in Hela cells when stimulated with serum (41). We wished to determine whether eIF4B Ser422 phosphorylation was similarly regulated in Ba/F3 cells when PKB was specifically activated by addition of 4-OHT. Ba/F3 myrPKB:ER cells were cytokine starved overnight and the phosphorylation status of eIF4B at Ser422 was compared after PKB activation when cells were pre-incubated with either rapamycin, PI3K inhibitor LY294002, MEK inhibitor U0126 or combinations of these inhibitors. Pre-incubation with rapamycin abrogated phosphorylation of S6 but again only very modestly inhibited eIF4B Ser422 phosphorylation (Fig. 2C, lane 3), whereas LY294002

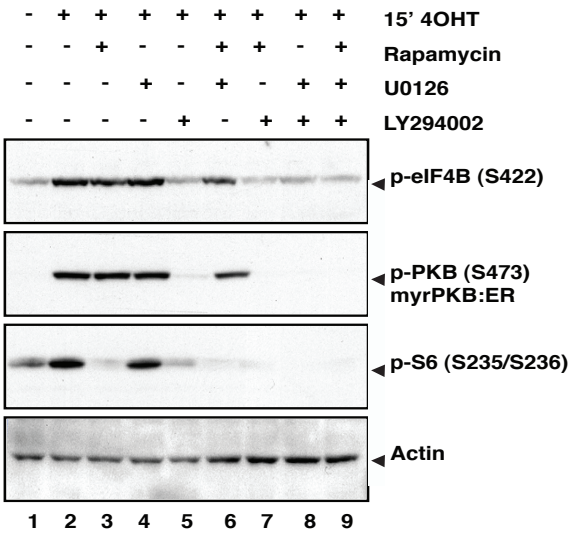


(***2 B)*** *Ba/F3-myrPKB:ER* cells were cytokine-starved and left untreated or treated with 4-OHT for the indicated times after or without pre-treatment with rapamycin (20 ng/ml), lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phopho-Foxo3a (T32), phospho-S6 (S235/S236) and actin.*

completely abrogated this (Fig. 2C, lane 5). LY294002 also inhibited phosphorylation of both PKB and S6 (Fig. 2C, lane 5), indicating that the myrPKB:ER protein is still dependent on basal PI3K activity. Pre-treatment of cells with U0126 had no effect on the phosphorylation of eIF4B at Ser422 in response to 4-OHT (Fig. 2C, lane 4) even when combined with rapamycin (Fig. 2C, lane 6). Taken together, these data indicate that PKB is directly responsible for eIF4B phosphorylation at Ser422 and this is not dependent on either p70S6K or MEK activity.

In vivo phosphorylation of eIF4B in response to insulin

PKBβ null mutant mice have been shown to be defective in their insulin response and suffer from diabetes (8). This crucial role that PKB plays in mediating the effects of insulin, led us to investigate the role of PKB in regulating insulin-stimulated eIF4B phosphorylation. A14 cells were serum starved overnight and the phosphorylation status of eIF4B at Ser422 was compared after stimulation with insulin when cells were pre-incubated with rapamycin, LY294002 or U0126. eIF4B Ser422 phosphorylation was increased after insulin stimulation (Fig. 3A, lane 2) and pre-treatment with either rapamycin or U0126 had no effect on this phosphorylation (Fig. 3A, lane 3 and 4). However, LY294002 was able to significantly decrease the phosphorylation on Ser422 (Fig. 3A, lane 5). A combination of rapamycin and U0126 did not result in inhibition of Ser422 phosphorylation (Fig. 3B, lane 6) in contrast to pre-treatment with LY294002 (Fig. 3B, lane 4). A combination of all three inhibitors completely abolished the phosphorylation of eIF4B at Ser422 in



(***2C)*** *Ba/F3-myrPKB:ER* cells were cytokine-starved and left untreated or treated with 4-OHT for 15 minutes after or without pre-treatment with rapamycin (20 ng/ml), U0126 (15 μM), LY294002 (50 μM) or combinations of these inhibiters, lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-S6 (S235/S236) and actin.*

response to insulin (Fig. 3B, lane 8). Taken together, these data indicate that insulin utilizes PKB and not p70S6K and RSK to regulate phosphorylation of eIF4B at Ser422.

To determine whether PKB activity is an absolute requirement for eIF4B Ser422 phosphorylation, A14 cells were transfected with myc-tagged dominant-negative PKB (mycPKBcaax) together with FLAG-tagged eIF4B. Cells were serum-starved overnight and subsequently stimulated with insulin before immunoprecipitating the FLAG-tagged protein. Insulin stimulated robust phosphorylation of Ser422 and inhibition of PKB abrogated this (Fig. 3C). This clearly indicates that PKB activity is indeed required for insulin induced phosphorylation of eIF4B on Ser422.

Regulation of phosphorylation of eIF4B at Ser406 in response to insulin

To determine whether eIF4B could additionally be phosphorylated by PKB on Ser406, we generated a FLAG-tagged eIF4B in which this residue had been mutated to alanine. A14 cells were transfected with wild type or mutant eIF4B, serum starved overnight and subsequently stimulated with insulin before immunoprecipitating the FLAG-tagged protein. Insulin stimulation resulted in phosphorylation of eIF4B as detected by both the phospho-eIF4B Ser422 antibody as well as the phospho-PKB substrate antibody (Fig. 4A, lanes 1 and 2). However, when Ser406 was mutated to an alanine this abolished reactivity with the phospho-PKB substrate antibody (Fig.

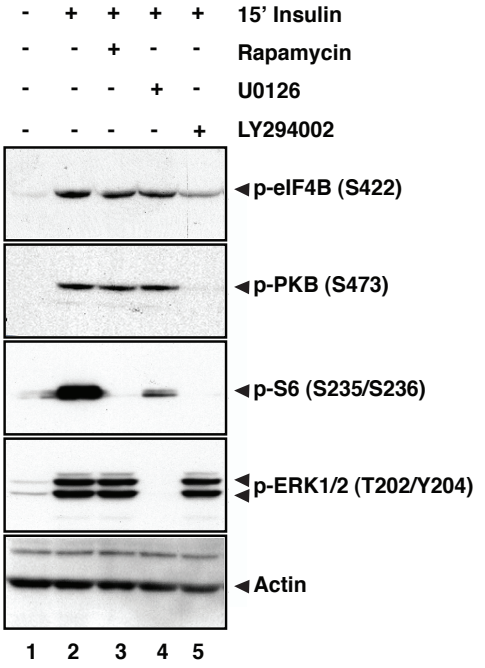
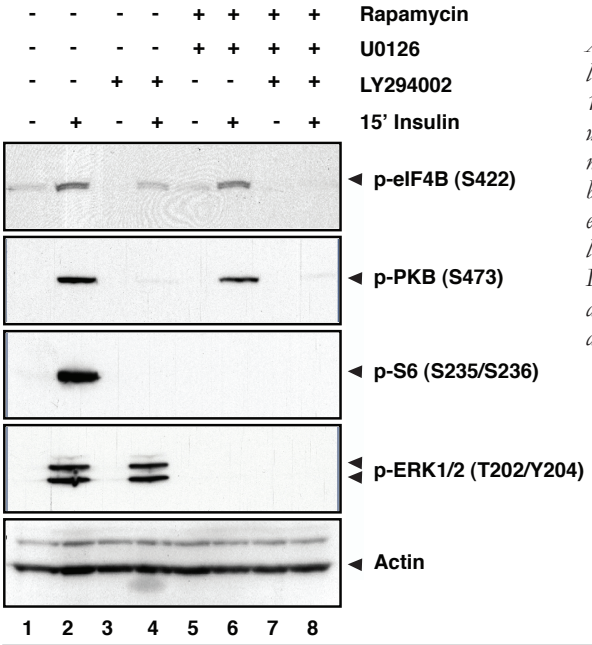
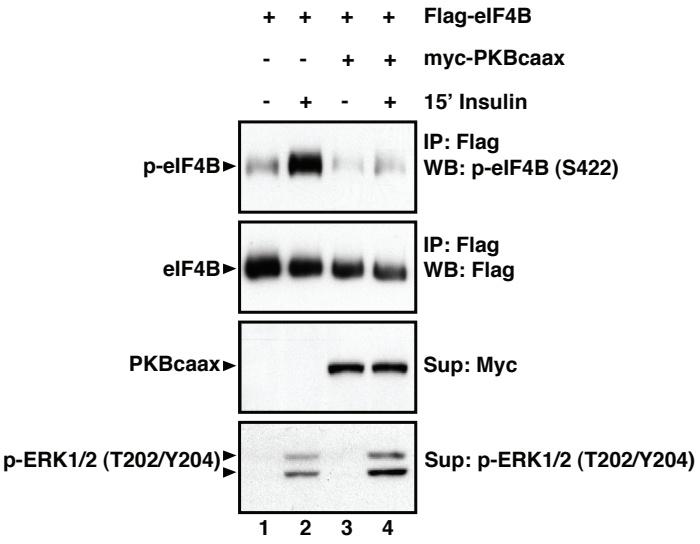


Figure 3 PKB activation sufficient for eIF4B phosphorylation of Ser422 after insulin stimulation of A14 cells.
(A) *A14 cells were serum starved overnight and left untreated or treated with insulin for 15 minutes after or without pre-treatment with rapamycin (20 ng/ml), U0126 (15 μM) or LY294002 (50 μM), lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-S6 (S235/S236) and phospho-ERK1/2 (T202/Y204) and actin.*



(3B)
A14 cells were serum starved overnight and left untreated or treated with insulin for 15 minutes after or without pre-treatment with LY294002 (50 μM), rapamycin (20 ng/ml) and U0126 (15 μM) or all of the before mentioned inhibitors together, lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-S6 (S235/S236) and phospho-ERK1/2 (T202/Y204) and actin.



(3C) *A14 cells were transfected with myc-tagged dominant-negative PKB (mycPKBcaax) together with FLAG-tagged eIF4B. Cells were serum-starved overnight and subsequently stimulated for 15 minutes with insulin before immunoprecipitating the FLAG-tagged protein. The immunoprecipitated FLAG-eIF4B protein was analyzed for levels of phospho-eIF4B (S422) and FLAG as a loading control. The whole cell lysate was analyzed for levels of mycPKBcaax using a myc antibody and phospho-ERK1/2 (T202/Y204) as a control for insulin stimulation.*

4A, lanes 3 and 4), while there was no effect on Ser422 phosphorylation. This demonstrates that the phospho-PKB antibody specifically recognises eIF4B Ser406 and allows us to make use of this to analyse Ser406 phosphorylation *in vivo*.

To define which signal transduction pathways regulate the eIF4B Ser406 phosphorylation *in vivo*, A14 cells were transfected with FLAG-tagged eIF4B, serum starved overnight and treated with either no inhibitors, LY294002, a combination of rapamycin and U0126 or all three inhibitors together before stimulation with insulin. Pre-treatment with LY294002 had no effect on the phosphorylation of eIF4B on Ser406 (Fig 4B, lane 3), whereas rapamycin and U0126 completely abolished phosphorylation of this residue (Fig. 4B, lanes 5). Since inhibition of the PI3K/PKB/mTOR/p70S6K pathway by LY294002 has no effect on phosphorylation of eIF4B at Ser406, this pathway is not required for phosphorylation of this residue. Thus, it appears that Ser406 phosphorylation is solely a MEK/ERK/RSK dependent event.

Phosphorylation of eIF4B regulates translational activity

It has been previously described that substitution of Ser422 to alanine results in loss of eIF4B activity in an *in vivo* translation assay (33) and that it interferes with the interaction of eIF4B with eIF3 (41). We wished to determine the effect on

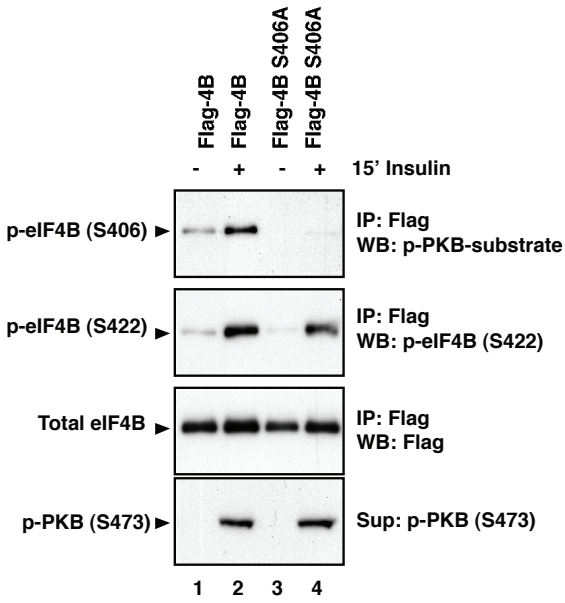
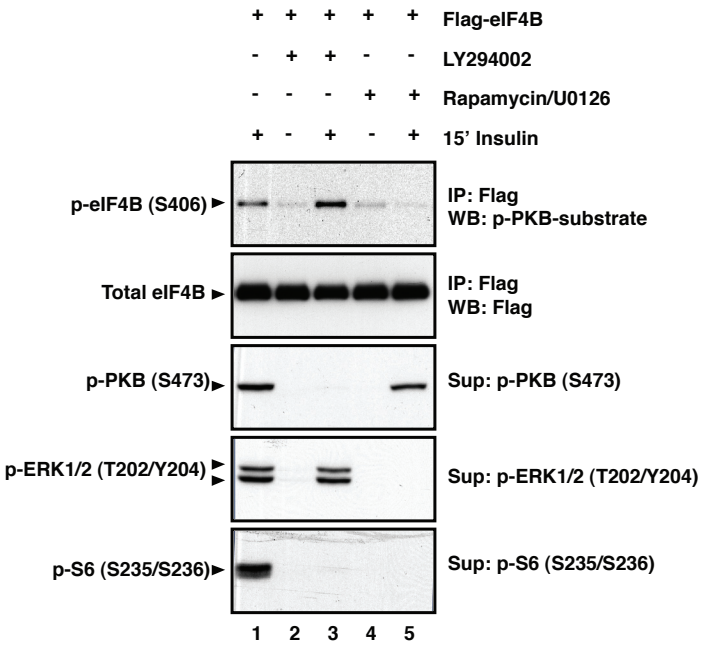


Figure 4. eIF4B phosphorylation of Ser406 after insulin stimulation is regulated by the MEK/ERK pathway

(A) *A14 cells were transfected with FLAG-tagged wild type eIF4B or mutant eIF4B in which Ser406 had been mutated to alanine. Cells were serum-starved overnight and subsequently stimulated for 15 minutes with insulin before immunoprecipitating the FLAG-tagged protein. The immunoprecipitated FLAG-eIF4B protein was analyzed for levels of phospho-PKB substrate, phospho-eIF4B (S422) and FLAG as a loading control. The whole cell lysate was analyzed for phospho-PKB (Ser473) as a control for insulin stimulation.*

translation of mutating Ser406, Ser422 or both of these sites to a non-phosphorylatable alanine. In order to study this, we made use of a recently developed translation control reporter system (TCRS) (43). This TCRS makes use of an evolutionary conserved upstream open reading frame (uORF) of the transcription factor C/EBPα. This uORF controls the ratio of two protein isoforms expressed from a single mRNA by regulated re-initiation (2): a Long Peptide (LP) expressed from a proximal initiation site and a Short Peptide (SP) expressed from a different reading frame at a distal site. At high translational activity the uORF is recognized and the truncated isoform is translated as a result of efficient re-initiation. The levels of the truncated isoforms are therefore directly proportional to the translational activity of the cell and a decrease in LP/SP ratio correlates with an increase in this activity. The Long and Short peptides contain a FLAG and myc tag respectively to distinguish between translated products.

To determine whether Ser406 and Ser422 phosphorylation affects the translational activity of cells, A14 cells were transiently transfected to express various mutant



(4B) *A14 cells were transfected with FLAG-tagged eIF4B. Cells were serum-starved overnight and subsequently stimulated for 15 minutes with insulin after or without pre-treatment with either LY294002 (50 μM) or a combination of rapamycin (20 ng/ml) and U0126 (15 μM) before immunoprecipitating the FLAG-tagged protein. The immunoprecipitated FLAG-eIF4B protein was analyzed for levels of phospho-PKB substrate and FLAG as a loading control. The whole cell lysate was analyzed for phospho-PKB (Ser473), phospho-S6 (S235/S236) and phospho-ERK1/2 (T202/Y204) as a control for the pre-treatment with the inhibitors and the insulin stimulation.*

forms of Flag-tagged eIF4B together with the TCRS vector. The day after transfection, A14 cells were washed and grown in 0.1% serum in the presence of insulin. As shown in Figure 5, expression of eIF4B in which either Ser406 or Ser422 was mutated to alanine resulted in a decrease in LP/SP ratio, this decrease was increased when both serines were mutated. Removing the functional translation *cis*-regulatory uORF in the TCRS (ΔuORF) abolished the expression of SP (lower panels). Taken together, these data indicate that phosphorylation of eIF4B on both Ser406 and Ser422 is an important mechanism by which AGC kinase family members can regulate translational activity.

DISCUSSION

In this study, we have demonstrated that PKB can phosphorylate eIF4B on Ser422 *in vitro*. Upon mitogen-stimulation phosphorylation on Ser442 is also regulated by PKB *in vivo*, since blocking PKB activity either by addition of dominant-negative

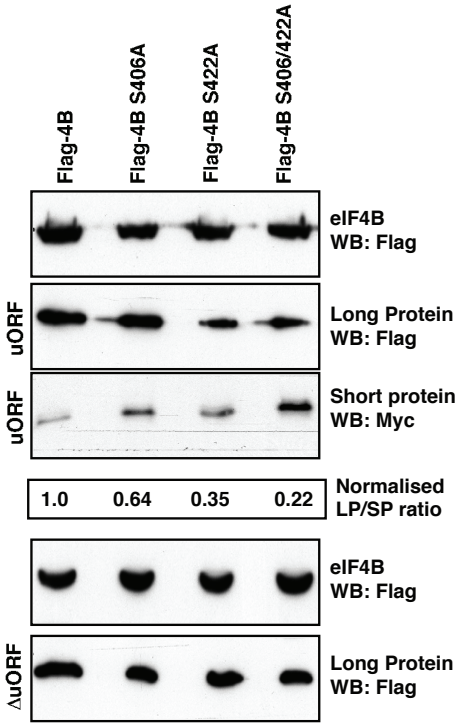


Figure 5. eIF4B phosphorylation of Ser406 and Ser422 regulates translational activity as monitored by a translation control reporter system (TCRS)

A14 cells were transiently transfected to overexpress various mutant forms of Flag-tagged eIF4B together with the TCRS vector. The day after transfection, A14 cells were washed and grown in 0.1% serum in the presence of insulin. The whole cell lysate was analyzed for total eIF4B protein and Long Protein (LP) by FLAG antibody. The Short protein was detected by Myc antibody. In the two lower panels, FLAG-tagged eIF4B was co-transfected together with a TCRS vector in which the functional translation cis-regulatory uORF had been removed, abolishing the expression of SP. The whole cell lysate was analyzed for total eIF4B protein and Long Protein (LP) by FLAG antibody.

