Analysis of the Protein Kinase B – Forkhead box O signaling pathway

Analyse van de proteine kinase B – Forkhead box O signaleringsroute

(met een samenvatting in het Nederlands)

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Table of contents

1 **7-34**
General introduction

2 **35-50**
Binding of protein kinase B to the plakin family member periplakin

3 **51-68**
In silico studies for discovery of novel PKB substrates and FOXO target genes

4 **69-78**
Protein kinase B phosphorylates pituitary-specific homeodomain transcription factors

5 **79-90**
Direct control of caveolin-1 expression by FOXO transcription factors

6 **91-96**
Concluding remarks

Summary **97-98**

Samenvatting **99**

Curriculum Vitae **100**

Dankwoord **101-102**

Abbreviations

4-OHT	4-hydroxy-tamoxifen
AFX	atypical fibroxanthoma
AR	androgen receptor
CDK	cyclin-dependent kinase
CREB	cAMP-responsive element binding protein
DAF	dauer formation
DB	DNA binding domain
DBE	DAF16-binding element
ECM	extracellular matrix
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ER	estrogen receptor
ERK	extracellular-regulated kinase
FKHR	Forkhead in rhabdomyosarcoma
FKHRL1	FKHR-like 1
FOXO	Forkhead box O
FSH	follicle-stimulating hormone
GH	growth hormone
GSK3	glycogen synthase kinase 3
HNF	hepatic nuclear factor
IGF1	insulin-like growth factor 1
IKK	IkappaB kinase
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MEF	mouse embryonic fibroblast
MLK	mixed-lineage kinase
MnSOD	manganese superoxide dismutase
mSOS	mammalian Son of Sevenless
mTOR	mammalian Target of Rapamycin
NLS	nuclear localization signal
NO	nitric oxide
PDGF	platelet-derived growth factor
PDK1	PI(3,4,5)P3-dependent kinase 1
PFK2	6-phosphofructo-2-kinase
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIP	phosphatidylinositol phosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PPL	periplakin
Rb	retinoblastoma tumor suppressor protein
RTK	receptor tyrosine kinase
S6K	S6 kinase
SEK1	stress-activated protein kinase ERK kinase 1
SH2	src homology 2
SGK	serum- and glucocorticoid-inducible kinase
TKO	triple knock-out
TNF	tumor necrosis factor
TSH	thyroid-stimulating hormone
WT	wild-type

Okay…… I think we should start (Hans)

Voor mijn ouders

Chapter

1

General introduction

Cellular processes such as proliferation, apoptosis and differentiation are the basis for normal cellular behavior and are regulated by a number of extracellular stimuli such as hormones, cytokines and growth factors. Via binding to (transmembrane) receptors these factors lead to regulation of intracellular signaling pathways, ultimately resulting in gene expression. When cells malfunction in one or more processes or when the balance between different processes is disturbed this can lead to abnormal cell behavior and pathological phenotypes such as cancer, obesity, diabetes and premature aging. This malfunctioning can arise when the activity of proteins involved in the pathways is changed due to altered expression, or mutations.

PI-3 kinase pathways

One of the major pathways involved in cellular processes is the phosphoinositide-3-kinase (PI3K)/protein kinase B (PKB/c-Akt) pathway. A well-studied extracellular stimulus for this pathway is insulin although there are numerous stimuli such as insulin-like growth factor (IGF), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). In haematopoietic cells this pathway can also be activated by a wide variety of extracellular agents such as antigens, interleukins (IL) and interferons (IFN) (reviewed in (92)). Also cellular adhesion to extracellular matrix via integrins leads to PI3K activation.

Here we will focus on insulin as a paradigm for PI3K/PKB activation. Upon binding of insulin to the insulin receptor, the receptor homodimerizes. Conformational changes within the intracellular domains of the receptor lead to auto-kinase activity and subsequently tyrosine residues in the carboxy-terminal part of the receptor become phosphorylated. Signaling proteins are then recruited to the phosphorylated residues at the cell membrane via binding to the receptor through SH2 domains. One of these proteins, p85, is the regulatory subunit of PI3K. Together with p110, the catalytic subunit, p85 forms an active PI3K complex at the plasma membrane. PI3K is a lipid kinase that can phosphorylate phosphatidylinositol lipids at the 3' position of the inositol ring. Phosphoinositol lipids that are phosphorylated on this position can serve as docking sites in the plasma membrane for proteins that contain specific domains i.e. the FYVE and PH domains, via which PI3K can exert its role in cellular processes (Figure 1).

The FYVE domain is named after the proteins in which this domain was first identified: Fab1p, YOTB, Vac1p and Early endosome antigen 1 (EEA1). This domain consists of a cysteine-rich zinc-finger-like motif