PKBcaax or PI3K inhibitor LY294002 was sufficient to abolish phosphorylation on this residue. In contrast mTOR inhibitor rapamycin and MEK inhibitor U0126 had no effect on Ser422 phosphorylation *in vivo*. Furthermore, we have identified a novel eIF4B phosphorylation site, Ser406, which is also phosphorylated upon mitogen-stimulation. Since Ser406 represents a consensus phosphorylation motif for AGC kinase family members and its phosphorylation can only be inhibited via inhibition of the MEK/ERK pathway, we propose that the phosphorylation of this residue is regulated by RSK (Fig. 6). Using a novel translational control reporter system (TCRS) phosphorylation of both residues was found to be physiologically relevant

in regulating the translational activity of eIF4B.

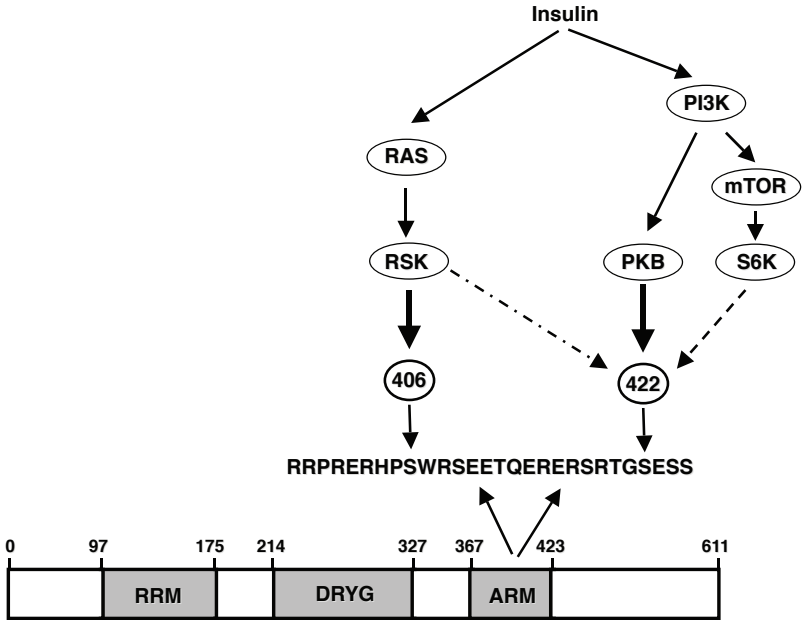


Figure 6. Signaling pathways involved in eIF4B Ser406 and Ser422 phosphorylation

Insulin activates the PI3K/PKB/mTOR pathway as well as the RAS/MEK/ERK pathway. Upon activation by insulin the RAS/MEK/ERK pathway is responsible for phosphorylation of the Ser406 residue whereas PKB phosphorylates Ser422. Both residues are important in the regulation of translation activation.

Previously, Shahbazian and co-workers reported that the phosphorylation of eIF4B on Ser422 is synergistically regulated by p70S6K and RSK upon serum-stimulation (41). Phosphorylation of this residue upon insulin-stimulation was reported to be solely dependent on p70S6K. However, in this study cells were pre-treated with rapamycin for an extended period of time (up to 18 hours). Recently, it has become clear that prolonged treatment with rapamycin can inhibit PKB activity in a cell type specific manner(39). Sarbassov and co-workers provided compelling evidence that mTOR in complex with Rictor (mTORC2) was sensitive to long-term rapamycin treatment and this complex was previously reported by the same group as the long sought after PDK2 kinase which phosphorylates PKB at Ser473 leading to its activation (39,40). In our study, cells were pre-treated with rapamycin for only a short period of time (less than 2 hours) to ensure that only mTOR in complex with Raptor (mTORC1), the upstream activator of p70S6K and not mTORC2 was inhibited. This had no effect on PKB activation as shown in Fig. 3A allowing us to specifically analyse the effect of mTOR/p70S6K inhibition.

Members of the AGC protein kinase family have highly homologous kinase domains

and similar substrate specificities, and can therefore be considered as potentially "promiscuous" when it comes to phosphorylation of target proteins. Care must therefore be taken in drawing conclusions from *in vitro* assays where, it is likely that various members of the AGC kinase family may phosphorylate substrates at the same site. *In vivo*, however, phosphorylation of substrates is likely to be a highly regulated process. In the case of eIF4B, three AGC kinase family members have now been shown to phosphorylate Ser422 *in vitro*, p70S6K, RSK and PKB respectively. Whereas insulin specifically utilizes PKB to phosphorylate this residue, serum may also utilize RSK to regulate Ser422 phosphorylation. Shahbazian and co-workers show after serum stimulation a temporal effect of MEK inhibitor U0126 and mTOR inhibitor rapamycin (41). U0126 effects the early phase of eIF4B phosphorylation whereas rapamycin effects the late phase. These data suggest that RSK is important for rapid regulation of eIF4B activity upon serum stimulation, and p70S6K (or PKB as we have indicated in the previous paragraph) is crucial for signal duration. In this study, we have shown that for insulin stimulation RSK does not play a role in Ser422 phosphorylation but this kinase is crucial in Ser406 phosphorylation. Therefore, it is safe to conclude that eIF4B phosphorylation and activation are regulated in a stimulus-dependent manner, and could be the reason why the Ser406 site was not identified by Raught and co-workers in their phosphomapping experiment after serum stimulation (33).

While our data demonstrate that p70S6K is not responsible for insulin-mediated Ser406/422 phosphorylation, this does not discount a role for this AGC family member in eIF4B phosphorylation. It has been demonstrated that under conditions of amino acid refeeding p70S6K is activated while PKB remains inactive (11). Under these situations it is likely that p70S6K can phosphorylate eIF4B allowing for regulation of protein translation. Regulation of eIF4B phosphorylation may be a fundamental process in the regulation of protein translation in response to diverse extracellular stimuli. Our data suggests that utilizing various AGC kinase family members allows this mechanism of translational control to be regulated through distinct stimulus-specific intracellular signalling pathways.

The effects of eIF4B phosphorylation on translation have, to some degree, been studied previously and eIF4B phosphorylation has been reported to correlate with high translational activity. In accordance with this, Holz *et al.* found that expression of a phosphomimetic form of eIF4B increased cap-dependent translation (15) and Shahbazian and co-workers found that phosphorylation of eIF4B on Ser422 stimulates its interaction with eIF3 (41). However, others observed an inhibition of translation upon eIF4B overexpression which was relieved when Ser422 was mutated into a non-phosphorylatable alanine (25,33). In this study, we also observe an increase in translational activity when Ser406 or/and Ser422 were mutated. It has been suggested that the relative amount of eIF4B expression is crucial for its effect on translation. Since eIF4B binds various members of the translation initiation complex including eIF3 and eIF4A, but also mRNA and 18S RNA, high expression

levels could disrupt the correct stoichiometry of complex formation resulting in an inhibitory effect. Our data demonstrate that phosphorylation of eIF4B in the ARM region indeed regulates translational activity. Since activation of AGC protein kinase family members has an overall positive effect on translation, it is thus likely that this phosphorylation results in activation of eIF4B.

In this study, we provide compelling evidence that phosphorylation of eIF4B can be regulated by both RAS and PKB signalling. Both pathways have been shown to be deregulated in a plethora of neoplasias. What role could eIF4B phosphorylation play in the process of transformation? eIF4B has been shown to play a critical role in stimulating the helicase activity of eIF4A to unwind inhibitory secondary structures in the 5' untranslated region of mRNAs. These highly structured mRNAs are poorly translated when the translation initiation activity is decreased (19). Highly structured mRNAs often encode those proteins that are components of pathways critical to cell growth, such as growth factors, transcription factors, tyrosine kinases and receptors (38). Rajasekhar *et al.* recently demonstrated that upon PKB and RAS signalling the profile of mRNA associated to polysomes was drastically altered, these mRNAs mainly encoded for the proteins mentioned (31). Therefore activation of eIF4B by dysregulated RAS and PKB signalling may be critical in the induction of cellular transformation.

Until recently, the effects PKB has on regulating translation were thought to be through increased mTOR activity. The inhibitory effects of rapamycin on PKB-induced transformation appeared to reveal the importance of mTOR as a downstream mediator of PKB signalling. However, the recent evidence that prolonged rapamycin treatment can itself inhibit PKB activation, re-emphasizes the importance of PKB itself as an oncogenic factor in regulating growth and proliferation. We suggest that oncogenic transformation as a result of uncontrolled PKB activity could be directly mediated by enhanced eIF4B activity, providing a novel rationale for the design of therapeutic strategies to inhibit tumour cell growth.

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CHAPTER 5

Comparative analysis of PI3K and PKB regulated gene expression profiles

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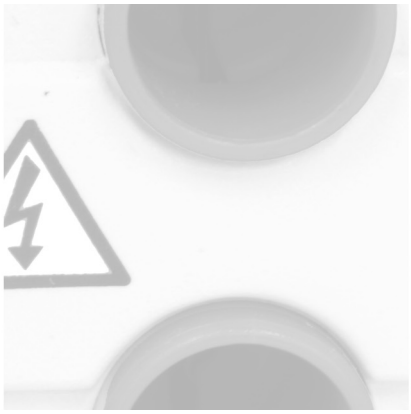
Work in progress




ABSTRACT

Mutations in the signal transduction pathways regulating Phosphoinositol-3 kinase (PI3K) and Protein Kinase B (PKB) activation are a hallmark of diverse neoplasias, providing both proliferative and antiapoptotic survival signals. Surprisingly, we previously observed that chronic PKB activation results in activation of programmed cell death. To determine the effect of chronic PI3K activation on cell survival we generated a comparable Ba/F3 cell line in which PI3K can also be directly and specifically induced. In contrast to PKB, activation of PI3K was shown to be sufficient for long-term cell survival. Using a microarray gene expression profiling approach we sought to uncover the molecular mechanisms underlying these different effects on cell survival. Our study revealed that while PI3K and PKB have many common transcriptional targets there are also distinct cellular processes which can be independently regulated. PI3K and PKB were both found to significantly regulate expression of genes implicated in cell cycle progression. Interestingly, we found that PKB specifically modulated expression of genes involved in the regulation of metabolism and oxidative phosphorylation. This PKB-specific gene set could be detrimental to cellular homeostasis resulting in the induction of apoptosis observed after chronic PKB activation. The use of transcriptional profiling in this manner allows detailed analysis of the architecture of signalling pathways to be undertaken. Furthermore, utilizing this approach we hope to reveal a PI3K/PKB "signalling signature" which might be helpful in the molecular characterisation of human neoplasias.

INTRODUCTION

Cancer is the result of a disturbed balance between cell division and growth on one hand, and programmed cell death on the other. During cancer development tumour cells acquire a number of phenotypic characteristics that allow them to proliferate both rapidly and limitlessly, invade the surrounding tissue, survive outside their normal micro-environment, and finally, metastasize to secondary sites. A cancerous phenotype is a result of accumulative carcinogenic mutations usually comprising of tumour suppressor gene loss and oncogene amplification. Dysregulation of the phosphoinositol-3 kinases (PI3K) signalling pathway is one of the most frequently targeted pathways in all sporadic human tumours. Taken together, mutations in one or more components of PI3K pathway are estimated to count for up to 30% of all human cancers (19).

PI3Ks phosphorylate phosphatidylinositol (PI) which is a minor component of eukaryotic cell membranes constituting approximately 8% of all phospholipids (4,15). The inositol head group of PI contains five free hydroxyl groups, only three (3', 4' and 5') of which can be phosphorylated *in vivo*. The 3' hydroxyl group can be phosphorylated by PI3Ks generating PI(3)P, PI(3,4)P_2 or PI(3,4,5)P_3. In a resting state, levels of 3-phosphoinositides are low but these levels are dramatically increased upon growth factor or cytokine stimulation. *In vivo*, the actions of PI3Ks are negatively regulated by the phosphoinositol phosphatases PTEN and SH2 domain containing inositol 5'phosphatase (SHIP). SHIP dephosphorylates PI(3,4,5)P_3 at the 5' phosphate whereas PTEN dephosphorylates it at the 3' phosphate. Inactivating mutations of PTEN or SHIP therefore lead to increased levels of this phospholipid (20). The loss of PTEN and subsequent activation of PI3K is a critical event in cancer onset and PTEN is thought to be the second most commonly mutated tumour suppressor in humans, after p53 (2).

The PI(3,4,5)P_3 lipids generated by PI3Ks have high affinity for a variety of cytoplasmic proteins resulting in their translocation to the plasma membrane. Phospholipid products generated by PI3Ks associate with proteins that contain one of a number of protein motifs that include the pleckstrin homology (PH) domain, SH2 domain, Phox homology (PX) domain, C2 domain and phosphotyrosine binding (PTB) domain (3). Translocation of these proteins to the membrane results in the formation of so-called 'signalosomes', where proteins associate and their activity is regulated.

The most well characterized effector of PI3K activity is Protein Kinase B (PKB/c-akt), a PH-domain containing serine/threonine kinase belonging to the AGC protein kinase family (5,22). Human tumours have been shown to exhibit frequent dysregulation of PKB activity. These alterations exist either as gene amplifications that lead to overexpression of PKB or hyperactivation of PKB due to dysregulation of its upstream mediators. Amplification of PKB isoform encoding genes occurred, among others, in ovarian carcinomas, non-hodgkin lymphoma, pancreatic carcinomas, hepatocellular carcinomas and colon cancers (reviewed extensively in

(2)).
In this study, we have investigated the effects of PI3K and PKB activation on gene expression using cytokine-dependent bone marrow-derived Ba/F3 cells. For this purpose, we developed a system where PI3K and PKB can be independently and inducibly activated. We have previously observed that chronic PKB activation was unable to maintain long-term cell survival, interestingly we now show that PI3K activation alone is sufficient for cell survival for an extended period of time. To investigate the functional differences underlying the effect PI3K and PKB activation have on survival, we performed a large scale microarray analysis of the genes expressed within 4 hours of the activation of these oncogenes. This approach allows us to distinguish which subsets of transcriptional targets of PI3K dependent or independent of PKB activity. This study provides novel insights into the intracellular signalling pathways regulated by PI3K and PKB activation. Identification of novel transcription targets of PI3K/PKB may also lead to the development of novel strategies for the treatment of human malignancies.

MATERIAL AND METHODS

Cell culture

Ba/F3 cells were cultured in RPMI 1640 medium with 8% Hyclone serum (Gibco, Paisley, UK) and recombinant mouse IL-3 produced in COS cells(9). For the generation of clonal Ba/F3 cells stably expressing myrPKB:ER* or myrP110α*: ER, the SRα-myrPKB:ER* construct and the SRα-myrP110α*:ER construct respectively were electroporated into Ba/F3 cells together with pSG5 conferring neomycin resistance and maintained in the presence of 1mg/ml G418 (Gibco, Paisley, UK) and IL-3. Clonal cell lines were generated by limited dilution. For cytokine withdrawal experiments, cells were washed twice with PBS and resuspended in AimV medium (Gibco, Paisley, UK).

Antibodies and reagents

The monoclonal antibody against phospho-PKB (Ser473) was from Cell Signaling Technologies (Hitchin, UK). Actin was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Phospho-GSK3α/β (Ser21/9), phospho-STAT5 (Tyr694) and Phospho-MAPK42/44(Thr202/Tyr204) were from New England Biolabs (Hitchin, UK). 4-hydroxytamoxifen (4-OHT) was purchased from Sigma (Seelze, Germany).

Western blotting

BaF3 cells were lysed in laemmli buffer (0.12M Tris HCL pH 6.8, 4% SDS, 20% Glycerol, 0.05 μg/μl bromophenol blue, and 35mM β-mercaptoethanol), boiled for 5 minutes and the protein concentration was determined. Equal amounts of sample were analyzed by SDS PAGE, electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA) and probed with the respective antibodies. Immunocomplexes were detected using enhanced chemiluminescence (ECL,

Amersham, Buckinghamshire, UK).

RNA isolation

Cells were stimulated as indicated and harvested at the respective times, washed twice with PBS, lysed in 1ml TRIZOL (Invitrogen, Breda, The Netherlands) and stored at -20°C. For isolation of total cellular RNA, samples were incubated for 3 minutes at room temperature, 0.2ml chloroform was added, vortexed and the suspension was incubated for 3 minutes at room temperature, followed by 15 minutes of centrifugation at 8000rpm at 4°C. Subsequently, the aqueous phase was transferred to new tubes, 0.5ml isopropanol was added and incubated for minimal 30 minutes at -20°C. Samples were centrifuged at 14000 rpm for 10 minutes at 4°C. The pellet was washed with 70% ethanol an dissolved in RNAse free water. DNAse treatment was performed using the DNAse Qiagenkit, after which it was purified with Qiagen Rna-easy spin columns.

Microarray analysis

We amplified mRNA by *in vitro* transcription using T7 RNA polymerase on 1 μg of total RNA. During *in vitro* transcription, 5-(3-aminoallyl)-UTP (Ambion) was incorporated into the single-stranded cRNA. We applied total RNA and cRNA quality control criteria in accordance with the Tumor Analysis Best Practices Working Group (nat rev genet 229). Cy3 and Cy5 fluorophores (amersham) were coupled to 500 ng of cRNA.

cRNA was hybridized (2 μg/channel in Agilent hybridisation chambers and oven) onto Corning UltraGAPS slides containing the Operon Mouse Genome Oligo Set V3. Hybridized slides were scanned on an Agilent scanner (G2565AA) at 100% laser power, 30% PMT. After data extraction using Imagene 6.1 (BioDiscovery), printtip Loess normalization was performed (Yang et al., 2002). Data was analysed per time point using ANOVA (R2.2.1/MAANOVA version 0.98-3) (http://www.r-project.org/). In a fixed effect analysis, sample, array and dye effects were modelled. P-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times globally. Genes with $p < 0.05$ after family wise error correction were considered significantly changed. Visualisation and cluster-analysis was done using GeneSpring 7.2 (Agilent).

RESULTS AND DISCUSSION

PI3K activation is sufficient for long-term survival

Ba/F3 cells are normally dependent on interleukin (IL-) 3 for their survival and proliferation. Stimulation of Ba/F3 cells with IL-3 results in the activation of multiple intracellular signal transduction pathways among which are the PI3K-PKB signalling module. In order to investigate the mechanisms by which PI3K and PKB differentially regulate cytokine mediated cellular functions, we generated two different Ba/F3 cell lines, one stably expressing an inducible active PKBα

(myrPKB:ER) and another stably expressing an inducible active PI3K (myrP110α*:ER). The activation of proteins coupled to the hormone binding domain (HBD) of the estrogen receptor (ER) is, in the absence of 4-hydroxytamoxifen (4-OHT), inhibited by heat-shock and chaperone proteins that associate with the fused estrogen receptor (ER) hormone-binding domain. In the presence of 4-OHT these inhibitory proteins dissociate allowing activation of the ER-coupled fusion protein. For the Ba/F3 myrPKB:ER cell line, we have previously shown that upon 4-OHT stimulation PKB becomes rapidly phosphorylated and activated (Chapter 2, Fig. 1A). PI3K activation in the Ba/F3 myrP110α:ER cell line functions in a similar manner. Activity of the PI3K catalytic subunit, p110α is normally dependent on its interaction with the inter-SH2 (iSH2) domain of the PI3K regulatory subunit, p85, therefore the iSH2 domain of p85α containing a myristoylation signal was fused at the N-terminus of p110α by a hinge domain. Similar to the Ba/F3 myrPKB:ER cell line, 4-OHT-mediated PI3K activation was sufficient to rapidly induce the phosphorylation of PKB and GSK-3 (Fig. 1A, top). In contrast to IL-3 stimulation, IL-3, 4-OHT treatment was unable to induce phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) MAPK or Signal transducer and activator of transcription (STAT) 5 whose activation is PI3K-independent (Fig. 1A, bottom).
Both PI3K and PKB are considered thought to be important factors contributing to cell survival and since our previous studies have demonstrated 4-OHT-mediated chronic PKB activation to be insufficient for long-term survival in Ba/F3 cells (Chapter 2, Fig. 2B), we wished to establish the effect of chronic PI3K activation.

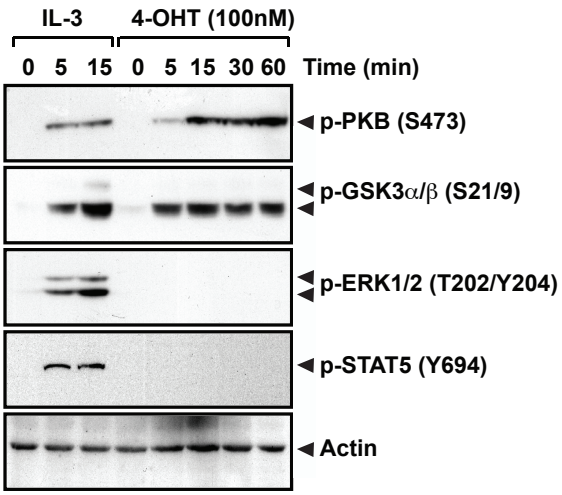


Figure 1. 4-OHT specifically activates myrP110α:ER
Ba/F3 myrP110α:ER cells were treated with either IL-3 or 4-OHT (100 nM) for the indicated times and lysed and equal amounts of protein were analyzed for levels of phosphorylated PKB (Ser473), phosphorylated GSK-3α/β (Ser21/Ser9), phosphorylated ERK1/2 (Thr202/Tyr204), phosphorylated STAT5 (Tyr694) and actin.

The ER:P110α* cell line was cytokine-starved and left untreated or treated them with IL-3, 4-OHT or both for up to 96 hours and measured the percentage of living cells every 24 hours. 4-OHT-mediated PI3K activation rescued Ba/F3 cells from cytokine withdrawal-induced apoptosis (Fig. 2). Importantly, in contrast to the anti-apoptotic effects observed after chronic PKB activation, these effects were not short lived and cells were able to survive for weeks in the absence of cytokine (data not shown).

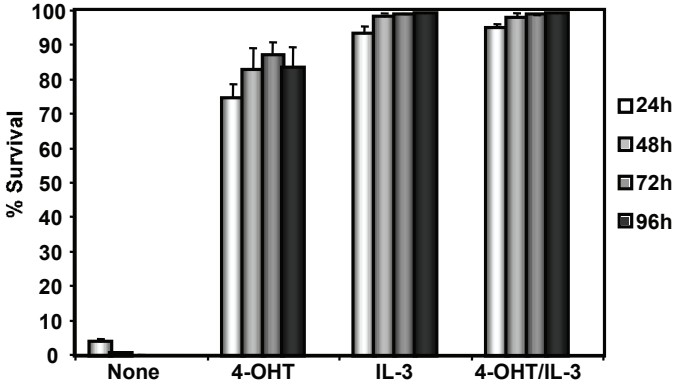


Figure 2. Chronic PI3K activation is sufficient for long-term survival
Ba/F3- myrP110α:ER cells were cytokine starved or treated with either IL-3, 4-OHT, or both for the indicated times. The percentage of living cells was determined by FACS analysis of the percentage of Annexin V–negative cells. Columns, average of three independent experiments; bars, SE.

Identification of genes regulated by PI3K and PKB

To identify differences in the transcriptional programs activated by PI3K and PKB, we again cytokine starved myrP110α*:ER or myrPKB:ER Ba/F3 cells respectively and stimulated these cells with 4-OHT for the indicated time points. Treatment of these cells for up to 24 hours with 4-OHT leads to the activation of PI3K and PKB (Fig. 3) respectively. RNA was isolated from all time points and RNA from 15 and

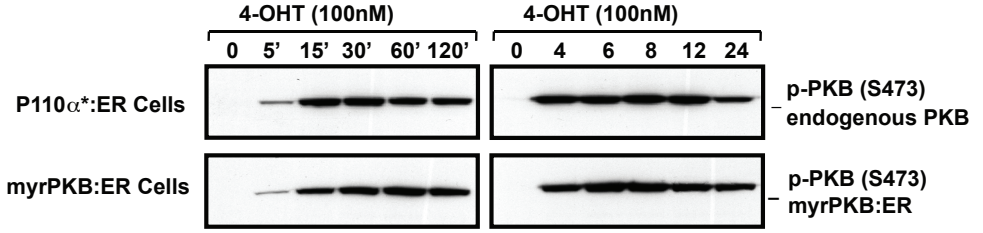


Figure 3. 4-OHT specifically activates myrPKB:ER* and myrP110α:ER
Ba/F3-myrPKB:ER and Ba/F3- myrP110α:ER cells were cytokine starved or treated with 4-OHT for the indicated times, lysed and equal amounts of protein were analyzed for levels of phosphorylated PKB (Ser473) and actin.*

30 minutes and 1, 2 and 4 hours was used to generate cRNA. The cRNA was hybridized onto Corning UltraGAPS slides containing the Operon Mouse Genome Oligo Set V3 as described in Material and Methods. This experiment was performed in quadruplicate. Using Genespring 7.2 software we identified 2019 genes that were significantly regulated in at least one of the two cell lines on at least one time point. These genes were subdivided in groups according to how they are regulated in the two cell lines (Fig. 4). Out of these groups, we further analyzed 6 subsets: in both cell lines at at least 5 out of 10 time points upregulated (Bothup) or downregulated (Bothdown), in the Ba/F3 myrP110α*:ER cell line at all time points upregulated (PI3Kup) or downregulated (PI3Kdown) and in the myrPKB:ER cell line at all time points upregulated (PKBup) or downregulated (PKBdown). In order to determine the physiological relevance of the genes in these groups, we made use of the "Webbased Gene Set Analysis Toolkit" (Webgestalt; http://bioinfo.vanderbilt.edu/webgestalt/) and organized the genes in these groups based on biochemical pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG). The output of this analysis is a KEGG table that shows all pathways in which at least 4 genes, involved in a specific pathway, are present in the submitted gene set. The hypergeometric test is used to determine statistical significance of enrichment of individual pathways (P value ≤ 0,01).

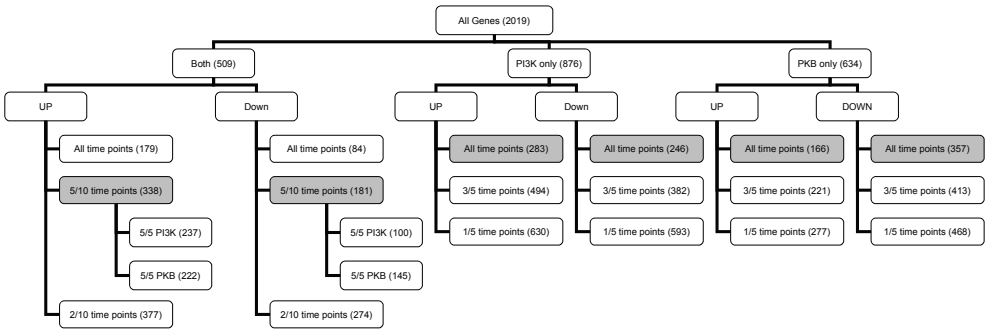


Figure 4. Schematic representation of the distribution of all 2019 significantly regulated genes

The groups that were used for further analysis are depicted in grey.

Genes significantly regulated by both PI3K and PKB activation

The genes in the group regulated by both PI3K and PKB activation may be considered to be particularly significant as they were identified in two independent cell lines and are likely to represent targets of cytokine-induced PI3K activation in Ba/F3 cells. In this gene group, six KEGG pathways were determined to be enriched in the Bothup list (Table 1) and none were determined to be enriched in the Bothdown list. Most evident was the identification of eleven genes involved in cell cycle progression (Table 2). The PI3K/PKB pathway has long been known to

be involved in cell cycle progression mostly by phosphorylation events as discussed in the introduction of this thesis (18). The cell cycle genes identified in this study have not been previously reported to be transcriptionally regulated by PI3K or PKB activation. Interestingly, Figure 5 shows schematically that most of these genes play a role in G1/S cell cycle progression and a role for PKB at this stage has been extensively described (18). For example, PKB phosphorylates $p27^{kip1}$ and $p21^{waf1/cip1}$ resulting in the nuclear exclusion of these cdk-inhibitors and subsequently cell cycle progression (31,32). PKB phosphorylation of FoxO transcription factors also

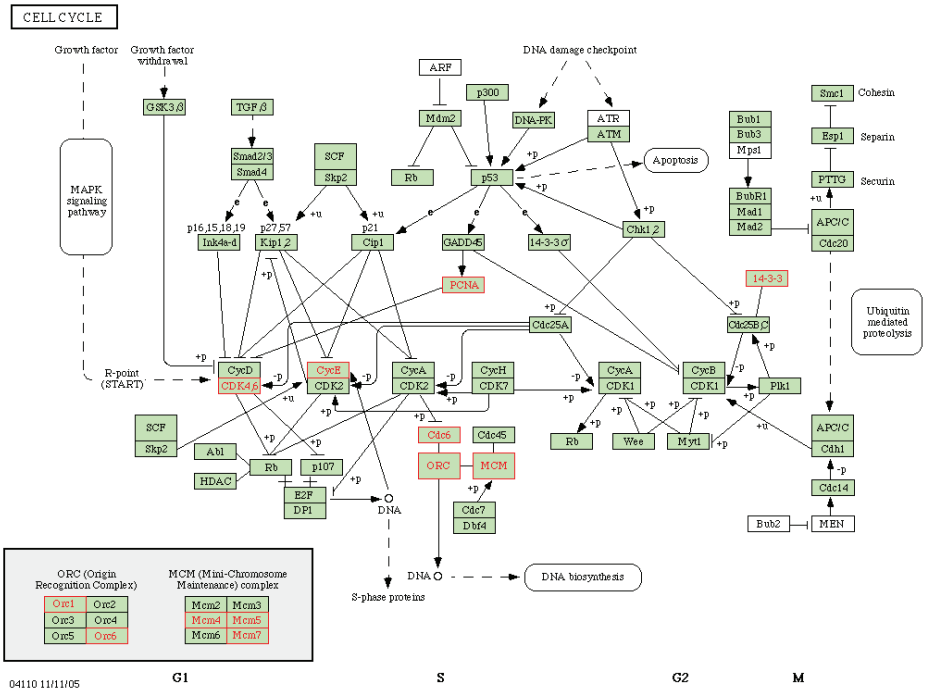


Figure 5. KEGG Map for the KEGG pathway "cell cycle"
Genes in the KEGG pathway cell cycle which are upregulated by both PI3K and PKB activation are indicated with a full circle.

inhibits the transcriptional upregulation $p27^{kip1}$ and the downregulation of D type cyclins (10,13,21,29). Furthermore, PKB phosphorylation and inhibition of glycogen synthase kinase-3 (GSK3) stabilizes cyclin D1 (1,8) (the role of PI3K and PKB in cell cycle progression is extensively reviewed in (18)). Interestingly, cyclin D2 was found to be regulated but only upon PI3K activation and not when PKB was activated, indicating that PKB activation alone is insufficient for transcriptional regulation of this gene (Supplemental data, Table. 3).
Thus, it appears that PI3K and PKB activation regulates G1/S transition on both the level of phosphorylation as well as on the level of transcriptional regulation. This is

further emphasized by the fact that three members of the family of Minichromosome Maintenance proteins (MCM) are transcriptionally upregulated by both PI3K and PKB activation. Interestingly, two other members of this family were picked up as potentially novel PKB phosphorylation substrates in our phosphoproteomic approach to identify novel PKB substrates (Chapter 3). Together, these data indicate that the regulation of proliferation is intricately regulated by PI3K and PKB.

Table 1 KEGG table of genes significantly upregulated by both PI3K and PKB activation

*338 transcripts were shown to be significantly upregulated by both PI3K and PKB activation in at least 5 out of the 10 of the studied time points. This constitutes of 245 known genes which were organized using the KEGG biochemical pathways. The KEGG Table shows KEGG pathways associated with the gene set (column 1), the number of genes in each pathway (column 2) and the Entrez Gene IDs for the genes (column 3). The 4th column gives the parameters for the enrichment of the KEGG pathway. O is the observed gene number in the KEGG pathway; E is the expected gene number in the KEGG pathway (Expected number of genes in a specific KEGG pathway for an interesting gene set= Total number of genes in the KEGG pathway for the reference set * Total number of genes in the interesting set / Total number of genes in the reference set); R is the ration of enrichment for the KEGG pathway (R=O/E); P is the p value indicating the significance of enrichment calculated from Hypergeometric test.*

KEGG pathway	# Genes	Entrez Gene IDs	Enrichment
Cell cycle	11	12447 12448 12567 17217 17218 17220 18392 18538 22628 23834 56452	O=11;E=0.59; R=18.69;P=2.16E-11
Pyrimidine metabolism	6	110074 16434 18102 18974 20135 51797	O=6;E=0.47; R=12.71;P=8.36E-06
MAPK signalling pathway	6	11651 11911 14828 15481 17869 67603	O=6;E=1.47;R=4.09; P=0.0037
Purine metabolism	5	11564 16434 18102 18974 20135	O=5;E=0.77;R=6.52; P=0.0011
Gap junction	5	22142 22145 22146 53857 67951	O=5;E=0.48; R=10.35;P=0.00013
Insulin signalling pathway	4	11651 13684 14104 19246	O=4;E=0.74;R=5.42; P=0.0066

Table 2 Genes in KEGG pathway cell cycle

The KEGG pathway cell cycle was significantly upregulated by both PI3K and PKB activation in at least 5 out of the 10 of the studied time points. This table shows the Entrez Gene IDs (column 1), the Gene symbol (column 2) and the Gene name (column 3) of the eleven genes in this KEGG pathway.