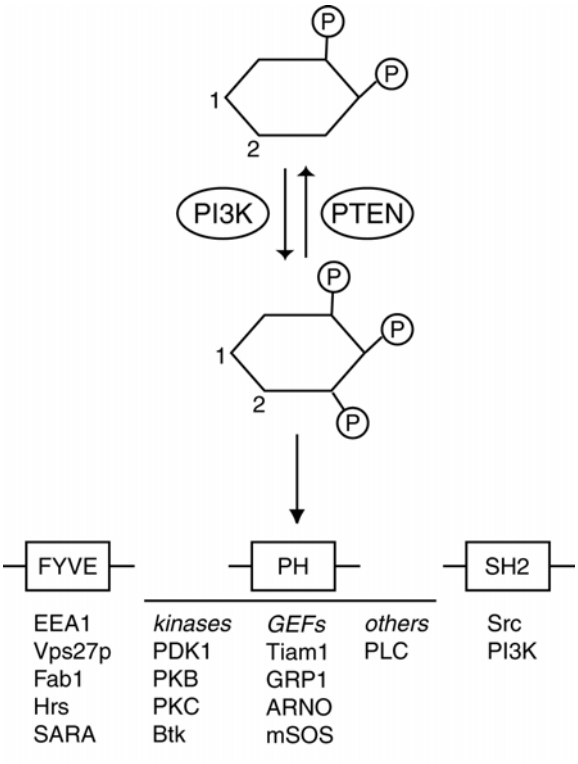

Figure 1: Effectors of PI(3,4,5)P3 lipids.
PI3K phosphorylates PI(4,5)P2, thereby creating PI(3,4,5)P3 lipids. This reaction is reverted by the lipid phosphatase PTEN. PI(3,4,5)P3 lipids can interacts with the depicted protein domains, that are present in the depicted proteins.

that coordinates two zinc atoms and which has basic amino acids surrounding the third cysteine residue. The basic amino acid residues together with the zinc atoms bind the acidic phosphate on the 3' position within the phosphoinositol lipid. Only monophosphatidic lipids can bind to FYVE domains. It has been demonstrated that PI3K has a role in Rab5-mediated endosome fusion through docking EEA1 (161). In yeast FYVE domain-containing proteins regulate vesicular trafficking. Vps27p for example is involved in endocytic and biosynthetic cargo sorting (136), and Fab1, a PI(3)P 5' kinase regulating vacuole size and cargo-selective sorting (120). Other examples of proteins that contain a FYVE domain are Hrs, which is a tyrosine kinase substrate that localizes to endosomes (17), and SARA, which is an adaptor protein that recruits transcription factors to the activated transforming growth factor β receptor (174). These proteins support a role for PI3K in vesicular trafficking and endosomal sorting.

Pleckstrin homology (PH) domains have approximately 100 amino acids that can bind both proteins and phosphoinositol lipids. These phospholipids must be phosphorylated on their 3' position (PI(3)P) but can also be phosphorylated on their 4', or 4' and 5' position (PI(3,4)P2 and PI(3,4,5)P3 respectively). The PH domain is present in numerous proteins and translocates them to the plasma membrane upon binding to PIP3 lipids. Proteins that harbour a PH domain and are activated by PI3K can be divided into three functional groups. The first group contains kinases such as various protein kinase C isoforms (PKC's), PKB, PI(3,4,5)P3-dependent kinase 1 (PDK1) and Bruton's tyrosine kinase (Btk). The PKC isoforms are activated either directly by PI3K or through PI3K-mediated activation of upstream kinases (42, 129). The second group consists of so-called guanine nucleotide exchange factors (GEFs). GEFs are proteins that can activate small GTPases by catalyzing the dissociation of GDP from the GTP-binding proteins. GTP can then bind and induce structural changes that allow binding to effector proteins. PH domain-containing GEFs include Tiam-1, leading to Rac-1 activation thereby leading to cytoskeletal rearrangements (141); ARNO and GRP-1, leading to Arf activation regulating vesicular trafficking (80, 178); and mSos which is the GEF for Ras and is inactivated by binding to PI(4,5)P2 (69, 141). The third group of PH domain containing proteins is a miscellaneous one, consisting of proteins including phospholipase C gamma (PLCγ) and structural proteins like spectrin and profilin.

Furthermore, it has been shown that besides FYVE- and PH domain containing proteins, other proteins can bind PI(3,4,5)P3 i.e. SH2 domain containing proteins Src and PI3K itself (24, 165). Binding to phospholipids could be competed away with phosphotyrosine residues suggesting that binding to the SH2 is mutually exclusive (142).

The action of PI3K can be counteracted by the lipid phosphatase PTEN ('phosphatase and tensin homologue deleted on chromosome ten', also known as MMAC1 (mutated in multiple advanced cancers) and TEP1 (transforming growth factor β-regulated and epithelial cell-enriched phosphatase 1)). It was identified as a tumor-suppressor gene located on chromosome 10q23.3 which is a region frequently mutated in several types of cancer such as high-grade glioblastoma, prostate and breast cancer (96). Homozygous knockout mice are embryonically lethal whereas heterozygous mice show a high level of tumorigenesis (167). PTEN is a dual-specificity phosphatase recognizing both protein substrates and PI(3,4,5)P3. The phosphatase activity of PTEN is only detected in the presence of acidic substrates, suggesting that it functions mainly via modifying phospholipids. Dephosphorylated lipids prevent translocation of PH domain-containing proteins to the plasma membrane. Besides PTEN, the phosphatases SHIP and SHIP-2 can also inhibit

PI3K signaling by dephosphorylating phosphoinositol lipids on their 5' position converting PI(3,4,5)P3 to PI(3,4)P2.

Protein kinase B

Protein kinase B (PKB) was originally identified by three independent groups. One group found that the kinase showed homology of the catalytic domain with those of PKA (65% similar) and PKC (75% similar) hence the name PKB (25). They also demonstrated that PKB is a downstream effector of PI3K activation (18). The second group identified the kinase in a similar fashion but named it RAC-PK (related to A and C protein kinases) (70). The third group found that PKB, or c-Akt, is the cellular homologue of v-Akt, the gene product of AKT-8, an acute transforming retrovirus that was isolated from a rodent T cell lymphoma (10, 162).

In mammalian cells PKB exists in three isoforms: PKBα (Akt), PKBβ (Akt2) and PKBγ (Akt3) (reviewed in (119)). The isoforms originate from different genes but show a very high homology on the protein level (i.e. more than 80%), and expression patterns differ between the different isoforms. PKBα and PKBβ are expressed ubiquitously with highest levels in brain, thymus, heart and lung. Expression of PKBγ is restricted to neuronal tissues and testes. Regulation of PKB expression is not well characterized but it appears that expression increases when cells become more terminally differentiated. It has been shown for several cell lines that upon terminal differentiation into myocytes, adipocytes and other cell types, PKB expression is elevated (2, 81, 102).

PKB contains two major domains. At the amino-terminus there is a PH domain. The PH domain of PKB contains seven β strands and two α helices. The β strands form two antiparallel β sheets that are capped by the carboxy-terminal α helix. 3' phosphorylated lipids bind the PH domain through specific hydrogen bonds in a positively charged pocket formed by the β1-β2, β3-β4 and β6-β7 loops. The β1-β2 loop is contributing to the inability of the PH domain to accommodate the 5' phosphate group whereas the amino acid residues in the bottom of the binding pocket, Lys14, Arg25, Tyr38, Arg48 and Arg86, can interact with the 3' and 4' phosphate groups of the phospholipids generated by PI3K (150). At the carboxy-terminus a glycine-rich region links the PH domain with the kinase domain, which shows a high similarity to those found in PKA and PKC. A hydrophobic regulatory region is also found at the carboxy-terminus of the protein.

The recruitment of PKB to the plasma membrane leads to phosphorylation of both threonine308 and serine473. These residues are located in the catalytic and regulatory domain, respectively. The equivalent phosphorylation sites are Thr309 and Ser474 in PKBβ, and Thr305 and Ser 472 in PKBγ. 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 its phosphorylation sites. Thr308 is phosphorylated by a kinase called PDK1. Subsequently, PKB can be phosphorylated on Ser473 by PDK1, or integrin-linked kinase 1 (ILK1) (33). However, it also has been shown that PKB can be phosphorylated in lipid rafts on residue 473 independently of PDK1 or ILK1 (55). This is suggested to occur via PKCα (131), although it has also been suggested that it can be a result of autophosphorylation (172).

PKB binding partners

Several proteins are known to form relatively stable complexes with PKB. Although some

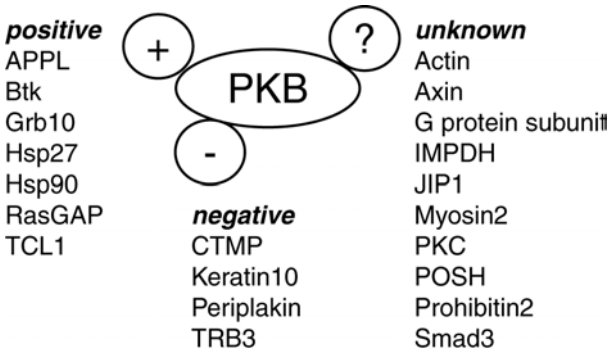

Figure 2: PKB binding proteins and their effect on PKB activity.

physiological substrates of PKB can also form a complex these proteins seem to have activity modulating roles (Figure 2).

The interacting proteins are depicted in Table 1 and the significance of these interactions is discussed below.

A number of proteins has been shown to bind to the PH domain of PKB. One of these proteins is **TCL1** that was originally identified as a proto-oncogene in T cell malignancies (179). Binding of TCL1 to the PH domain of PKB leads to an increase in PKB activity suggesting that this interaction might mimic binding of PKB to phospholipids. Laine and colleagues showed that TCL1 and its family members can interact with both PKBα and PKBβ isoforms (94). Oligomerization of PKB is facilitated by TCL1 leading to PKB phosphorylation and enhanced GSK3β and BAD phosphorylation. Pekarsky and colleagues also observed the interaction of TCL1 with PKB, however only with the α isoform (134). In the presence of TCL1, they also found enhanced PKB phosphorylation, GSK3β inactivation, and an increase in nuclear localization of PKB. Another protein interacting with the PH domain of PKB is inosine-5' monophosphate dehydrogenase (**IMPDH**), which was identified in a yeast-two-hybrid (Y2H) assay utilizing the PH domain of PKB (65). Just as for TCL1 it is not known which region or amino acids within the PH domain account for this binding. Co-transfection studies revealed binding of IMPDH with constitutively active PKB and not with kinase-inactive PKB. Furthermore, no effect on PKB activity is known but the interaction does lead to an increase in IMPDH activity thereby regulating the synthesis of GTP. **Myosin2** also binds to the PH domain of PKB and this binding competes with PI(4,5)P2 lipids (168). This suggests that myosin2 might block translocation of PKB to the plasma membrane by means of lipid binding, but no effect on PKB activity has been shown. Various **PKC** family members including PKCζ, PKCα, and PKCδ interact with PKB, and with the amino-terminus of the PH domain in particular (82, 83, 85). However, this interaction has only been demonstrated *in vitro*, and no *in vivo* functional studies have been reported. Konishi also observed PKB interaction with **Gβγ protein subunit** (83). But as for the PKB-PKC interactions this binding has not yet been studied in further detail. In an Y2H screen performed with the PH domain of PKB another binding partner was identified termed **periplakin** ((175) and Chapter 2). This protein is a member of the plakin family and can also bind to the cytosketal protein vimentin. The interaction of periplakin with PKB and vimentin can lead to an inhibition of PKB-mediated FOXO phosphorylation and modulation of transcriptional activity. The interaction and its effect of periplakin with PKB are further described in Chapter 2 of this thesis. PKB has also been shown to associate with the actin cytoskeleton through binding to **actin** (20). This binding is transient and dependent on growth factor stimulation and subsequent PKB phosphorylation. Another interesting protein that binds to the PH domain of PKB is JNK interacting protein 1 (**JIP1**) (77). In neurons the excitotoxin kainate activates the JNK pathway and induces apoptosis. JIP1 binds the PH domain of PKBα better than PKBβ, and both proteins are colocalized at the plasma membane. Upon treatment with kainite this

Table 1: Binding partners of PKB. Listed are the described PKB associating proteins, whether the interaction was shown for endogenous proteins (endo), the effect of binding (on PKB activity) and the corresponding reference.