Entrez Gene ID	Symbol	Gene name
12447	Ccne1	cyclin E1
12448	Ccne2	cyclin E2
12567	Cdk4	cyclin-dependent kinase 4
17217	Mcm4	minichromosome maintenance deficient 4 homolog (S. cerevisiae)
17218	Mcm5	minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)
17220	Mcm7	minichromosome maintenance deficient 7 (S. cerevisiae)
18392	Orc1l	origin recognition complex, subunit 1-like (S. cerevisiae)
18538	Pcna	proliferating cell nuclear antigen
22628	Ywhag	3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide
23834	Cdc6	cell division cycle 6 homolog (S. cerevisiae)
56452	Orc6l	origin recognition complex, subunit 6-like (S. cerevisiae)

Genes significantly regulated by PI3K but not PKB activation

The group of genes significantly regulated by PI3K activation and not by PKB activation could contribute to the difference observed in cell survival upon PI3K and PKB activation. KEGG analysis of this gene subset showed eight KEGG pathways to be enriched in the PI3Kup list (Table 3) and three were determined to be enriched in the PI3Kdown list (Table 4). Seven genes were involved in the MAPK signalling pathway (Table 5). As shown in Table 1 the MAPK signalling pathway was also found to be enriched in the Bothup list. In this list six genes were shown to be upregulated by both PI3K and PKB activation (Table 1). Figure 6 schematically shows that the genes involved in the MAPK signalling pathway regulated by PI3K activation are distinct from does that are activated by both PI3K and PKB activation. Interestingly, whereas the genes upregulated by PI3K activation are genes for which overexpression and subsequent activation would result in the activation of MAPK signalling, the genes upregulated by both are mostly inhibitory and could play a role in signal termination after cytokine stimulation. Upregulation of genes involved in the activation of MAPK signalling pathway by PI3K could potentially result in increased activity of this pathway. Interestingly, studies performed in our laboratory have shown that PI3K activation, in contrast to PKB activation, is able to modestly induce ERK1/2 phosphorylation (data not shown). ERK1/2 activation has been implicated in the transcriptional regulation of c-myc and interestingly, c-myc was

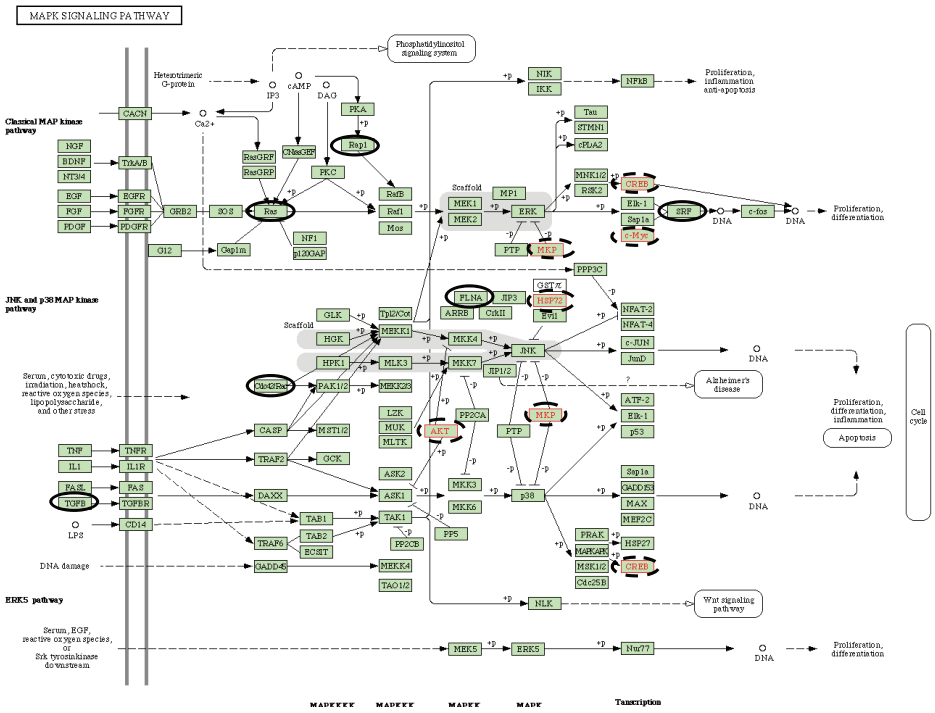


Figure 6. KEGG Map for the KEGG pathway "MAPK signalling"
Genes in the KEGG pathway cell cycle which are upregulated by PI3K activation are indicated with a full circle. Genes in the KEGG pathway cell cycle which are upregulated by both PI3K and PKB activation are indicated with a dashed circle.

shown to be transcriptionally upregulated by PI3K activation and downregulated by PKB activation (supplemental data Table 7). The expression of the c-myc proto-oncogene is elevated or deregulated in a wide range of human cancers and is often associated with an aggressive tumour (24,28). *In vitro* experiments have shown that constitutive overexpression of c-myc can immortalize rat fibroblasts and prevent withdrawal from the cell cycle (23). Even though oncogenic activation of c-myc alone causes uncontrolled proliferation, similar for most oncogenes cellular transformation seemed to require additional oncogenic lesions (12,17). Interestingly, c-myc protein levels have already been shown to be regulated by both PI3K signalling as well as MAPK signalling (14,30). ERK1/2 phosphorylates c-Myc on Ser62 which is necessary for the subsequent phosphorylation by GSK3β on Thr58 which in turn provides the key signal for degradation of c-Myc. PI3K/PKB signalling inhibits GSK3β signalling, thereby stabilizing c-Myc. It would be interesting to examine what the effect of these transcriptional and post-translational events have on the

c-myc protein levels in our cell systems. Taken together, the induction of c-myc transcription is potentially a mechanism for the differential effect PI3K and PKB activation have on cell survival.

Table 3 KEGG table of genes significantly upregulated by PI3K activation

283 transcripts were shown to be significantly upregulated by PI3K activation in 5 out of 5 of the studied time points. This constitutes of 219 known genes which were organized using the biochemical pathways from KEGG. See table 1.

KEGG pathway	# Genes	Entrez Gene IDs	Enrichment
Focal adhesion	8	11461 11464 12540 16653 19217 215449 21894 22340	O=8;E=0.97; R=8.22;P=6.81E-06
MAPK signalling pathway	7	12540 15526 16653 192176 20807 215449 21803	O=7;E=1.31; R=5.34;P=0.00038
Purine metabolism	6	14450 16434 18971 18972 19075 231327	O=6;E=0.68; R=8.76;P=6.96E-05
Pyrimidine metabolism	6	16434 18971 18972 19075 21915 320685	O=6;E=0.42; R=14.22;P=4.40E-06
Regulation of actin cytoskeleton	6	108100 11461 11464 12540 16653 18643	O=6;E=0.97; R=6.17;P=0.00047
Leukocyte transendothelial migration	5	11461 11464 12540 13057 215449	O=5;E=0.56; R=8.99;P=0.00025
Tight junction	4	11461 11464 12540 16653	O=4;E=0.56; R=7.13;P=0.0025
Adherens junction	4	108100 11461 11464 12540	O=4;E=0.36; R=11.19;P=0.00047

Table 4 KEGG table of genes significantly downregulated by PI3K activation

246 transcripts were shown to be significantly downregulated by PI3K activation in 5 out of 5 of the studied time points. This constitutes of 205 known genes which were organized using the biochemical pathways from KEGG. See table 1.

KEGG pathway	# Genes	Entrez Gene IDs	Enrichment
Valine, leucine and isoleucine degradation	4	110821 11992 15356 67041	O=4;E=0.23; R=17.22;P=8.82E-05
Adipocytokine signalling pathway	4	14081 16452 18035 20848	O=4;E=0.31; R=12.85;P=0.00028
Jak-STAT signalling pathway	4	13857 16452 20848 20851	O=4;E=0.67; R=5.94;P=0.0048

Table 5 Genes in KEGG pathway MAPK signalling pathway

The KEGG pathway MAPK signalling pathway was significantly upregulated by PI3K activation in 5 out of 5 of the studied time points. This table shows the Entrez Gene IDs (column 1), the Gene symbol (column 2) and the Gene name (column 3) of the seven genes in this KEGG pathway.

Entrez Gene ID	Symbol	Gene name
12540	Cdc42	cell division cycle 42 homolog (S. cerevisiae)
15526	Hspa9a	heat shock protein 9A
16653	Kras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
192176	Flna	filamin, alpha
20807	Srf	serum response factor
215449	Rap1b	RAS related protein 1b
21803	Tgfb1	transforming growth factor, beta 1

Genes significantly regulated by PKB but not PI3K activation

The group of genes regulated by PKB activation alone could be considered physiologically irrelevant since PKB activation is always preceded by PI3K activation. However in tumours in which PKB is hyperactivated due to gene amplification (2) these genes might represent interesting anti-cancer therapeutic targets. KEGG analysis revealed five KEGG pathways to be enriched in the PKBup list (Table 6) and four were determined to be enriched in the PKBdown list (Table 7). The KEGG pathway analysis shows that PKB activation alone results in substantial changes in metabolic regulatory genes (Table 6 and 7). Mitochondrial respiration malfunction and increased glycolysis are frequently observed in cancer cells (7,11,26,27). Here, we specifically show an increase in mitochondrial proteins such as ATP synthases, NADH dehydrogenases and cytochrome c (Table 8). NADH dehydrogenases and cytochrome c are part of the electron transport chain necessary for ATP synthesis. ATP synthases are crucial for maintaining the mitochondrial membrane potential which in turn has been shown to be crucial for PKB mediated survival (25). Furthermore, decreased expression of genes involved in glycolysis and gluconeogenesis was also observed (Table 9). Gluconeogenesis results in the generation of glucose from non-sugar carbon substrates such as pyruvate, lactate, glycerol, and amino acids (primarily alanine and glutamine) (16). Interestingly, enolase, a cytoplasmic glycolytic enzyme important in the formation of phosphoenolpyruvate was shown to be downregulated in human cancers (6). These alterations in cellular metabolism were only observed when PKB was activated and not when PI3K was activated. A difference in cellular metabolism could also explain the differential effect of PI3K and PKB activation on cellular survival and the state of oxidative stress observed in cell in which PKB is chronically activated.

Table 6 KEGG table of genes significantly upregulated by PKB activation

166 transcripts were shown to be significantly upregulated by PKB activation in 5 out of 5 of the studied time points. This constitutes of 112 known genes which were organized using the biochemical pathways from KEGG. See table 1.

KEGG pathway	# Genes	Entrez Gene IDs	Enrichment
Oxidative phosphorylation	8	11951 11958 11984 170658 228033 66445 68194 69875	O=8;E=0.29; R=27.89;P=5.48E-10
Cell cycle	6	12428 12649 17120 18817 21781 433759	O=6;E=0.27; R=22.22;P=3.52E-10
Proteasome	5	17463 19184 23996 26440 59029	O=5;E=0.077; R=65.08;P=1.36E-08
Biosynthesis of steroids	4	110196 13360 17855 20775	O=4;E=0.041; R=97.61;P=7.25E-08
ATP synthesis	4	11951 11958 11984 228033	O=4;E=0.10; R=40.05;P=3.13E-06

Table 7 KEGG table of genes significantly downregulated by PKB activation

357 transcripts were shown to be significantly downregulated by PKB activation in 5 out of 5 of the studied time points. This constitutes of 144 known genes which were organized using the biochemical pathways from KEGG. See table 1.

KEGG pathway	# Genes	Entrez Gene IDs	Enrichment
Glycolysis / Gluconeogenesis	10	11669 11674 13806 4751 18641 18648 18655 8746 21991 407972	O=10;E=0.18; R=55.71;P=2.58E-15
Carbon fixation	4	11674 18655 18746 1991	O=4;E=0.072; R=55.71;P=7.60E-07
Insulin signalling pathway	4	16333 18641 18746 20411	O=4;E=0.43; R=9.21;P=0.00098
Fructose and mannose metabolism	4	108155 11674 18641 21991	O=4;E=0.17; R=24.03;P=2.41E-05

Table 8 Genes in KEGG pathway Oxidative phosphorylation

The KEGG pathway oxidative phosphorylation was significantly upregulated by PKB activation in 5 out of 5 of the studied time points. This table shows the Entrez Gene IDs (column 1), the Gene symbol (column 2) and the Gene name (column 3) of the eight genes in this KEGG pathway.

Entrez Gene ID	Symbol	Gene name
11951	Atp5g1	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 1
11958	Atp5k	ATP synthase, H+ transporting, mitochondrial F1F0 complex, subunit e
11984	Atp6v0c	ATPase, H+ transporting, lysosomal V0 subunit C
170658	BC002163	cDNA sequence BC002163
228033	Atp5g3	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c (subunit 9), isoform 3
66445	Cyc1	cytochrome c-1
68194	Ndufb4	NADH dehydrogenase (ubiquinone) 1 beta subcomplex 4
69875	Ndufa11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11

Table 9 Genes in KEGG pathway Glycolysis / Gluconeogenesis

The KEGG pathway Glycolysis / Gluconeogenesis was significantly downregulated by PKB activation in 5 out of 5 of the studied time points. This table shows the Entrez Gene IDs (column 1), the Gene symbol (column 2) and the Gene name (column 3) of the ten genes in this KEGG pathway.

Entrez Gene ID	Symbol	Gene name
11669	Aldh2	aldehyde dehydrogenase 2, mitochondrial
11674	Aldoa	aldolase 1, A isoform
13806	Eno1	enolase 1, alpha non-neuron
14751	Gpi1	glucose phosphate isomerase 1
18641	Pfkl	phosphofructokinase, liver, B-type
18648	Pgam1	phosphoglycerate mutase 1
18655	Pgk1	phosphoglycerate kinase 1
18746	Pkm2	pyruvate kinase, muscle
21991	Tpi1	triosephosphate isomerase 1
407972	Gapdh	glyceraldehyde-3-phosphate dehydrogenase

In this study we have made an attempt to distinguish between PI3K and PKB activation on the level of the regulation of gene expression. Our analysis demonstrates that while PI3K and PKB activate a large group of overlapping genes (25%), both kinases can also regulate a group of genes independently of each other. Our study shows confirms that this signalling module plays an important role in cellular proliferation by regulating genes involved in cell cycle progression. Furthermore, PI3K activation specifically regulates a number of genes involved in MAPK signalling and PKB activation results in changes in cellular metabolism. We hypothesize that these changes in signalling play a major part in the differential effect PI3K and PKB activation have on long-term cell survival.

In conclusion, the data presented here provide the first global characterisation of PI3K and PKB regulated gene expression. For verification of the microarray data, we will focus on investigating genes that are regulated two-fold or more (supplemental data). In the future it would be interesting to compare our data set with previously published gene expression profiles from human tumour samples to establish whether it is possible to identify specific PI3K/PKB signatures. Identification of such a PI3K/PKB "signalling signature" might allow stratification of current therapeutic strategies for such tumours.

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SUPPLEMENTAL DATA

To refine the number of identified genes we would verify, we limited the list to genes that were regulated two-fold or more in at least one of the studied time points (Supplemental data Table 1-7). Supplemental Figure 1 shows the distribution of the two-fold or more regulated genes.

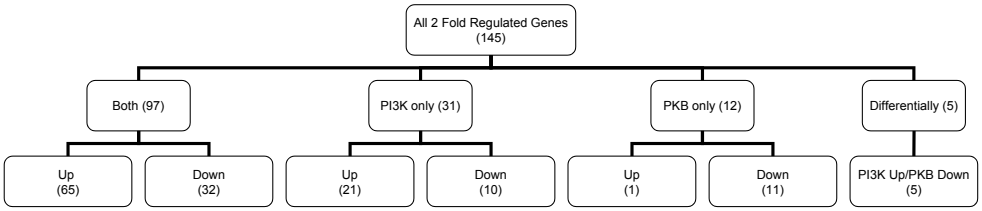


Figure 1 Schematic representation of all two-fold or more regulated genes

Table 1 Genes ≥2 fold upregulated by both PI3K and PKB activation
65 genes are ≥2 fold significantly upregulated by both PI3K and PKB activation. The table shows the Gene symbol (column 1), the Entrez Gene IDs for the genes (column 2), the Gene name (column 3) and the mouse gene identifier (MGI) (column 4). The 5th column gives the fold change (FC) after PKB activation and the 6th column gives the FC after PI3K activation at the latest time point (4 hours) unless the fold change is higher at another time point in the time course then t1he time point is annotated between brackets ().

Symbol	Entrez Gene IDs	Gene name	MGI	FC PKB	FC PI3K
Psmc3ip	19183	proteasome (prosome, macropain) 26S subunit, ATPase 3, interacting protein	1098610	2.1	3.0
Cycs	13063	cytochrome c, somatic	88578	2.3	3.7
Tuba4	22145	tubulin, alpha 4	1095410	1.9	3.5
Srm	20810	spermidine synthase	102690	1.7	4.4
Rcor2	104383	REST corepressor 2	1859854	1.6	3.7
Hspa8	15481	heat shock protein 8	105384	2.0	3.5
Timm8a	30058	translocase of inner mitochondrial membrane 8 homolog a (yeast)	1353433	1.7	3.0
Orc1l	18392	origin recognition complex, subunit 1-like (S.cereviaiae)	1328337	1.5 (120)	3.5
Ccne1	12447	cyclin E1	88316	1.6	3.0
Hells	15201	helicase, lymphoid specific	106209	1.6	4.0
Uhrf1	18140	ubiquitin-like, containing PHD and RING finger domains, 1	1338889	1.7	4.1

Cdr2	12585	cerebellar degeneration-related 2	1100885	2.4	2.6
Myohd1	66196	myosin head domain containing 1	1913446	1.4	2.7
Zmynd19	67187	zinc finger, MYND domain containing 19	1914437	1.4 (120)	2.5
Ctps	51797	cytidine 5'-triphosphate synthase	1858304	1.8	2.8
Aars	234734	alanyl-tRNA synthetase	2384560	1.5 (60)	2.6
Nol5a	67134	nucleolar protein 5A	1914384	1.6	2.9
Hsp110	15505	heat shock protein 110	105053	2.0	2.7
Cdc6	23834	cell division cycle 6 homolog (S. cerevisiae)	1345150	1.6	3.0
Tipin	66131	timeless interacting protein	1921571	1.6 (120)	2.7
Nap1l1	53605	nucleosome assembly protein 1-like 1	1855693	1.4	2.5
Pcna	18538	proliferating cell nuclear antigen	97503	1.6	2.7
Mcm4	17217	minichromosome maintenance deficient 4 homolog (S. cerevisiae)	103199	1.6	2.6
Fen1	14156	flap structure specific endonuclease 1	102779	1.9	2.9
Sfrs2	20382	splicing factor, arginine/serine-rich 2 (SC-35)	98284	1.7	2.5
Pyp	67895	pyrophosphatase	97831	1.8	2.8
Actb	11461	actin, beta, cytoplasmic	87904	1.5	2.5
Dnclc1	56455	dynein, cytoplasmic, light chain 1	1861457	2.2	2.0
Polr3e	26939	polymerase (RNA) III (DNA directed) polypeptide E	1349452	1.4	2.5
Isg20l1	68048	interferon stimulated exonuclease gene 20-like 1	1915298	1.5	2.3
Rrs1	59014	RRS1 ribosome biogenesis regulator homolog (S. cerevisiae)	1929721	1.7	2.4
Cpne1	266692	RNA binding motif protein 12	1922960	1.6	2.1
Bop1	12181	block of proliferation 1	1334460	1.5	2.5
Comtd1	69156	catechol-O-methyltransferase domain containing 1	1916406	1.5	2.0
Bysl	53414	bystin-like	1858419	1.6	2.2
Hspa5	14828	heat shock 70kD protein 5 (glucose-regulated protein)	95835	2.1	1.7
Hspca	15519	heat shock protein 1, alpha	96250	1.5	2.2
Fasn	14104	fatty acid synthase	95485	1.6	2.2

Sfrs3	20383	splicing factor, arginine/serine-rich 3 (SRp20)	98285	1.7	2.3
Hspd1	15510	heat shock protein 1 (chaperonin)	96242	1.8	2.5
Reln	19699	reelin	103022	1.7	2.2
Dnaja1	15502	DnaJ (Hsp40) homolog, subfamily A, member 1	1270129	1.6 1.8	2.1 2.3
C1qbp	12261	complement component 1, q subcomponent binding protein	1194505	1.4	2.0
Ptp4a1	19243	protein tyrosine phosphatase 4a1	1277096	1.5	2.0
Nol1	110109	nucleolar protein 1	107891	1.4	2.0
Odc1	18263	ornithine decarboxylase, structural 1	97402	1.7	2.4
Mcm5	17218	minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	103197	1.5	2.1
Cacybp	12301	calcyclin binding protein	1270839	1.6	2.2
Hnrpab	15384	heterogeneous nuclear ribonucleoprotein A/B	1330294	1.6	2.1
Rrm2	20135	ribonucleotide reductase M2	98181	1.6	2.4
Ranbp1	19385	RAN binding protein 1	96269	1.8	2.4
Lyar	17089	Ly1 antibody reactive clone	107470	1.6	2.3
Grwd1	101612	glutamate-rich WD repeat containing 1	2141989	1.5	2.3
Fubp1	51886	far upstream element (FUSE) binding protein 1	1196294	1.8	2.2
Pdxp	57028	pyridoxal (pyridoxine, vitamin B6) phosphatase	1919282	1.5	2.3
Sfrs7	225027	splicing factor, arginine/serine-rich 7	1926232	1.7	2.2
Gps1	209318	G protein pathway suppressor 1	2384801	1.5	2.4
Nasp	50927	nuclear autoantigenic sperm protein (histone-binding)	1355328	1.7	2.5
Kctd15	233107	potassium channel tetramerisation domain containing 15	2385276	1.3 (120)	2.1
Mrpl12	56282	mitochondrial ribosomal protein L12	1926273	1.5	2.1
Dut	110074	deoxyuridine triphosphatase	1346051	1.5	2.1
Ccne2	12448	cyclin E2	1329034	1.5	2.4
Orc6l	56452	origin recognition complex, subunit 6-like (S. cerevisiae)		1.4	2.1
Tubb6	67951	tubulin, beta 6	1915201	1.8	2.4
Idh3a	67834	isocitrate dehydrogenase 3 (NAD+) alpha	1915084	1.8	2.0

Table 2 Genes ≥2 Fold downregulated by both PI3K and PKB activation

32 genes are ≥2 fold significantly downregulated by both PI3K and PKB activation. The table shows the Gene symbol (column 1), the Entrez Gene IDs for the genes (column 2), the Gene name (column 3) and the mouse gene identifier (MGI) (column 4). The 5th column gives the fold change (FC) after PKB activation and the 6th column gives the FC after PI3K activation at the latest time point (4 hours) unless the fold change is higher at another time point in the time course then the timepoint is annotated between brackets ().