Protein	Endo	Effect	Reference
Actin	Yes	Localization of PKB to cytoskeleton	(20)
APPL	Yes	Potentiates PKB activity	(108, 185)
Axin	Yes	Regulates Wnt signaling?	(47)
Btk	Yes	Enhances PKB activation by H2O2	(100)
CTMP	No	Inhibits PKB activity	(103)
Gβγ protein subunit	No	?	(83)
Grb10	Yes	Translocation PKB to membrane	(68)
Hsp27	No	Activates PKB	(84)
Hsp90	Yes	PKB phosphorylation, activity, stability	(154)
IMPDH	No	Regulation of GTP synthesis?	(65)
JIP1	No	Prevents JNK activation	(77)
Keratin10	Yes	Sequesters and inhibits PKB	(128)
Myosin2	No	?	(168)
Periplakin	Yes	Inhibits PKB nuclear activity	(175)
PKC	No	?	(82, 83, 85)
POSH	No	Blocks MLK-MKK-JNK complex formation	(44)
Prohibitin2	No	MyoD activation and muscle differentiation	(164)
RasGAP	Yes	Enhances PKB activation	(188)
Smad3	Yes	Sequesters and inhibits Smad3	(26, 144)
TCL1	Yes	Increases PKB activity and nuclear translocation	(134)
TRB3	Yes	Inhibits PKB activity	(40)

binding is disrupted, leading to increased binding of JIP1 with JNK and having JNK activation and subsequently apoptosis as a consequence. Thus activation of PKB leads to inhibition of JNK activation via JIP1 binding. This suggests that PKB can regulate signaling by binding to proteins that otherwise would interact with and activate other signaling pathways. Through Y2H analysis with the GAP domain of **RasGAP**, this protein has been identified as yet another interacting protein with the PH domain of PKB (188). The authors also found that RasGAP enhanced phosphorylation of Ser473 within PKB and that this occurred via ILK1 thereby regulating PKB-dependent cell survival.

Besides interacting with the PH domain, proteins can also bind to other regions of PKB. Heat shock proteins (Hsp) such as **Hsp27** can bind PKB upon H_2O_2 or heat shock treatment. Under these conditions PKB becomes activated and increased binding with Hsp27 is observed (84). **Hsp90** is another PKB binding partner when in complex with Cdc37 (154). Within PKB, amino acids 229 – 309 are responsible for this interaction and disruption of the interaction by use of a truncated form of PKB decreases kinase phosphorylation and subsequent activation leading to apoptosis. Hsp90 inhibitors induce inhibition of PKB activation and a subsequent loss of cyclin D1/3 expression in HER2 overexpressing breast cancer cells (8). These inhibitors disrupt binding of Hsp90 with PKB resulting in proteasome-dependent degradation of PKB (7). These and other data suggest that PKB in complex with Hsp90 and Cdc37 may facilitate its activation by preventing both dephosphorylation and protein degradation, and in addition possibly recruit upstream kinases or downstream target proteins

such as eNOS (45). Another binding partner of PKB is **APPL** (i.e. adaptor protein containing PH domain, PTB domain, and leucine zipper motif) (108). This protein was identified in a yeast-two-hybrid screen with full-length PKB as bait. The authors also show interaction of APPL with the PI3K catalytic subunit p110 suggesting it may function as an adapter to tether PKB and PI3K. APPL can suppress androgen receptor (AR) transactivation by interacting and activating PKB (185). This suppressive effect can be blocked by dominant-negative PKB or PI3K inhibitors. **Grb10** is also a positive regulator of PKB signaling (68), which was identified as a downstream modulator of PKB. Grb10 enhances PKB activity by translocation to the cell membrane via its SH2 domain. In H2O2-induced signaling activation of PKB is in part dependent on **Btk**. The mechanism through which this occurs remains to be elucidated. The kinase Btk can bind endogenous PKB and although both proteins colocalize in non-stimulated conditions, the interaction is induced upon oxidative stress (100). Both kinases colocalize in the perinuclear region and membrane ruffles. An interaction partner of PKB that is questionable is **axin**, since it has been described that PKB can bind a complex with axin, in which also GSK3β is present, in the presence of Dvl (47). Furthermore, PKB can enhance Wnt signaling, which is suggested to require binding of PKB to the axin complex. Whether the complex is needed for PKB-dependent phosphorylation of GSK3 is not known. Besides the described JIP1 binding PKB is also able to modulate stress signaling via direct binding to the JNK scaffold **POSH** (plenty of SH3 domains) (44). PKB can interact with the carboxyterminus of POSH thereby negatively regulating complex formation of POSH with MLK, MAPKK and JNK and subsequent downstream signaling. Another PKB binding protein, interacting with the carboxy-terminus of PKB, is **Smad3** (26, 144). TGFβ stimulation results in Smad3 binding to Smad4, which translocates to the nucleus to induce transcription. Smad3 can also be sequestered by PKB leading to inhibition of Smad3 phosphorylation and nuclear translocation. The interaction with Smad3 has no direct effect on PKB signaling itself.

Besides periplakin, there are also other binding partners of PKB that negatively regulate PKB activity. **TRB3**, a homologue from the drosophila protein tribbles that is involved in mitotic regulation, binds PKB and the binding leads to a decrease in growth factor-induced PKB phosphorylation (40). TRB3 knockdown induces phosphorylation of PKB resulting in inhibition of downstream targets such as GSK3 and FOXO1. In hepatocytes, ectopic expression of TRB3 induces a blockage of insulin-induced PKB-mediated glucose transport. It has been shown that TRB3 can be transcriptionally activated by fasting through a PGC1/PPARα-dependent mechanism (86). Carboxyl-terminal modulator protein (**CTMP**) binds to the regulatory carboxy-terminus of PKB (103). Both proteins colocalize and form a complex at the plasma membrane in serum-starved cells. Phosphorylation of Ser473 and to a lesser extent Thr308, is inhibited by the interaction with CTMP. This leads to a decrease in PKB activity and in phosphorylation of downstream effectors. Stable expression of CTMP shows a regression of the v-Akt-transforming phenotype compared to wild-type cells and in addition the tumorigenic capacity of these transformed cells is less in the presence of CTMP. The cytoskeletal protein **keratin10** is a negative regulator of cell proliferation (127), through binding to both PKB and PKCζ (128). Binding leads to sequestration of these kinases to the cytoskeleton, thereby inhibiting intracellular translocation and activation. This observation of decreased PKB activity is also seen in transgenic mice that ectopically express keratin10 (153).

PKB substrates

Many substrates have been described for PKB (Figure 3). These substrates all carry a so-called PKB motif consisting of Arg-Xaa-Arg-Yaa-Yaa-Ser/Thr in which the Xaa stands for any amino acid, Yaa for preferably small residues other than glycine. Although this motif (in short R-X-R-X-X-S/T) is the consensus phosphorylation motif some substrates have been described that modestly diverge from this consensus sequence, for instance FOXA2/HNF3β (R-R-X-X-T) (182). All known substrates are listed below in Table 2 and are described in further detail below.

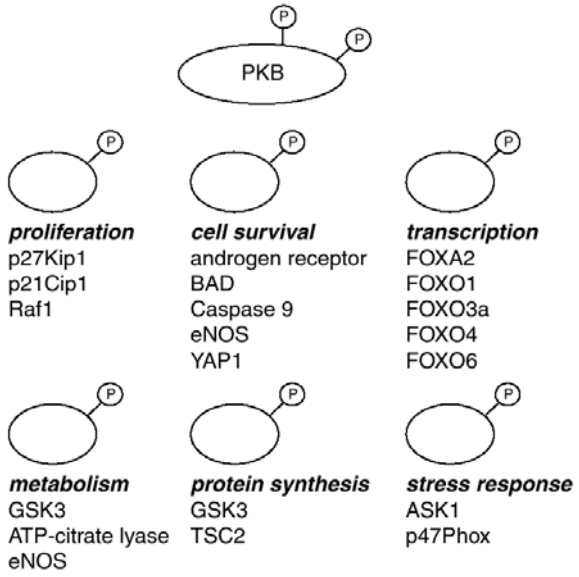

Figure 3: Processes regulated by PKB substrates.

A well-studied process regulated by PKB is cell survival. PKB can directly phosphorylate **BAD**, a Bcl2 family member that promotes apoptosis by interacting with BclXL in the mitochondrial membrane (31). This interaction of BAD and BclXL leads to a release of Cytochrome c from the mitochondrial compartment into the cytoplasm where it can bind to Apaf1 thereby activating apoptotic proteases called caspases. Phosphorylation of BAD on Ser112 and Ser136 sequesters BAD through interaction with 14-3-3 proteins, preventing binding to BclXL (reviewed in (39)). Whereas Ser112 is mainly phosphorylated by Raf1, PKB phosphorylates BAD on residue Ser136 and this phosphorylation can be blocked by dominant-negative PKB or the PI3K inhibitor wortmannin (54). BAD can also be phosphorylated on residues Ser112 and Ser136 by Pak (p21-activated kinase 1) (156). Furthermore, PKB can lead to Pak phosphorylation upon oncogenic Ras stimulation thereby activating Pak and subsequently indirectly regulating BAD (171). In addition, PKB can act downstream of the Cytochrome c release by phosphorylating **caspase 9** (19). Ser196 is the PKB-responsive residue and phosphorylation leads to an inhibition of caspase activity through an undefined mechanism. Importantly, the Ser196 site is not conserved in rodent species suggesting that caspase 9 is not a critical effector in apoptotic regulation by PKB. Recently a novel PKB substrate involved in cell survival has been described namely X-linked inhibitor of apoptosis (**XIAP**) (29). PKB is able to bind XIAP and phosphorylate it on Ser87 leading to stabilization of XIAP through inhibition of ubiquitination and subsequent degradation. The authors demonstrated that inhibition of XIAP expression can inhibit PKB-induced protection from cisplatin-mediated apoptosis. Another substrate of PKB is apoptosis-signal kinase 1 (**ASK1**) (76), which can be phosphorylated on Ser83. This kinase is involved in JNK and p38 stress signaling by stimulating MAPKKs. Phosphorylation results in inhibition of the ASK1 kinase activity thereby decreasing JNK activity. JNK has been described to promote apoptosis in some situations and this inhibition by PKB via ASK1 suggests an additional manner for modulating cellular survival. Regulation of the transcription factor p53 is critical in cellular processes such as cell growth and apoptosis. One manner in which p53 can be modulated is by

Table 2: Substrates of PKB. Depicted are the described substrates of PKB, their PKB motif in proteins, the effect of phosphorylation by PKB and in which processes the proteins are involved.