Symbol	Entrez Gene IDs	Gene name	MGI	FC PKB	FC PI3K
Hist1h1c	50708	histone 1, H1c	1931526	-2.7	-3.8
Hbp1	73389	high mobility group box transcription factor 1	894659	-3.0	-2.4
Nme7	171567	non-metastatic cells 7, protein expressed in	2449121	-3.2	-2.5
Ypel3	66090	yippee-like 3 (Drosophila)	1913340	-4.2	-4.2
Cmas	12764	cytidine monophospho-N-acetylneuraminic acid synthetase	1337124	-2.4	-3.0
Wdr45	54636	WD repeat domain 45	1859606	-2.6	-2.3
Abtb1	80283	ankyrin repeat and BTB (POZ) domain containing 1	1933148	-2.7	-2.3
Ccl9	20308	chemokine (C-C motif) ligand 9	104533	-1.7	-2.6
Tmprss7	208171	transmembrane serine protease 7	2686594	-2.6	-2.0
Frmd6	319710	FERM domain containing 6	2442579	-1.6	-2.2 (120)
Zfp99	67235	zinc finger protein 99	1914485	-2.0 (60)	-2.2 (60)
Tnfaip8l2	69769	tumor necrosis factor, alpha-induced protein 8-like 2	1917019	-2.2	-1.7
Ppp1r10	52040	protein phosphatase 1, regulatory subunit 10	1289273	-1.5 (120)	-2.1
Rgs2	19735	regulator of G-protein signaling 2	1098271	-1.9	-2.4 (120)
Avil	11567	advillin	1333798	-2.2	-2.0
Calcoco1	67488	calcium binding and coiled coil domain 1	1914738	-2.2	-2.0
Pck2	74551	phosphoenolpyruvate carboxykinase 2 (mitochondrial)	1860456	-1.3	-2.2
Fars2	69955	phenylalanine-tRNA synthetase 2 (mitochondrial)	1917205	-1.4	-2.0
Sipa1l1	217692	signal-induced proliferation-associated 1 like 1	2443679	-1.6	-2.1
Arhgap18	73910	Rho GTPase activating protein 18	1921160	-1.7	-2.4
Klk22	13646	kallikrein 22	95291	-2.2	-1.9
Mxi1	17859	Max interacting protein 1	97245	-1.9	-2.1
Pink1	68943	PTEN induced putative kinase 1	1916193	-2.3	-1.9

Txnip	56338	thioredoxin interacting protein	1889549	-2.4	-1.3 (240) /+1.4 (60)
Clk1	12747	CDC-like kinase 1	107403	-1.9	-2.1 (120)
Tbc1d17	233204	TBC1 domain family, member 17	2449973	-1.6	-2.2
Flcn	216805	folliculin	2442184	-2.0	-2.1
Wbscr27	79565	Williams Beuren syndrome chromosome region 27 (human)	1933146	-2.2	-1.7
Sat1	20229	spermidine/spermine N1-acetyl transferase 1	98233	-2.0	-1.5
Slc7a7	20540	solute carrier family 7 (cationic amino acid transporter, y+ system), member 7	1337120	-2.3	-1.7
Sla2	77799	Src-like-adaptor 2	1925049	-1.8 (120)	-2.0 (120)
Maf1	68877	MAF1 homolog (S. cerevisiae)	1916127	-2.1	-1.4

Table 3 Genes ≥2 Fold upregulated by PI3K activation

21 genes are ≥2 fold significantly upregulated by PI3K activation. The table shows the Gene symbol (column 1), the Entrez Gene IDs for the genes (column 2), the Gene name (column 3) and the mouse gene identifier (MGI) (column 4).

The 5th column gives the fold change (FC) after PI3K activation at the latest time point (4 hours) unless the fold change is higher at another time point in the time course then the timepoint is annotated between brackets ().

Symbol	Entrez Gene IDs	Gene name	MGI	FC
Eif5	217869	eukaryotic translation initiation factor 5	95309	3.0
Fosl1	14283	fos-like antigen 1	107179	2.8
D18Wsu98e	28062	thioredoxin-like 4	1351613	3.0
Ris2	67177	retroviral integration site 2	1914427	2.9
Gart	14450	phosphoribosylglycinamide formyltransferase	95654	2.7
Actb	14461	actin, beta, cytoplasmic	87904	2.1
Gzmb	14939	granzyme B	109267	2.1 (60)
Pold2	18972	polymerase (DNA directed), delta 2, regulatory subunit	1097163	2.0
Ccnd2	12444	cyclin D2	88314	2.5
Exo1	26909	exonuclease 1	1349427	2.3 (120)
E2f1	13555	E2F transcription factor 1	101941	2.1
Camkk2	207565	calcium/calmodulin-dependent protein kinase kinase 2, beta	2444812	2.0
Shmt1	20425	serine hydroxymethyl transferase 1 (soluble)	98299	2.2
Dhfr	13361	dihydrofolate reductase	94890	2.0
Gnl3	30877	guanine nucleotide binding protein-like 3 (nucleolar)	1353651	2.0
Pprc1	226169	peroxisome proliferative activated receptor, gamma, coactivator-related 1	2385096	2.2
Stk16	20872	serine/threonine kinase 16	1313271	2.1
Srf	20807	serum response factor	106658	2.1 (120)
Msto1	229524	misato homolog 1 (Drosophila)	2385175	2.2
Mrps18b	66973	mitochondrial ribosomal protein S18B	1914223	2.1
Mybbp1a	18432	MYB binding protein (P160) 1a	106181	2.1

Table 4 Genes ≥2 Fold downregulated by PI3K activation

10 genes are ≥2 fold significantly downregulated by PI3K activation. The table shows the Gene symbol (column 1), the Entrez Gene IDs for the genes (column 2), the Gene name (column 3) and the mouse gene identifier (MGI) (column 4). The 5th column gives the fold change (FC) after PI3K activation at the latest time point (4 hours) unless the fold change is higher at another time point in the time course then the timepoint is annotated between brackets ().

Symbol	Entrez Gene IDs	Gene name	MGI	FC
Tspan8	216350	tetraspanin 8	2384918	-2.7
Itsn1	16443	intersectin 1 (SH3 domain protein 1A)	1338069	-3.3
Tsc22d3	14605	TSC22 domain family 3	1196284	-2.3
Fars2	69955	phenylalanine-tRNA synthetase 2 (mitochondrial)	1917205	-2.1
Clcn3	12725	chloride channel 3	103555	-2.3
Osgepl1	72085	O-sialoglycoprotein endopeptidase-like 1	1919335	-2.2
Pde3b	18576	phosphodiesterase 3B, cGMP-inhibited	1333863	-2.2
Acsl1	14081	acyl-CoA synthetase long-chain family member 1	102797	-2.0
Phlpp	98432	PH domain and leucine rich repeat protein phosphatase	2138327	-2.2
Smc4l1	70099	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	1917349	-2.0

Table 5 Genes ≥2 Fold upregulated by PKB activation

1 gene is ≥2 fold significantly upregulated by PKB activation. The table shows the Gene symbol (column 1), the Entrez Gene IDs for the genes (column 2), the Gene name (column 3) and the mouse gene identifier (MGI) (column 4).

The 5th column gives the fold change (FC) after PKB activation at the latest time point (4 hours) unless the fold change is higher at another time point in the time course then the timepoint is annotated between brackets ().

Symbol	Entrez Gene IDs	Gene name	MGI	FC
Fgf15	14170	fibroblast growth factor 15	1096383	2.9

Table 6 Genes ≥2 Fold downregulated by PKB activation

11 genes are ≥2 fold significantly downregulated by PKB activation. The table shows the Gene symbol (column 1), the Entrez Gene IDs for the genes (column 2), the Gene name (column 3) and the mouse gene identifier (MGI) (column 4). The 5th column gives the fold change (FC) after PKB activation at the latest time point (4 hours) unless the fold change is higher at another time point in the time course then the timepoint is annotated between brackets ().

Symbol	Entrez Gene IDs	Gene name	MGI	FC
Bnip3	12176	BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3	109326	-2.6
Pfkl	18641	phosphofructokinase, liver, B-type	97547	-2.6 (120)
Muc20	224116	mucin 20	2385039	-3.4
Etv5	104156	Ets variant gene 5	1096867	-3.3
Slco4a1	108115	solute carrier organic anion transporter family, member 4a1	1351866	-2.3 (120)
Rgs11	50782	regulator of G-protein signaling 11	1354739	-2.5 (30)
Slc2a3	20527	solute carrier family 2 (facilitated glucose transporter), member 3	95757	-2.4
Pgam1	18648	phosphoglycerate mutase 1	97552	-2.1 (120)
Gna15	14676	guanine nucleotide binding protein, alpha 15	95770	-2.0 (120)
Aldoa	11674	aldolase 1, A isoform	87994	-2.1 (60)
St3gal2	20444	ST3 beta-galactoside alpha-2,3-sialyltransferase 2	99427	-2.0

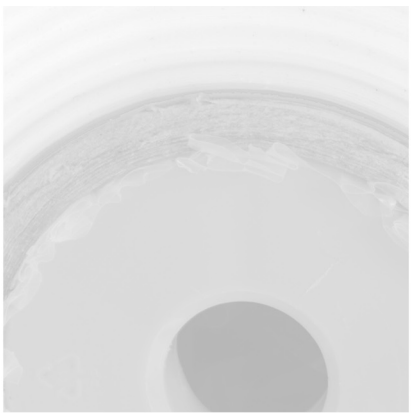
Table 7 Genes ≥2 Fold differentially regulated by both PI3K and PKB activation

5 genes are ≥2 fold significantly differentially regulated by both PI3K and PKB activation. The table shows the Gene symbol (column 1), the Entrez Gene IDs for the genes (column 2), the Gene name (column 3) and the mouse gene identifier (MGI) (column 4). The 5th column gives the fold change (FC) after PKB activation and the 6th column gives the FC after PI3K activation at the latest time point (4 hours) unless the fold change is higher at another time point in the time course then the timepoint is annotated between brackets ().

Symbol	Entrez Gene IDs	Gene name	MGI	FC PKB	FC PI3K
NM_ 010495		inhibitor of DNA binding 1 (Id1), mRNA		-1.5 (30)	2.0
Tuba8	53857	tubulin, alpha 8	1858225	-1.7	2.4
Dusp6	67603	dual specificity phosphatase 6	1914853	-5.7 (120)	1.6 (60)
F2r	14062	coagulation factor II (thrombin) receptor	101802	-3.5	1.5 (60)
Myc	17869	myelocytomatosis oncogene	97250	-2.0	1.7

CHAPTER 6

Discussion


The identification and characterisation of PI3K/PKB-mediated signalling events opens opportunities for development of previously unrecognised targets for anti-cancer therapeutics. In this thesis we have identified and characterised novel signalling components downstream of PKB. We have specifically focussed on the role of PKB in cell survival and have performed phosphoproteomic and microarray analysis to identify novel downstream phosphorylation and transcriptional targets. This thesis is part of ongoing research into clarifying the specific role PKB plays in oncogenic transformation.

Regulation of Foxo3a protein levels

In chapter two we demonstrate that chronic PKB activation is in itself insufficient for cell survival. Higher levels of oxidative stress caused by prolonged PKB activation resulted in increased levels of Foxo3a leading to expression of its transcriptional targets Bim and p27kip1 (Chapter 2, Fig 4A). Up-regulation of these proteins ultimately results in cell cycle arrest and subsequently apoptosis. We concluded that the observed increased Foxo3a expression was caused by transcriptional up-regulation since both Foxo3a promotor activity as well as mRNA levels were shown to be increased upon PKB activation.

In cells ectopically expressing Foxo3a a modest increase in protein levels upon H_2O_2 treatment could also be observed (data not shown). While these enhanced protein levels might still be a result of transcriptional activation, Foxo3a turnover could also be affected. In order to investigate this further, we treated Ba/F3 cells with the proteasome inhibitor MG132. When MG132 was added in the presence of IL-3, endogenous Foxo3a protein levels were dramatically increased within four hours of treatment, indicating that under normal homeostatic conditions Foxo3a proteins are rapidly degraded (Fig. 1A). However, addition of MG132 in combination with 4-OHT-mediated chronic PKB activation did not give rise to a further increase of Foxo3a protein levels compared to PKB activation alone (Fig. 1B). This suggests

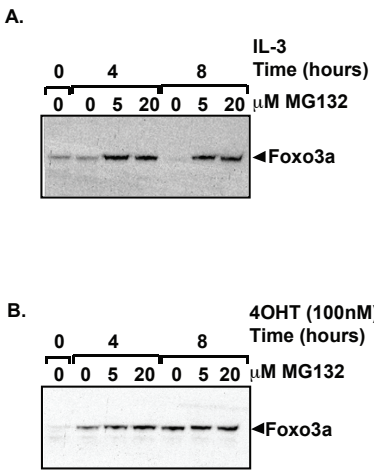


Figure 1: PKB activation inhibits Foxo3a proteasomal degradation

Ba/F3 myrPKB:ER cells were cultured for the indicated times in the presence of IL-3 (Fig. 1A) or 4-OHT (Fig. 1B) with or without the indicated concentrations of proteasome inhibitor MG132 added to the medium, lysed and equal amounts of protein were analyzed for levels of Foxo3a.*

that chronic PKB activation might already result in the blockage of proteasomal degradation of this transcription factor. This leads us to the conclusion that the observed increase of Foxo3a protein levels upon PKB activation is not solely a result of transcriptional up-regulation but there are probably also alterations in its proteosomal degradation.

In contrast, it has been previously shown that phosphorylation of Foxo3a by PKB leads to its degradation (6). The proteasome has an important role in the degradation of normal, damaged, mutant, or misfolded proteins. This includes the degradation of normal and regulatory proteins in the cellular metabolism and additionally the removal of damaged proteins as a stress response (13). But how could chronic PKB activation inhibit proteasomal function? We have established that chronic PKB activation results in a state of cellular oxidative stress (Chapter 2). Oxidants produce modifications to proteins leading to loss of function (or gain of undesirable function). These oxidized protein derivatives tend to aggregate, and accumulation of such aggregates may lead to cell death. The degradation of non-functional, oxidized proteins by the proteasome is an essential part of the antioxidant defences of cells (1,2,7). Two well-characterised proteasome components, the 11S and the 19S regulators, form together with the 20S 'core' proteasome, the ATP- and ubiquitin-dependent 26S proteasome (12). Relatively mild oxidative stress rapidly (but reversibly) inactivates both the ubiquitin activating/conjugating system and 26S proteasome activity. It was found that the 26S proteasome is much more susceptible to oxidative stress than the 20S proteasome (8). It is proposed that aging, and various degenerative diseases, result in increased oxidative stress, leading to elevated levels of protein oxidation, cross-linking, and aggregation. Since these products of severe oxidative stress also inhibit the 20S proteasome, they result in a vicious cycle of progressively worsening accumulation of cytotoxic protein oxidation products.

The apparent rapid turnover of Foxo3a under normal conditions is regulated by the proteasome and the increased oxidative state of the cell may inhibit proteasome activity in general. However, it is also possible that chronic PKB activation specifically alters Foxo3a targeting to the proteasome for example by inhibiting ubiquitination of this transcription factor. Furthermore, recently the proteasome inhibitor bortezomib was shown to increase oxidative stress in cells through a yet unknown mechanism (5). Therefore, addition of MG132 could also result in increased Foxo3a levels via oxidative stress-mediated transcriptional upregulation. This needs to be further investigated. It also appears that there is a maximum level of Foxo3a protein since higher concentrations of MG132 and longer periods of proteasome inhibition do not result in increasing levels of Foxo3a protein. It is likely that Ba/F3 cells enter an apoptotic program when a certain threshold level of Foxo3a protein is reached.

We have already clearly demonstrated that the regulation of Foxo3a protein levels can have important implications for its activity. It would be interesting to be able to distinguish between transcriptional and proteasomal regulation of Foxo3a protein levels. Therefore, we would like to further investigate the regulation of Foxo3a

protein levels by the proteasomal system under conditions of chronic PKB activation and oxidative stress and to study the physiological implications of these fluctuating Foxo3a protein levels. The recent finding that FoxO transcription factors are tumour suppressors (4) has only heightened interest in the molecular mechanisms regulating FoxO expression.

mTOR and regulation of PKB activity

The discovery of mTORC2 as the long sought after PDK-2 that phosphorylates PKB at Ser473 by Sarbassov and co-workers (10) and the finding that this complex is sensitive to prolonged rapamycin treatment in a cell type specific manner (9), has prompted the re-examination of previous studies in which rapamycin was used. In cells that express rapamycin-sensitive mTORC2 the assembly and thus activity of the complex is suppressed. Rapamycin blocks the interaction of rictor to newly synthesized mTOR, whereas the interaction between raptor and mTOR is weakened immediately (9). However, for reasons yet unknown the level of this inhibition is cell type specific for the mTORC2 complex. It has been suggested that in some cell lines an unidentified protein or a post-translational modification shields the inhibitory site on mTOR. Interestingly, rapamycin-sensitive mTORC2 is often observed in haematopoietic cell lines. Therefore, we were interested in the effect of long-term rapamycin treatment in the bone marrow-derived cell line that we have used as a model system in this thesis. Pre-treatment of Ba/F3 myrPKB:ER* cells with rapamycin completely inhibits the phosphorylation of PKB on Ser473, indicating that mTORC2 in these cells is indeed rapamycin-sensitive (Fig. 2). In Chapter 2 we investigated cell survival in the presence or absence of rapamycin (Chapter 2, Fig. 2C). We measured apoptosis after 4-OHT-mediated PKB activation in the presence or absence of rapamycin. In contrast to cells cultured in the presence of IL-3, rapamycin was able to inhibit the rescue of cytokine-starved cells by PKB activation. Taking into account that rapamycin blocks PKB activation as indicated by the lack of PKB phosphorylation on Ser473, it is perhaps not surprising that the effect of PKB activation on survival is inhibited. However, long-term rapamycin treatment in combination with IL-3 does not lead to increased apoptosis even though phosphorylation of PKB on Ser473 is inhibited when cells pre-treated with rapamycin are stimulated with IL-3 (Chapter 2, Fig. 2C). It could be that there is still some residual PKB activity otherwise it would appear that PKB activity is actually not *per se* required for cytokine-mediated survival. In contrast, we have previously shown that PI3K inhibition in cytokine treated Ba/F3 cells is sufficient to induce apoptosis. This implies that even though PKB is an important mediator of PI3K function, there are other effectors downstream of PI3K that in some way regulate cell survival. In Chapter 5, even though it is a work in progress, we have made an important step in distinguishing PI3K and PKB mediated signal transduction pathways. Furthermore, it would be interesting to investigate the effects of PI3K activation under conditions of PKB inhibition by rapamycin in processes such as

survival and metabolism.

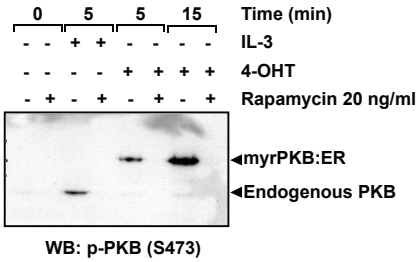


Figure 2: Pre-treatment of Ba/F3 cells with mTOR inhibitor rapamycin inhibits PKB phosphorylation on Ser473

Ba/F3 myrPKB:ER cells were cultured for 24 hours in the presence or absence of mTOR inhibitor rapamycin, then the cells were cytokine starved again in the presence or absence of rapamycin, subsequently cells were either left untreated or stimulated with IL-3 or 4-OHT for the indicated times, lysed and equal amounts of protein were analyzed for levels of phosphorylated PKB (Ser473).*

Lost in Translation

eIF4B phosphorylation sites are regulated in a hierarchical manner

In chapter four of this thesis, we have identified eIF4B Ser422 as a novel PKB substrate site and phosphorylation of Ser406 was found to be regulated by RSK. In the addition to the experiments presented in Chapter 4 of this thesis we also analysed phosphorylation of eIF4B and eIF4B in which Ser422 was mutated to an alanine by PKB in an *in vitro* kinase assay. COS cells were transfected with FLAG-tagged human eIF4B isoforms, immunoprecipitated and incubated together with active PKB in a kinase assay and phosphorylation was detected using the phospho-PKB substrate antibody. As shown in Figure 3, in an *in vitro* kinase assay Ser406 of eIF4B is in fact a substrate for PKB in contrast to what we have found *in vivo* upon cellular stimulation with insulin. The high degree of homology between the kinase

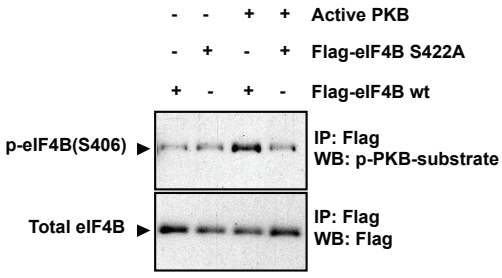


Figure 3: PKB phosphorylates eIF4B on Ser422 in vitro

FLAG-tagged eIF4B or FLAG-tagged eIF4B in which Ser422 was mutated to alanine was phosphorylated by PKB in an in vitro kinase assay. Proteins were incubated without active PKB present as a control. Samples were analyzed for levels of phospho-PKB substrate and FLAG.

domains of members of the AGC protein kinase family can result in "promiscuous" activity when it comes to phosphorylation of substrates. Therefore in *in vitro* kinase assays members of the AGC kinase family may phosphorylate substrates at the same site. *In vivo*, however, phosphorylation of these sites is a highly specific and regulated process. This data clearly highlights the importance of confirming *in vitro* phosphorylation experiments in intact cells.

Interestingly, when eIF4B Ser422 was mutated to an alanine PKB was no longer able to phosphorylate Ser406. To investigate this further we transfected A14 cells with either eIF4B or eIF4B in which either serine 406 or 422 is mutated to an alanine and cells were stimulated with insulin after overnight serum starvation. Phosphorylation on serine 422 is increased by insulin stimulation regardless of the phosphorylation status of serine 406 (Fig. 4, second panel) In contrast, phosphorylation on serine 422 appears to inhibit phosphorylation of serine 406 since mutation of serine 422 to a non-phosphorylatable alanine results in a high basal phosphorylation of Ser406 compared to eIF4B in unstimulated cells (Fig. 4, upper panel). It could be that the phosphorylation status of serine 422 affects the accessibility of this region of the eIF4B. Stimulation of cells with insulin did not result in a further increase in serine 406 phosphorylation. These data suggest that "priming" of Ser422 phosphorylation can regulate subsequent Ser406 phosphorylation. This has important consequences for regulation of eIF4B activity *in vivo* suggesting that multiple kinase inputs can modulate translational activity.

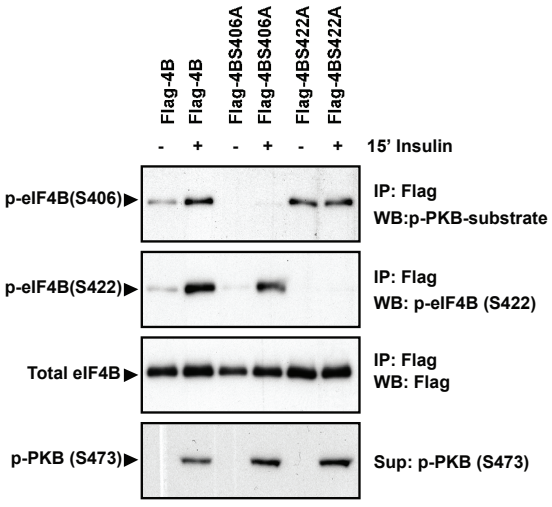


Figure 4: Mutation of eIF4B on Ser422 blocks the induction of Ser406 phosphorylation

A14 cells were transfected with FLAG-tagged wild type eIF4B or mutant eIF4B in which Ser406 or 422 had been mutated to alanine. Cells were serum-starved overnight and subsequently stimulated for 15 minutes with insulin before immunoprecipitating the FLAG-tagged protein. The immunoprecipitated FLAG-eIF4B protein was analyzed for levels of phospho-PKB substrate, phospho-eIF4B (Ser422) and FLAG as a loading control. The whole cell lysate was analyzed for phospho-PKB (Ser473) as a control for insulin stimulation.

Cell type specific regulation of eIF4B phosphorylation

In Ba/F3 myrPKB:ER* cells we showed that phosphorylation of Ser422 on eIF4B is regulated by PKB however, we also wanted to determine how this phosphorylation site is regulated by cytokine stimulation. We cytokine-starved cells overnight and stimulated them with either IL-3 or 4-OHT in the presence or absence of either MEK inhibitor U0126 or Rapamycin. Again we observed that pre-treatment with rapamycin only slightly reduces phosphorylation of eIF4B on serine 422 in response to either IL-3 or 4-OHT (Fig. 5). However in this cell system U0126 alone was

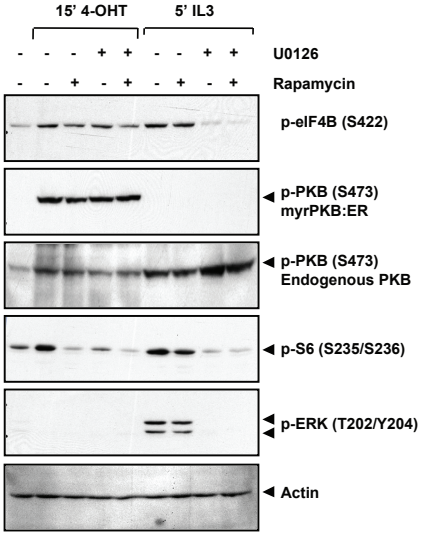


Figure 5: Phosphorylation of eIF4B on Ser422 in response to IL-3 is regulated by ERK1/2 activation

Ba/F3 myrPKB:ER cells were cytokine starved and left untreated or treated with either IL-3 or 4-OHT (100nm) for 5 or 15 minutes respectively after or without pre-treatment with either or both rapamycin (20 ng/ml) or U0126 (15 μM), lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (Ser422), phospho-PKB (Ser473), phospho-S6 (Ser235/Ser236), phospho-ERK1/2 (T202/Y204) and actin.

sufficient to inhibit IL-3 induced eIF4B phosphorylation, as well as S6 phosphorylation. Thus, these data support previous studies by Shahbazian et al who have shown that p70SK and RSK work together in controlling the phosphorylation status of eIF4B on serine 422 in Hela cells stimulated with serum (11). Recently it was also demonstrated that inhibition of ERK alone was capable of diminishing eIF4B phosphorylation on serine 422 in HT-29 human colorectal adenocarcinoma cells (3) and in haematopoietic stem cells (our own unpublished data). Taken together, our data show that *in vivo* phosphorylation of eIF4B is a highly regulated process that occurs in a cell type and stimulus specific manner. The use of various AGC kinase family members allows this mechanism of translational control to be regulated through distinct stimulus-specific intracellular signalling pathways.

Concluding remarks

Interest in the role of PKB in neoplastic transformation has increased enormously over the past decade, and it is now evident that activation of the PKB pathway is one of the most common molecular alterations in human malignancy. Importantly, many consequences of hyperactivated PKB signalling are considered as hallmarks of cancer. In this thesis we have identified novel signalling components downstream of PKB as part of ongoing research into the specific role PKB plays in oncogenesis. We have shown that PKB can regulate translation through phosphorylation of eIF4B. Translational control plays a major role in regulating protein expression, which is crucial for cell growth, survival and cellular homeostasis. Important cellular signalling pathways such as the RAS/MEK/ERK pathway and the PI3K/PKB/mTOR pathway control the translational machinery by phosphorylating a large number of translation initiation factors. The PI3K/PKB/mTOR signalling pathway is intimately implicated in cancer development and progression, as many of its components are either mutated or amplified in human cancers and these changes deregulate protein translation. Understanding the molecular basis of translational deregulation in cancer will hopefully contribute to the development of novel anticancer therapeutic strategies.

Our large-scale microarray screen of PI3K and PKB regulated genes gives us an overview of the processes regulated by these prominent proto-oncogenes. The upregulation of a large group of genes involved in the regulation of cell cycle progression makes sense since cancer is frequently considered to be a disease of the cell cycle. Verification of these genes on the transcriptional and protein expression level will help elucidate the role of PI3K and PKB in the deregulation of cell division in human cancer. The microarray analysis has also demonstrated that PKB activation independently of PI3K activation has a significant effect on cellular metabolism. These alterations in metabolism could possibly be the cause of the state of oxidative stress in cells in which PKB is chronically activated, subsequently resulting in the upregulation of Foxo3a protein and its pro-apoptotic transcriptional targets. In future experiments we will attempt to elucidate the crucial players at the transcriptional level in the cellular functions of these two proto-oncogenes.

Although many new roles for PKB in regulating cellular homeostasis have emerged over the last few years, it is still unclear whether all functions attributed to this kinase are truly mediated by it. The limitations in the approaches used to investigate the function of PKB complicate the verification of these processes *in vivo*. It is important that future work in this area is aimed at developing pharmacological reagents and both genetic and biochemical approaches that not only identify novel substrates for PKB but also verify whether the physiological functions already ascribed to PKB have been appropriately assigned. While the research described in this thesis is a start, the generation of a potent and specific PKB inhibitor, or even an activator, would certainly revolutionise the study of the processes mediated by this important player in intracellular signal transduction.

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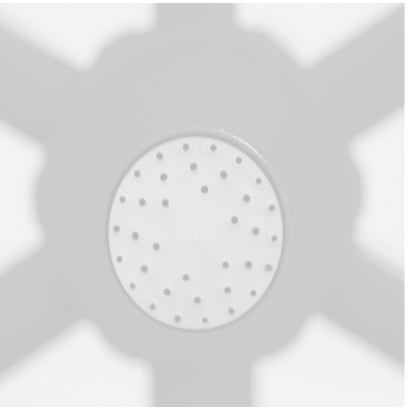


SUMMARY

Cancer is the result of a disturbed balance between cell division and growth on one hand, and programmed cell death on the other. Crucial cellular processes such as proliferation, apoptosis and differentiation are regulated by a complex network of signal transduction pathways. Therefore, cancer can be viewed as a disease of deregulated signal transduction: cancer cells grow when they should not and do not die (i.e. undergo apoptosis) when they should. This malignant transformation is driven by accumulative genetic alterations including tumour suppressor gene loss, oncogene amplification and chromosomal derangements. Elucidation of disturbed signal transduction pathways involved in the carcinogenic process, both at the protein and the genomic levels, is therefore critical for the identification of new targets for cancer treatment.

The phosphoinositol-3 kinase (PI3K)-protein kinase B (PKB) signalling pathway is crucial to many aspects of cell growth and survival. Deregulation of PI3K and PKB occurs by a variety of mechanisms in a large percentage of human tumours. Illustrative of this fact is that increased cellular metabolism is an important hallmark of cellular transformation. Transformed cells generally exhibit greatly increased levels of nutrient utilization compared with their non-transformed counterparts; in particular, glucose metabolism is increased. PI3K and PKB have been implicated in regulation of these metabolic processes. Furthermore, aberrant PKB signalling not only plays a role in cancer onset and progression but it has also been suggested to play a role in other pathological conditions such as obesity, diabetes and premature aging. Together, these facts have made the elucidation of the PI3K-PKB pathway the subject of many studies.

In recent years, targeted deletion of specific isoforms of PKB in mice has proved to be a powerful tool for elucidating the physiological roles of PKB proteins, as well as the redundancy and specific functions of the different isoforms. These mice knockout studies support a role of PKB in metabolism, glycolysis, insulin signalling, growth, vascularisation and lipid metabolism. Some of the PKB substrates that mediate these processes have been identified. For example, it has been shown that PKB directly phosphorylates and inhibits members of the FOXO subfamily of forkhead transcription factors. Interestingly, broad somatic deletion of FOXO transcription factors results in a progressive cancer-prone condition, indicating that these proteins function as tumour suppressors. Regulation of the activity of FOXO transcription factors is thus a critical means through which PKB modulates cellular homeostasis.

The continued elucidation of the role of the PI3K-PKB pathway in cell growth, survival, and proliferation has shed light on why regulation of this pathway is often altered within tumours. The prevalence of a hyperactivated PI3K-PKB pathway in human cancers suggests that cancer cells might be more sensitive to inhibitors of this pathway than normal cells. The continuing efforts to develop specific, high-affinity inhibitors against the PI3K-PKB pathway have the potential to yield new

therapeutics to treat human cancer. The identification and characterisation of PI3K/PKB-mediated signalling events opens opportunities for development of previously unrecognised targets for anti-cancer therapeutics.

Therefore in this thesis we have focussed on the identification and characterisation of novel signalling components downstream of PKB. We have specifically focussed on the role of PKB in cell survival and have performed phosphoproteomic and microarray analysis to identify novel downstream phosphorylation and transcriptional targets. This thesis is part of ongoing research into clarifying the specific role PKB plays in oncogenic transformation.

In **chapter two,** we investigated the effect of chronic PKB activation on cellular survival and proliferation using cytokine-dependent bone marrow-derived Ba/F3 cells in which PKBα activation can be directly, and specifically, induced by addition of 4-hydroxytamoxifen (4-OHT). Direct activation of PKB rescued Ba/F3 cells from cytokine-withdrawal-induced apoptosis, however, surprisingly, these anti-apoptotic effects were short-lived, cells only being protected for up to 48 hours. We observed that activation of PKB in survival factor deprived cells led to a dramatic increase of Foxo3a on both the transcriptional and protein level leading to expression of its transcriptional targets Bim and $p27^{kip1}$. High levels of PKB activity result in increased aerobic glycolysis and mitochondrial activity resulting in overproduction of reactive oxygen species (ROS). To determine whether oxidative stress might itself be responsible for Foxo3a upregulation we added N-acetylcysteine (NAC), a thiol-containing radical oxygen scavenger to the culture medium. This prolonged the life-span of cells treated with 4-OHT and prevented the upregulation of Foxo3a protein levels caused by PKB activation suggesting that deregulated PKB activation leads to oxidative stress resulting in Foxo3a upregulation and subsequently cell death. Taken together, our data in this chapter provide novel insights into the molecular consequences of uncontrolled PKB activation.

Chapter three describes a phosphoproteomic approach to identify novel PKB substrates. To define the precise role protein kinases play in cellular physiology, it is necessary to have a clear picture of the *in vivo* substrates through which those kinases exert their actions. For PKB there have been many reports describing *in vitro* substrates, however many of these remain to be validated *in vivo*. Validation of "proper" PKB substrates is complicated by the lack of specific inhibitors for this kinase. In order to identify novel PKB substrates BaF3 myrPKB:ER* cells were stimulated with 4-OHT in order to induce phosphorylation of PKB substrates. Phosphorylated proteins were separated by phospho-Ser/Thr affinity purification, analyzed by 2D SDS-PAGE and western blotting utilizing an antibody raised against the minimal PKB consensus phosphorylation site (RXRXXS/T). Hybridisation with this phospho-PKB substrate antibody resulted in the identification of several proteins whose phosphorylation was regulated upon specific PKB activation. These included eukaryotic translation initiation factor 4B (eIF4B), 5'-Phosphoribosyl N-formylglycinamide amidotransferase (FGARAT), Minichromosome Maintenance

proteins (MCM) 3, MCM6, Lamin B1 and Moesin. We discuss the role of those identified proteins as novel *in vivo* PKB targets and the feasibility of this approach for phosphoprotein characterization.