PKB substrates	RxRxxS/T	Effect	Process	Reference
14-3-3ζ	GARRS**S**[58]	-	Homodimerization	(138, 183)
AHNAK	RHRSN**S**[5535]	?	?	(166)
Androgen receptor	RAREA**S**[213] RMRHL**S**[793]	-	Apoptosis	(99, 181)
ASK1	RGRGS**S**[83]	-	Stress signaling	(76)
Ataxin-1	RKRRW**S**[776]	+	Neurodegeneration	(21)
ATP-citrate lyase	PSRTA**S**[454]	?	Metabolism	(12)
Bad	RGRSR**S**[136]	-	Apoptosis	(31)
BRCA1	RKRRP**T**[509]	?	DNA damage checkpoint	(1)
Caspase 9	RRRFS**S**[196]	-	Apoptosis	(19)
CHK1	RPRVT**S**[280]	-	DNA damage checkpoint	(78)
Cot1/TPL2	QPRCQ**S**[400]	+	NFκB signaling	(74)
EDG1	RSRRL**T**[236]	+	Migration, chemotaxis	(95)
eNOS	RIRTQ**S**[1177]	+	Nitric oxide metabolism	(38)
FOXA2	RRSY**T**[157]	-	Transcription	(182)
FOXO1	RPRSC**T**[24] RRRAA**S**[256] RPRTS**S**[319]	-	Transcription	(145)
FOXO3a	RPRSC**T**[32] RRRAV**S**[253] RSRTN**S**[315]	-	Transcription	(15)
FOXO4	RPRSC**T**[28] RRRAA**S**[193] RPRSS**S**[258]	-	Transcription	(88)
FOXO6	RPRSC**T**[26] RRRAV**S**[184]	-	Transcription	(67)
Gab2	RERKS**S**[159]	-	ErbB-R dephosphorylation	(101)
GABA(A) receptor	LRRRA**S**[410]	+	Synaptic strength	(180)
GSK3	RARTS**S**[9]	-	Glycogen metabolism	(27)
hTERT	RIRGK**S**[824]	+	Telomerase activity	(75)
Huntingtin	RSRSG**S**[421]	-	Survival of striatal neurons	(63)
IKKα	RERLG**T**[23]	+	NFκB signaling	(124)
IRS1	RPRSK**S**[265] RSRTE**S**[302] RVRAS**S**[325] RHRGS**S**[358]	+	Insulin signaling	(132)
Mdm2	RRRAI**S**[166] RKRHK**S**[186]	+	p53 degradation	(191)
Nur77	RGRLP**S**[350]	-	Orphan receptor signaling	(133)
p21Cip1	RKRRQ**T**[145]	+	Cell cycle arrest	(190)
p27Kip1	RKRPA**T**[157]	-	Cell cycle arrest	(160)
p47Phox	PPRRS**S**[304] AYRRN**S**[328]	+	NAPDH oxidase activity, oxygen radical formation	(22, 60)
PDE-3b	RPRRR**S**[273]	+	14-3-3 binding, cAMP/cGMP	(79)
PRAS40	RPRLN**T**[246]	+	14-3-3 binding	(91)
PTP1B	RYRDV**S**[50]	-	Insulin signaling	(143)
Rac	RIRPL**S**[71]	-	Migration	(93)
Raf1	RQRST**S**[259]	-	ERK pathway, proliferation	(192)
TSC2	RKRST**S**[924] RGRSK**T**[1518]	-	mTOR signaling, protein synthesis	(66, 137)
XIAP	RHRKV**S**[87]	+	Cell survival	(29)
YAP	HVRAH**S**[127]	+	14-3-3 binding, apoptosis	(9)

ubiquitination and degradation. The **Mdm2** gene product derives from a gene that was originally identified as an amplified gene in spontaneously transformed cells and encodes an E3 ubiquitin ligase. PKB can bind and phosphorylate MDM2 on Ser166 and Ser186, which leads to a nuclear localization (105, 191). Subsequently Mdm2 interacts with p53 and acetyl transferase p300 thereby inducing p53 ubiquitination and degradation. Nuclear localization of Mdm2 can suppress the sensitization of etoposide-induced apoptosis mediated by dominant-negative PKB (191). Mayo and colleagues show that dominant-negative PKB inhibits nuclear localization of Mdm2 thereby stabilizing p53 and increasing its transcriptional activity (105). The human homologue Hdm2 is also phosphorylated on the same residues but there is evidence for an additional phosphorylation site for PKB (5). Interestingly, phosphorylation of Mdm2 by PKB leads to a decrease of auto-ubiquitination resulting in Mdm2 stabilization (43) Besides regulating the stability of Mdm2 and p53, via Mdm2, PKB also regulates the stability of the **androgen receptor** (AR) in a negative manner (98). Upon HER2/neu induction activated PKB can phosphorylate the AR on Ser213 and Ser793 (99, 181). This phosphorylation leads to an inhibition of AR-mediated apoptosis by suppressing transactivation of the AR and interactions between the AR and AR co-regulators (99). Thus PKB can inhibit AR signaling via degradation of the AR and inhibition of its activity. In T cells, apoptosis induced by antigen receptor activation requires the immediate-early gene **Nur77,** that codes for an orphan nuclear receptor. It is present at high levels in immature thymocytes and apoptotic T cell hybridomas. It has been shown that Ser350 within the DNA binding domain of Nur77 can be phosphorylated leading to an inhibition of DNA binding (57). PKB interacts with Nur77 and phosphorylates the receptor at this serine residue thereby inhibiting its activity and subsequently apoptosis (133). PKB can also phosphorylate the protein **YAP** that stands for Yes-associated protein (9), which binds the Src family tyrosine kinase Yes, and is involved in p53 and p73 transcription factor-mediated apoptosis. YAP is phosphorylated by PKB on Ser127 leading to an increased binding to 14-3-3 proteins. This binding leads to a cytoplasmic localization and a loss of YAP in the nucleus where it normally functions as a co-activator of transcription factor p73 in transcribing pro-apoptotic genes. An additional way of transcriptional regulation by PKB is via the transcription factor family FOXO, which will be discussed later.