In **chapter four,** we further characterise eukaryotic translation initiation factor 4B (eIF4B) as a novel substrate of PKB. eIF4B plays a critical role in stimulating the helicase activity of eIF4A to unwind inhibitory secondary structures in mRNAs and recruit the 40S ribosomal subunit to those mRNAs. eIF4B is a multiphosphorylated protein and phosphorylation can control its functional activity. PKB was found to phosphorylate eIF4B on Ser422 *in vitro* and *in vivo* after mitogen-stimulation. Furthermore, we identified Ser406 as a novel mitogen-regulated phosphorylation site. Phosphorylation of Ser406 was found to be regulated by p90 ribosomal S6 kinase (RSK) *in vivo*. Utilising a translational control reporter system (TCRS), phosphorylation of both residues was found to be physiologically relevant in regulating the translational activity of eIF4B. These data provide novel insight into complex multi-kinase regulation of eIF4B phosphorylation and reveal an important mechanism by which PKB can regulate translation, potentially critical for the transforming capacity of this AGC kinase family member.

In **chapter five**, we show that whereas PKB activation alone is insufficient for long-term cell survival, PI3K activation is sufficient. Using a microarray gene expression profiling approach we sought to uncover the molecular mechanisms underlying these different effects on cell survival. Our study revealed that while PI3K and PKB have many common transcriptional targets there are also distinct cellular processes which can be independently regulated. PI3K and PKB were both found to significantly regulate expression of genes implicated in cell cycle progression. The upregulation of a large group of genes involved in the regulation of cell cycle progression makes sense since cancer is frequently considered to be a disease of the cell cycle. Verification of these genes on the transcriptional and protein expression level will help elucidate the role of PI3K and PKB in the deregulation of cell division in human cancer. Interestingly, we also found that PKB specifically modulated expression of genes involved in the regulation of metabolism and oxidative phosphorylation. This PKB-specific gene set could be detrimental to cellular homeostasis resulting in the induction of apoptosis observed after chronic PKB activation. The use of transcriptional profiling in this manner allows detailed analysis of the architecture of signalling pathways to be undertaken. Furthermore, utilizing this approach we hope to reveal a PI3K/PKB "signalling signature" which might be helpful in the molecular characterisation of human neoplasias.

For the future it is important that work on the PI3K-PKB pathway is aimed at developing pharmacological reagents and both genetic and biochemical approaches that not only identify novel substrates for PKB but also verify whet=her the physiological functions already ascribed to PKB have been appropriately assigned. The research described in this thesis makes an important start.

NEDERLANDSE SAMENVATTING (voor de leek)

In het dagelijkse leven worden wij als mensen steeds geconfronteerd met keuzes. Bij de meeste keuzes staan we niet eens meer stil. Je kunt naar je werk lopen, fietsen, of je kunt de trein of de auto nemen. Meestal neem je het vervoersmiddel dat je elke dag neemt en denk je er verder niet meer over na. Waar je waarschijnlijk ook niet bij stilstaat is dat jij niet de enige in je lijf bent die steeds keuzes maakt. De cellen waaruit je opgebouwd bent, nemen ook keuzes. Cellen hebben simpel gezegd maar drie keuze mogelijkheden. De eerste keuze mogelijkheid is doodgaan. Ja, voor cellen is dat een 'keuze', zelfmoord is onder cellen nog steeds de grootste doodsoorzaak. We noemen het alleen liever gereguleerde celdood of apoptose. De tweede keuze mogelijkheid is delen. In principe blijft in een volwassen mens het aantal cellen waaruit hij/zij bestaat gelijk. Als er eens een paar cellen afsterven, dan gaan de overgebleven cellen groeien en delen tot de normale hoeveelheid cellen weer bereikt is. Maar ook bij het volwassen worden is celdeling van groot belang. We beginnen namelijk als één enkele cel: de bevruchte eicel. Nu zijn we een volwassen persoon die bestaat uit miljarden cellen. De derde keuze mogelijkheid is differentiëren. Wij mensen zijn gelukkig niet zomaar een vormloze klont cellen. We bestaan uit verschillende celtypen. Die diversiteit in cellen zorgt ervoor dat we eruitzien zoals we er nu uitzien en dat we kunnen zien, horen, een hart hebben enz.

Met deze samenvatting hoop ik jullie wat meer inzicht te geven in hoe keuzes op celniveau gemaakt worden, oftewel in hoe het besluitvormingsproces verloopt. En hoe belangrijk het voor ons als mensen is dat die keuzes goed gemaakt worden.

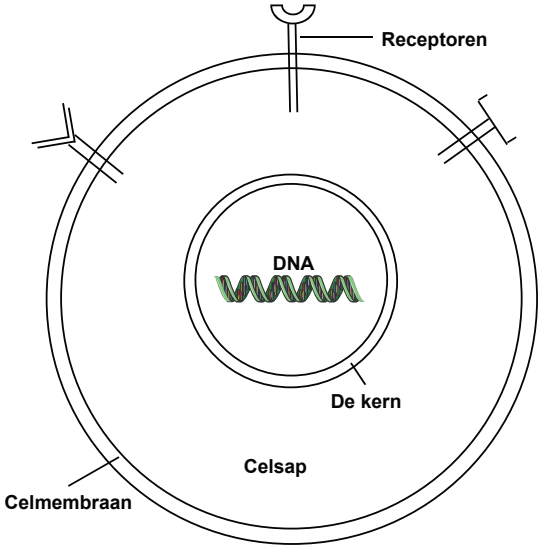
Om te begrijpen hoe keuzes op celniveau gemaakt worden moet ik eerst wat meer vertellen over ons lichaam en welke rol cellen in ons lichaam spelen. Vervolgens zal ik wat meer vertellen over cellen zelf en over hoe het besluitvormingsproces in het werk gaat.

Er zijn organismen die uit maar één cel bestaan, zoals bacteriën en gisten. In zo'n ééncellig organisme (die je kunt vergelijken met een soort eenmansbedrijfje) doet die ene cel alles wat nodig is om te blijven leven, zoals het opnemen van voedingsstoffen, het omzetten van deze stoffen in levensenergie en het uitscheiden van afvalstoffen. Het menselijk lichaam is opgebouwd uit miljarden cellen. In het menselijke lichaam zijn cellen geen autonome eenheden. Elke cel heeft zijn eigen specifieke plaats en functie. Je kunt dit vergelijken met een werknemer in bijvoorbeeld een groot bedrijf of bij de overheid. Zo zijn er o.a. spiercellen, hersencellen, huidcellen, botcellen, bloedcellen en zenuwcellen. Deze verschillende cellen ontstaan via een complex proces dat differentiatie wordt genoemd uit slechts één enkele embryonale 'stamcel' (de bevruchte eicel).

Wat is nu eigenlijk een cel?

Een cel bestaat hoofdzakelijk uit eiwitten, suikers en vetten. Eiwitten zijn de werkpaarden van de cel. In een cel spelen zich een groot aantal processen af. Er moet bijvoorbeeld zuurstof worden omgezet in energie, er moet contact gehouden

worden met naburige cellen, er moet snel gereageerd kunnen worden op veranderende omstandigheden, er moeten bouwstenen worden aangemaakt en afgebroken en ga zo maar door. Al deze processen worden uitgevoerd door eiwitten. In één cel zijn duizenden van deze eiwitten werkzaam in honderden diverse processen.
Eiwitten bevinden zich veelal in het celsap. Dit celsap wordt omsloten door een membraan. Dit is een soort vlies met onder andere communicatiemiddelen met de buitenwereld (=receptoren, een soort voelsprieten). In het binnenste van een cel bevindt zich een archief (=kern) met genetische informatie (=DNA) die nodig is om de cel zelf en dus het organisme als geheel te laten functioneren (Figuur 1).



Figuur 1 Schematisch overzicht van de cel

Het DNA kun je onderverdelen in genen. Genen zijn stukken DNA, die een code zijn voor de aanmaak van een bepaald eiwit. Je kunt genen zien als handleidingen voor het maken van eiwitten. Stel dat je een computer wilt maken en je koopt hiervoor een handleiding. Zo'n handleiding lijkt niet meer dan inkt op papier, tegelijkertijd is het een code voor de handelingen die je moet verrichten om de computer te bouwen. Zo werkt ook een cel als hij eiwitten wil maken. Hij leest hiervoor de juiste handleiding (gen) en hij weet dan welke bouwstenen hij moet gebruiken om het juiste eiwit te bouwen. De hele verzameling van genen is dus een blauwdruk van een cel. De codes voor de aanmaak van alle eiwitten staan erin. Het is belangrijk dat alle genen worden verdubbeld op het moment dat een cel gaat delen, zodat beide cellen uiteindelijk weer een volledige set van genen en dus een volledige blauwdruk meekrijgen.

Hoe vormen al die losse cellen samen een mens?

Vaak zijn gelijksoortige cellen georganiseerd in organen: verschillende type levercellen vormen de lever, huidcellen vormen de huid en spiercellen vormen onze spieren. Cellen moeten goed en nauwkeurig met elkaar samenwerken om het orgaan en het totale lichaam juist te laten functioneren. Zoals in iedere complexe organisatie is communicatie van cruciaal belang voor deze goede samenwerking. Cellen beïnvloeden elkaars gedrag door het uitzenden van signaalstoffen of signaalmoleculen. Die signaalstoffen worden ook wel hormonen genoemd. Sommige hormonen reizen niet zo ver en bereiken alleen naburige cellen. Andere hormonen reizen via de bloedbaan en worden door cellen elders in het lichaam herkend via zogenaamde receptoren. Deze 'voelsprieten' zitten meestal aan de buitenkant van de cel op het celmembraan. Dit systeem werkt volgens een 'slot-sleutel principe': alleen de juiste sleutel (signaalmolecuul/hormoon) brengt beweging in het slot (de receptor), bij een verkeerde sleutel blijft het slot dicht. Om een idee te geven van de complexiteit van dit systeem: er bestaan wel duizenden verschillenden soorten receptoren en net zoveel signaalmoleculen/hormonen. Een bekend voorbeeld van zo'n verreizend molecuul is epo. Bij hoogtestages is de zuurstofspanning veel lager dan normaal. Onder invloed daarvan gaan de nieren meer epo produceren. Epo zet rode bloedcellen aan tot deling, zodat het bloed meer zuurstof kan vervoeren naar de spieren. Valsspelende wielrenners spuiten ook wel epo in bij zich zelf om dit effect op een snellere manier te bewerkstelligen.

Vaak krijgen cellen een hele waaier aan signalen tegelijk binnen. Het is erg belangrijk dat een cel al deze signalen op de juiste wijze interpreteert. Cellen maken daarom gebruik van netwerken van met elkaar communicerende eiwitten. Op deze manier worden de binnenkomende signalen geïntegreerd. Dit leidt uiteindelijk tot een bepaald besluit van de cel (bijvoorbeeld delen of doodgaan). Deze besluiten hebben als uiteindelijk doel het lichaam adequaat te laten functioneren.

Hoe kan het dat alle cellen hetzelfde DNA hebben en er toch verschillende typen cellen zijn?

Het DNA dat zich in de celkern bevindt, is de code voor de aanmaak van eiwitten. Ieder verschillend celtype brengt zijn eigen specifieke combinatie van eiwitten tot expressie, maar elke cel bevat als het goed is hetzelfde DNA. Daarom wordt DNA wordt ook wel de 'blauwdruk van het leven' genoemd. Iedere cel van het menselijke lichaam bevat 23 paar chromosomen die opgebouwd zijn uit dit DNA. In het menselijke genoom (= het geheel aan erfelijk materiaal) zitten zo'n 20.000-25.000 genen. Een gen is een stuk DNA dat alle informatie bevat voor het maken van een specifiek eiwit. Zoals gezegd heeft de cel niet alle eiwitten tegelijkertijd nodig. Sommige eiwitten bevorderen de celdeling terwijl andere deze juist remmen. Tijdens de celdeling moeten de eiwitten van de laatste categorie dus niet gemaakt worden. Daarnaast zijn er eiwitten die juist wel of niet in een bepaald celtype voorkomen. Zo wordt het zuurstof bindende eiwit hemoglobine uitsluitend in rode bloedcellen

gemaakt. Iedere situatie in ieder celtype vereist zo een specifieke set van eiwitten. Een belangrijke vraag binnen de moleculaire biologie is hoe deze specificiteit in de aanmaak van nieuwe eiwitten (=eiwitsynthese) gerealiseerd wordt. Om iets meer te weten te komen over specificiteit in de eiwitsynthese is het belangrijk om te weten hoe een gen nu 'vertaald' kan worden in een eiwit.

Hoe wordt een gen vertaald in een eiwit?

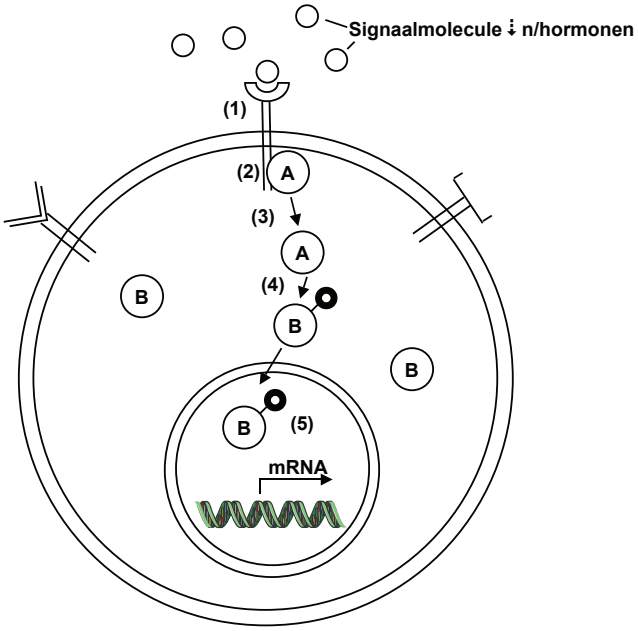
Allereerst wordt er een kopie (mRNA) van het gen gemaakt. Het gen wordt als het ware overgeschreven. Net als materiaal uit een archief, mag je DNA dus niet zomaar 'meenemen' maar moet je er eerst een kopie van maken om ervoor te zorgen, dat de originele informatie (DNA) goed bewaard blijft. Dit proces heet transcriptie en wordt uitgevoerd door eiwitten genaamd transcriptiefactoren. De DNA kopie (mRNA) wordt vervolgens omgezet oftewel vertaald naar een eiwit. Dit proces wordt translatie genoemd.

Eiwitsynthese is gekoppeld aan de signalen van buiten de cel. Een cel is namelijk niet autonoom maar werkt samen met andere cellen in het uitoefen van een bepaalde functie in een organisme. Signalen zoals bijvoorbeeld groeifactoren kunnen binden aan receptoren in de celmembraan. Vanaf de celmembraan wordt het signaal door eiwitten doorgegeven naar de celkern, waar transcriptiefactoren de mRNA's maken van de eiwitten die nodig zijn voor dit specifieke signaal. Welke transcriptiefactoren zogezegd aan of uit worden gezet, wordt bepaald door externe signalen. Dit bepaalt welke specifieke set mRNA's en dus eiwitten er worden gemaakt. Die bepalen vervolgens wat de cel gaat doen. Wordt de bloedstamcel een rode bloedcel of een bloedplaatje? Gaat de oude maagwandcel dood? Gaat de naburige maagwandcel delen om de leegte op te vullen?

Rest nog één vraag: Hoe wordt nou precies een signaal van buiten de cel via de receptor doorgegeven naar de kern?

Zoals reeds eerder vermeld wordt dit signaaltransductie genoemd, een moeilijke benaming voor signaaloverdracht. Cellen maken gebruik van netwerken van met elkaar communicerende eiwitten. Dus niet alleen cellen communiceren met elkaar maar de eiwitten in de cel ook. Eiwitten doen dit bijvoorbeeld door hun chemische activiteit (hun kunstje) op elkaar los te laten. Ze koppelen bijvoorbeeld een extra chemische groep aan een eiwit, waardoor dat eiwit zijn eigenlijke functie anders gaat vervullen. Op deze manier kan een eiwit de werking van een ander eiwit reguleren. Proteïne kinase B (PKB), het eiwit waar dit boekje vol van staat, is bijvoorbeeld in staat een fosfaat groep aan eiwitten te koppelen. Een deel van de eiwitten waarbij PKB dit doet zou zonder deze fosfaat groep de cel doden, het zijn zogenaamde 'moordeiwitten'. De fosfaat groep houdt deze eiwitten in toom met als resultaat dat de cel blijft leven.

Hoe leidt dit nu tot veranderingen in de cel?
Een signaalmolecuul bindt aan een receptor (Figuur 2, stap 1).De receptor is normaal gekoppeld aan eiwit A (stap 2). Door de binding met het signaalmolecuul verandert de receptor van vorm en laat eiwit A los (stap 3). De actieve/chemische kant van eiwit A is daarom nu los in de cel en hangt niet meer aan het membraan. In de cel botst eiwit A tegen eiwit B en verandert dat eiwit door er een extra chemische groep aan te zetten (stap 4). Door die extra chemische groep wordt eiwit B, dat toevallig een transcriptiefactor is, actief (stap 5). Een transcriptiefactor is zoals al eerder gezegd een factor die DNA kan overschrijven naar mRNA, wat uiteindelijk vertaald wordt naar eiwit. Een actieve transcriptiefactor zorgt er dus voor dat er een eiwit wordt gemaakt dat eerst niet werd gemaakt.