Another 14-3-3 associating protein, **PRAS40** (proline-rich Akt substrate), was identified in a screen for insulin-responsive 14-3-3 interacting proteins (91). Phosphorylation of Thr246 by PKB induces binding of PRAS40 to 14-3-3 and the presence of proline-rich regions in the aminoterminus of the proteins suggests that it also may act as a binding partner for SH3 or WW domain-containing proteins thereby modulating their activity. Apart from phosphorylating 14-3-3 associating proteins such as BAD, YAP and PRAS40, PKB is able to phosphorylate 14-3-3 itself as well, in particular **14-3-3ζ** (138). Powell and colleagues show interaction of PKB with 14-3-3ζ and phosphorylation of Ser58. Phosphorylation of this residue is involved in regulating homodimerization that in turn might be involved in regulating the availability of 14-3-3 binding to other proteins (183).

Another process regulated by PKB is proliferation. Cells have a cell cycle that can be divided into four distinct phases. In the S phase DNA is replicated whereas in the M phase cells divide. The G1 and G2 phases are phases before S phase and M phase respectively. Cell cycle progression is tightly regulated by complexes of cyclins and cyclin-dependent kinases (CDKs). These proteins are themselves regulated by a family of CDK inhibitor proteins including

p21Cip1, p27Kip1, p57Kip2 and p16Ink4a. PKB regulates **p21Cip1** by phosphorylation on Thr145 upon cellular activation by HER2/neu (190). This leads to a p21Cip1 translocation from the nuclear compartment to the cytoplasm thereby stimulating proliferation. Blocking the PKB pathway by ectopic expression of dominant-negative PKB restores the nuclear localization of p21Cip1 and subsequent cell cycle inhibition. In the cytoplasm p21Cip1 can also interact with ASK1 leading to an inhibition of ASK1 activity thereby inhibiting apoptosis (4). Huang and colleagues showed that this interaction is induced by rapamycin and that rapamycin-induced cell death is prevented only in cells expressing p21Cip1 (62). PKB can also associate with **p27Kip1** and phosphorylate it on residue Thr157 (160). As for p21Cip1, phosphorylation of p27Kip1 leads to its cytoplasmic translocation. Cells expressing mutant p27Kip1 (T157A) are not susceptible to rescue of anti-proliferative effects by active PKB. Besides regulating CDK inhibitors PKB can also exert its proliferative effect by acting on **cyclin D1/3** levels. PKB leads to cyclin D translation through an unknown mechanism (112), and the protein is also stabilized by PKB (35). Cyclin D is also destabilized and targeted to the proteasome through binding and phosphorylation by glycogen synthase kinase 3β (GSK3β). GSK3 plays an important role in various cellular processes such as glycogen metabolism by inhibiting glycogen synthase. This phosphorylation of **GSK3** by PKB occurs on Ser21 in GSK3α and Ser9 in GSK3β thereby inhibiting its activity (27). PKB is both necessary and sufficient for GSK3 inactivation by insulin as determined using PKB mutants (176). Endothelial nitric oxide synthase (**eNOS**) is involved in the production of nitric oxide (NO), thereby regulating angiogenesis. In tumors this leads to an increased supply of nutrients to the cancer cells and therefore promotes their survival. PKB phosphorylates eNOS on Ser1177 leading to eNOS activation and increased NO production (38). This phosphorylation is possibly facilitated via complex formation of PKB, eNOS and Hsp90 (45).

Besides acting on GSK3, PKB can also regulate additional proteins involved in metabolism. **ATP-citrate lyase** is a cytosolic enzyme that catalyzes the conversion of citrate derived from the Krebs cycle into oxaloacetate and acetyl-CoA in an ATP-dependent manner. It has been reported that insulin can induce phosphorylation of ATP-citrate lyase on Ser454 and that this is mediated by a cytosolic kinase (135, 187). Berwick and colleagues showed that PKB can directly phosphorylate ATP-citrate lyase on Ser454 suggesting that this is the kinase mediating the insulin-induced phosphorylation (12). It also has been suggested that the insulin-induced phosphorylation and activation of the enzyme 6-phosphofructo-2-kinase (**PFK2**) on Ser466 and Ser483 is mediated by PKB in addition to other protein kinases such as p70S6K and MAPK (34). Using kinase-dead mutants of PDK1 and PKB however, Bertrand and colleagues showed that the PFK2 phosphorylation is dependent on PDK1 and not mediated by PKB (11).

PKB is also implicated in cell-specific processes. Human neutrophils are haematopoetic cells involved in the innate immune response against invading microorganisms. To efficiently kill them neutrophils have the ability to generate toxic oxygen radicals by the NAPDH oxidase enzymatic complex. This complex is composed of multiple proteins among which p47Phox. PKB can interact with **p47Phox** and phosphorylate it on residues Ser304 and Ser328 leading to formation and activation of the NADPH oxidase complex and the formation of oxygen radicals such as hydrogen peroxide and O_2^- (22, 60).