Figuur 2 Schematisch overzicht van signaaltransductie

Als dit eiwit bijvoorbeeld hemoglobine is dan kan dit een onderdeel zijn in de ontwikkeling van deze cel naar een rode bloedcel. Als het een eiwit is dat nodig is voor celdeling, kan dit proces leiden tot deling. Maar het kan ook een zogeheten zelfmoord eiwit zijn, dat bijvoorbeeld begint met het kapot knippen van het DNA wat dan het begin is van de dood van de cel.
De regulatie van celdood, celdeling en celdifferentiatie is een delicaat proces. Een goede celdifferentiatie is belangrijk, omdat we alle verschillende celtypes nodig hebben om als mens goed te functioneren. Zonder voldoende hersencellen zaten we hier nu niet. Ook zijn fouten in het celdifferentiatieproces één van de grootste

oorzaken van leukemie bij kinderen.
Als mensen zijn we gewend om dood als iets slechts te zien. De gereguleerde dood van cellen is echter nodig voor het goede functioneren van ons lichaam. Tijdens de embryonale ontwikkeling moeten de cellen in de vliezen tussen de vingertjes afsterven anders zien de handjes van een baby eruit als die van een kikker. Verder geven op deze manier oude cellen hun taak over aan nieuwe frisse cellen, voordat ze echt hun functie niet meer kunnen doen. Ze gaan als het ware met pensioen in plaats van dat ze er tijdens het uitvoeren van hun taak erbij neervallen.
Bij fouten in de celdeling denkt men het eerste aan één ziekte: kanker. Als er te veel celdeling plaatsvindt, kan er een tumor ontstaan. Een cel kan bijvoorbeeld in afwezigheid van de juiste signalen gaan delen. De cel wijkt af van zijn normale functie op dat moment. Dit kan gebeuren door een mutatie in het DNA waardoor er een transcriptiefactor geproduceerd wordt die niet meer chemisch gemodificeerd hoeft te worden om actief te zijn, maar die altijd een eiwit maakt dat aanzet tot celdeling. Zo'n fout in de signaaltransductie kan dan leiden tot een tumor.
Gelukkig blijft het overgrote deel van onze cel netjes in het gareel. De juiste keuzes worden gemaakt. Er gaat echter geregeld wel eens wat mis op celniveau ook bij mensen die niet ziek zijn en die geen kanker krijgen. In de meeste gevallen werken de cellen in het lichaam samen om de 'bad guys' aan te pakken. Kortom, na al die jaren onderzoek is het lichaam nog steeds een wonder en zelfs aan schijnbaar simpele keuzes gaat een heel proces vooraf.

Mijn proefschrift
Wat heb ik nu precies gedaan/ontdekt in de afgelopen jaren? Ik heb gewerkt aan een eiwit genaamd PKB. Wanneer dit eiwit actief is, zet het een fosfaat groep aan andere eiwitten. Op deze manier reguleert PKB andere eiwitten, waaronder zogenaamde 'moordeiwitten'. Ik heb ontdekt dat alhoewel PKB een rol speelt in het remmen van deze moordeiwitten, PKB activiteit alleen niet genoeg is voor het langdurig levend blijven van cellen. Verder heb ik een eiwit (eIF4B) gevonden waaraan PKB een fosfaat groep zet. Het was nog niet bekend dat PKB dat bij dit eiwit kon doen. Aangezien eIF4B een rol speelt bij translatie (het omzetten van mRNA naar eiwit) is dit een erg belangrijke bevinding voor de verduidelijking van de functie van PKB in de cel. Als laatste heb ik gekeken van welke genen middels het proces transcriptie mRNA wordt gemaakt als PKB actief is. Deze informatie is vergeleken met de genen waarvan mRNA wordt gemaakt als PI3K (eiwitbroertje van PKB) actief wordt. Dit om een onderscheid te kunnen maken tussen de functies die beide eiwitten in de cel vervullen. Aangezien PKB (en ook PI3K) in tumoren vaak actiever is dan in normale cellen, kan informatie over het precieze functioneren van PKB in de cel gebruikt worden voor bijvoorbeeld een betere typering van tumoren en een meer doelgerichte behandeling daarvan. Dit proefschrift zet een belangrijke stap in die richting, maar de rest is werk voor in de toekomst.

DANKWOORD

"Zo moet het eigenlijk altijd zijn.....het leven, een feestelijk en hartstochtelijk spel gespeeld met nieuwe en oude vrienden"

Frederique Spigt in Wie is de mol? 2006

Het mogen schrijven van een dankwoord is misschien wel het leukste aspect van promoveren. Ik realiseer me dat dit een erg gewaagde uitspraak is, maar als je het objectief bekijkt: wanneer zal ik ooit nog de kans krijgen om iedereen in mijn leven te bedanken? Mijn kansen op het winnen van een olympische medaille, Wimbledon, een Oscar of de Nobelprijs zijn nu toch wel grotendeels verkeken. Ik schrijf dit zonder enige wroeging daarover, maar ik ben me wel ten volle bewust hoe speciaal het is dat ik deze kans krijg. Verder zal dit deel van mijn proefschrift het meest gelezen worden en misschien zelfs wel, al hoop ik toch echt van niet, ook het meest bediscussieerd.

Paul Er is veel veranderd in de jaren dat ik voor je heb gewerkt. Drie verhuizingen, je werd professor en het lab ging van signaaltransductie lab naar stamcel-immuno lab. Als ik in de afgelopen vier en een half jaar één ding van je heb geleerd is het wel dat als je iets wil dat je er dan ook echt voor moet gaan en als je iets vindt, je er ook echt voor moet staan. Mijn dank daarvoor.

Kim Je was drie jaar lang mijn alwetende buurvrouw oftewel superpostdocos. En om het nu maar op papier te zetten, zodat je het nooit meer zelf hoeft te vragen: je vertrek naar Organon liet een leegte achter op het lab. Persoonlijk beschouw ik mijn vriendschap met jou als een van de hoogtepunten van mijn AIO-tijd. Ik ben blij dat we onze filmdates, kolonistenavondjes en roddelkletsjes voort hebben kunnen zetten. Enne: Wann könnten EJ und ich nun mit dir in die Heimat gehen? Zo, kom daar nu nog maar eens onderuit!
Kristan Leuk hoor, zo'n lief, klein, blond en onschuldig vermomming, waar kan ik die kopen? Lijkt me best handig! Bedankt voor het verbreden van mijn culturele horizon, zingen op de 'Sound of Music' en natuurlijk mijn 'mood-clock', die mede door jou vaak op 'blij meisje' stond. In de laatste jaren was jij degene die mij het meeste uitdaagde, je had altijd goede vragen en suggesties over mijn proeven of een artikel gelezen dat ik nog niet eens had ontdekt. Als die Foxos nou eens een keer mee zouden werken, dan zou dat helemaal perfect zijn. Ach, je kan anders ook nog altijd weer een keer aan p70SsucksKinase gaan werken. Voor mij ben je in ieder geval de grootste kleine AIO van de afdeling immunologie!
Kim en Kristan Ik ben blij en trots dat ik op 7 juni mijn labmama en mijn labzusje achter me heb staan. Bedankt dat jullie mijn paranimfen willen zijn.

De Coffers! *Christian* Nog even en jij mag ook je dankwoord schrijven, denk daar

maar aan als je het met het einde in zicht even niet meer ziet zitten. Bij deze wil ik je bedanken voor je oprechte interesse, de kolonistenavondjes en je onverwachte enthousiasme voor de eigenwijsjes en andere door mij verzonnen gekkigheden. Ik wacht op je boekje! *Jorg* Onze eigen labpuppy, Adonis, bijna net zo hot als Foxp3 of waren dat jouw eigen woorden? Een ding is zeker, er is nog een hele wereld van 'scoopers' die door jou 'gescoopd' kunnen gaan worden. Go for it! *Loes* Wie moet er nu toch je liefdesleven coördineren nu ik niet meer op het lab ben? Of gaat het misschien allemaal wel een stuk beter als ik me er niet mee bemoei. Veel succes met de laatste loodjes! *Tessa* Halfzusje! Succes met Lair en de stamcellen. Zal ik voor jou nog even een routebeschrijving naar het Academiegebouw maken? *Cornelieke* Door jou waren zelfs drie verhuizingen geen reden meer voor verlenging. Ik heb met veel plezier met jou aan het 'Mega Microarray experiment' gewerkt. Veel succes met het in het gareel houden van het lab. *Miranda* Realiseer je je eigenlijk wel dat sinds ik je Lara het Labschaap heb gegeven, je carrière toch echt in de lift zit? Ik acht hiermee dan ook de kracht van mascottes bewezen. Veel succes met je kudde analisten en natuurlijk de stammies. *Jeffrey* Man, wat kan jij een onzin uitkramen! En van blotjes kun je met photoshop ook echt kunst maken, het ziet er geweldig uit, maar of het nu echt de bedoeling is? Je was een fijne labtafel-buurman. Succes met de wetenschap of wordt het toch de aandelenhandel? *Liesbeth* Brabant, carnaval, de harmonie... Jouw verhalen deden me altijd weer denken aan 'thuis'. Je was een fijne collega. Houdoe! *Edwin* Veel succes met het zoeken naar de groene cellen (=speld) in je muizen (=hooiberg). Newbies *Lianne en Rhandy* Het wordt toch weer een meidenlab op de eerste! Werk ze! *Hanneke* Dat nieuwsgierige hoofdje dat opkeek de eerste keer dat ik het lab op liep, zal ik niet snel vergeten. Enorme kletskous! Bedankt voor de 'Chi', het doen van alle rottige labklusjes en het zelfgemaakte ijs niet te vergeten. Ben je niet trots op me dat ik niet eens iets zeg over het feit dat je meer verdient dan de meeste AIO's en analisten en dat je een afscheidskado en feest kreeg en vervolgens toch gewoon bleef! Wanneer mag ik weer komen Bonanza-en en bramen-ijs eten? *Robert* Bedankt voor je inzet. Het was een genot om je als student te hebben. Ik ben blij dat student zijn bij een laatstejaars AIO je niet heeft afgeschrikt om verder te gaan in de wetenschap. Succes!

Ik begon mijn AIO-tijd bij de afdeling Longziekten, daarom wil ik graag de volgende mensen bedanken: *Bart, Caroline, Corneli, Deon, Evert, Falco, Henk, Jan, Jeroen, Lei, Madelon, Marcela en Vera*. En de volgende personen in het bijzonder: *Alex* Samen uit, samen thuis zat er jammer genoeg niet in. Ik heb van geen enkele 'journal club' zoveel geleerd als van de artikel besprekingen die ik met jou had. Bedankt voor je aanstekelijke enthousiasme voor de wetenschap, maar misschien nog wel meer voor de geweldige Indiase muziekjes, die ik nog vaak beluister! *Erik-Jan* Nog altijd ben je mijn favoriete OSM collega! Of moet ik zeggen collegos? Bedankt voor alle gezelligheid op het lab en ik ben blij dat we nog steeds vriendjes zijn. En je las het al: We gaan nu echt! *Laurien* Altijd goedlachs en bereid om iedereen

te helpen. Geen wonder dat iedereen met je samen wil werken. Bedankt voor je hulp met mijn sollicitatie presentatie. Veel plezier straks met de kleine (ik ben heel goed in het uitkiezen van leuke knuffels!). Maar we blijven wel kolonisten, toch? *Leo* Je promotorschap was van korte duur. Ik heb met veel plezier op jouw afdeling gewerkt. *Lisa B.* It was heaps of fun to have you in the lab! Thank you for all the new music (Missy Higgins!). *Lisa F.* Bedankt voor het wegwijs maken in de wereld van de 2D gelen, zoals je ziet heeft het ook nog wat opgeleverd! *Willem* Soms is promoveren gewoon heel erg hard werken, jij deed dat als geen ander. Ik heb daar enorm veel respect voor, ga nu ook maar lekker wat leuke dingen doen, zoals vaderzijn of zo.

Mijn AIO-loopbaan eindigde ik bij de afdeling Ieniemienie (=Immunologie). Ik heb de immunologie tijdens mijn studie altijd weten te ontwijken, maar zo is het leven, dat waar je voor wegloopt komt altijd weer op je pad terecht. De T-cellen hebben mijn hart niet weten te veroveren, de afdeling immunologie daarintegen wel. Ineens had ik een hele kudde AIO's om me heen, die allemaal in het zelfde schuitje zaten, geweldig! Ik wil graag de volgende mensen bedanken: Ana, Christine, Debbie, Elise, Floor P1, Floor P2, Frans, Gerrit, Henk, Henk Jan, Ineke, Ingrid, Jan, Joke, Jolanda, José, Kees, Kiki, Kristin, Leonie, Marco, Margreet, Marieke van L., Miranda B., Nening, Paul, Petra, Rogier, Ronald, Samantha, Simone, Sigrid, Suzanne, Talitha, Vivienne, Walter. En natuurlijk oud-ieniemienie-logen: *Miranda van B.* Ja, nu heb ik eindelijk tijd voor yoga! *Marielle* Dat het je gelukt is om een boekje af te maken, terwijl die Coffer AIO's de oren van je kop tetterden, vind ik nog steeds erg knap. *Robert Jan* Elke gelijkenis met het lettertype dat in jouw boekje is gebruikt berust op puur toeval, toch? *Paul* en *Nienke* Bedankt voor alle promotie/proefschrift-tips.
En niet te vergeten: *Frank* 'Het is niet de AIO, het is niet de begeleider, maar het is het proces.' 'Ziek zijn overkomt je, maar verzuim is een keuze.' 'Eigenlijk zouden we AIO's helemaal niet moeten betalen, ze zijn tenslotte PhD Students.' Zo kan ik nog wel even doorgaan wat betreft prikkelende uitspraken uit uw mond. Het is goed om te zien dat zelfs een afdelingshoofd nog een goed gevoel voor humor heeft. Ik heb met veel plezier op uw afdeling gewerkt, mijn dank daarvoor. *Linde, Jeanette, Saskia E. en Anton* bedankt voor de dinsdagochtend input in mijn projecten. *Linde* Ook bedankt voor de klare taal tijdens mijn voortgangsgesprekken: 'Waarom werk je daar nou nog aan, dat krijg je toch nooit meer af!' *Saskia ter B. en Yvonne* Bedankt voor al het geregel, vooral ook met de promotie. En *Saskia,* over de stoutste vrouw gesproken, ik weet wel wie dat bij de afdeling immunologie is. *Jantine* Bedankt voor alle 'phosphorylatie-hulp'. *Eva* Straks gezellig samen in de trein naar Den Haag! Leuk! *Charlotte* Met wie moet ik nu mijn tweede pannenkoek delen? *Marieke B.* Onwijze roddel! Wel lekker leven in de brouwerij blijven brengen daar op de tweede, hoor! *Lydia* Samen gestudeerd, samen student-assistent en toen op dezelfde afdeling AIO, volgens mij komen we elkaar nog wel eens ergens tegen. Bedankt voor de afleiding tijdens het schrijfproces. Ik zal de Wie is de mol?-competitie en de GA-

koffie missen, maar voor de UMC bingo kom ik in november terug!

De volgende mensen wil ik graag bedanken voor hun wetenschappelijke betrokkenheid bij het tot stand komen van dit proefschrift: *Eric Lam* In your lab everything always works! What's your secret? Thank you for all your help with chapter 2. Also a big thank you to *Karen, Rosaline and Ines. Boudewijn, Arjan* en *Niels* Bedankt voor alle mass spec hulp. *Arjan* Een gesprek met jou werkte altijd als een motiverende adrenaline stoot. Bedankt voor de discussies, je behulpzaamheid en je enthousiasme. *Frank Holstege* en *Marian* Bedankt voor het doen van het 'Mega Microarray experiment'. *Marian* Bedankt ook voor het uitleggen van Genespring en meer, zodat ik door de genen het hoofdstuk weer een beetje kon zien. *Dave Hall* Thank you for the enlightening discussions on chapter two.

Bedankt ook aan al mijn 'oude' en 'nieuwe' vriendjes en vriendinnetjes (waarvan sommige reeds hiervoor bij naam genoemd) die ik schaamteloos heb verwaarloosd om dit boekje te kunnen voltooien. Ik zal het nooit meer doen, ik beloof het. Bedankt voor alle afleiding in de vorm van leuke activiteiten zoals bijklets-café-avonden, bingo-en, bioscoopbezoeken, bowlen, concerten, DJ-en op PANN-Feest of in de Rits, kaartavonden, kolonisten, lekker eten in knusse restaurantjes, Lost kijken, theater, The L Word avondjes, L2 Convention (incl. twee keer op de foto met Laurel!), weekendjes weg en meer, veel veel meer. Bedankt ook voor jullie vriendschap, interesse en jullie geduld. En voor de niet-biologen/AIO's: Bedankt dat jullie mij er steeds weer aan herinnerden dat er meer is in het leven dan promoveren! Op 7 juni is het feest en jullie zijn uitgenodigd!

Als laatste wil ik graag mijn familie bedanken: *Mama* en *papa* Bedankt, omdat niemand mij ooit nog zoveel zal kunnen geven als jullie hebben gedaan. Ik heb jullie lief. *John, Karin* en *Robert* Mijn grote broers en grote zus. Zo anders en toch vaak schokkend hetzelfde. Best leuk dat jullie stukjes van mij zijn! En nu ik dan als enige in ons gezin ga promoveren, ben ik wel een beetje een groot zusje, toch? *Silvia*, *Michelle* en de mini's *Martijn* en *Nadine* Bedankt voor de gezelligheid. Eindelijk weer tijd om te poolen en/of een logeerpartijtje! *Mum* en *Dad* Bedankt voor alle steun, interesse en natuurlijk alle leuke dingen in Limbo-land! Lieve *Oma* Eigenlijk de oma van Sue, maar nu ook een beetje die van mij!

Lieve *Sue-ie!* Vriendinnetje zijn van iemand die aan het promoveren is, is niet altijd leuk. Samen zijn met iemand die gepromoveerd is, is vast veel leuker! Zullen we dat samen gaan ontdekken? Ja! Eindelijk weer meer samen, want mijn leven is leuker met jou. Bedankt dat je er altijd voor mij was/bent. Ik hou van jou!

CURRICULUM VITAE

Ankie van Gorp werd geboren op 8 februari 1979 te Breda. Na het behalen van haar VWO diploma aan het St. Odulphuslyceum in Tilburg, begon zij in 1997 aan de studie Biologie aan de Universiteit Utrecht. Als onderdeel van haar studie deed zij onderzoekservaring op tijdens twee stages. Een eerste stage werd afgelegd bij de afdeling Moleculaire Microbiologie van de Universiteit Utrecht onder supervisie van Prof Dr. J. Tommassen en Dr. M. El Khattabi. Vervolgens liep zij stage bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum Utrecht onder supervisie van Prof. Dr. J.L. Bos en Dr. J.M. Enserink. In 2002 behaalde zij haar doctoraal examen en startte zij haar promotieonderzoek bij de afdeling Longziekten en later de afdeling Immunologie van het Universitair Medisch Centrum Utrecht, onder begeleiding van Prof. Dr. P.J. Coffer. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.
Vanaf 1 april 2007 is zij werkzaam als wetenschappelijk onderzoeker bij het Nederlands Forensisch Instituut in Den Haag. Daar coördineert zij het DNA-onderzoek bij strafzaken en volgt zij een opleiding tot DNA-deskundige.