In neurons, PKB exerts another cell type-specific function. It phosphorylates subunit β2 of the type A γ-aminobutyric acid receptor

(**$GABA_AR$**), which is a ligand-gated Cl-channel and the major receptor mediating fast inhibitory transmission in the brain (180). This phosphorylation occurs at Ser410, and increases the number of $GABA_A$Rs at the cell membrane hence increasing its synaptic transmission potential.

Chemotaxis in endothelial cells occurs via the G-protein coupled receptor **EDG1** and can be induced by sphingosine-1-phosphate (S1P). S1P treatment activates PKB that subsequently binds EDG1 and phosphorylates Thr236 in the third intracellular loop of the receptor (95). This is not necessary for Gi-dependent signaling but essential for Rac activation, cortical actin assembly and chemotaxis. Mutant EDG1 (T236A) sequesters PKB and acts as a dominant-negative receptor to inhibit S1P-induced Rac activation.

Small GTPases are also targeted by PKB. **Rac1** belongs to the family of Rho GTPases and plays an important role in the control of cell shape, adhesion and migration. Rac1 is phosphorylated by PKB on Ser71 and this inhibits the GTP binding whereas the GTPase activity remains the same (93). Another protein that is phoshorylated by PKB is **Raf1** (192). Raf1 is the key mediator that signals from the small GTPase Ras to MEK and ERK, and can regulate proliferation, apoptosis and differentiation. This pathway can act synergistically with or in opposition to the PI3K/PKB pathway. Phosphorylation of Ser259 within Raf1 can modulate its kinase activity (111), through induced binding to 14-3-3 (107). PKB can bind and phosphorylate Raf1 thereby inhibiting its activity (192). The balance and interaction between the PI3K/PKB and Raf/MAPK pathway on the level of PKB and Raf1 determine the temporal phosphorylation pattern of MAPK and guide the fate of muscle cells to either proliferate or differentiate (147).

Besides regulating the MAPK pathway, PKB can also modulate the NFκB transcriptional pathway. It can do so by targeting **Cot** ((cancer Osaka thyroid) or Tpl2 (tumor progression locus 2)) (74), a protein originally identified in a screen for transforming genes in a human thyroid carcinoma cell line (109). Cot is a MAPKKK and can activate both the canonical MAPK and JNK pathways by acting through MEK1 and SEK1 respectively (151). Furthermore, Cot has been implicated in the NFκB pathway by activating the IKK complex. PKB interacts with Cot and phosphorylates residues Ser400 and Ser413 (74). This does not change its kinase activity but phosphorylation on Ser400 does lead to activation of IKK kinases. PKB can also directly phosphorylate **IKKα** on Thr23 leading to a subsequent activation (124).

By phosphorylating **Gab2**, PKB can mediate a negative feedback thereby regulating mitogenic signaling (101). Gab2 belongs to a family of scaffolding proteins. Upon cytokine and antigen stimulation it becomes tyrosine phosphorylated and can bind a variety of proteins including SHP2 and Shc. PKB is able to bind to Gab2 and phosphorylate it on Ser159 (101). This phosphorylation leads to a decrease in tyrosine phosphorylated Gab2. Removing the negative feedback by mutating this serine to alanine leads to an enhancement of cytokine signaling and releases a potent transforming activity in fibroblasts.

FOXO transcription factors

FOXO transcription factors are part of the superfamily of FOX (forkhead box) factors. They all have a winged-helix domain in common that is responsible for the interaction with DNA (72). This domain contains 110 amino acids forming three α-helices at the aminoterminus, three β-sheets and two large loops at the carboxyterminus. One small subfamily is that of the FOXO proteins that has the orthologue DAF16 in *Caenorhabditis elegans* (*C. elegans*) and bind to promoter

sequences similar to the described consensus sequence 5'-TTGTTTAC-3' (50).

The nematode worm *C. elegans* can enter a so-called dauer stage when environmental conditions are unfavorable such as low food or high population density. This stage is a developmental arrest that is characterized by a low metabolic activity and increased resistance to (oxidative) stress, and that allows the worm to live up to ten times longer than a normal adult. One of the proteins responsible for the induction and maintenance of dauer is DAF16. This transcription factor is inhibited by DAF2 signaling, an insulin receptor-like protein, and the mediators of this DAF2-induced inhibition of DAF16 activity show high homology with the mammalian PI3K/PKB pathway: Age1/DAF23 (PI3K), DAF18 (PTEN), PDK1 and Akt1/Akt2 (PKB) (122, 125, 126).

There are three mammalian classical FOXO transcription factors: FOXO1, FOXO3a and FOXO4, also known as FKHR, FKHRL1 and AFX respectively, and the recently described FOXO6. These factors were found to be part of genomic fusions. The translocation t(2;13)(q35;q14) is a fusion of the Pax3 gene to the 3'-end of FOXO1 and is found in alveolar rhabdomyosarcoma (52, 159). In a small subset of the same tumor type the translocation t(1;13)(p36;q14) fuses the Pax7 gene to the same FOXO1 part (32). Both Pax proteins are paired box transcription factors and both fusions preserve an intact Pax DNA binding domain. In acute lymphoblastic leukemia the chromosomal translocation t(X;11)(q13;q23) causes a fusion of the mixed-lineage leukemia (MLL) gene with FOXO4 (13, 130). The same MLL gene, which codes for a trithorax-related transcription factor, is found to be fused to FOXO3a in the t(6;11)(q21;q23) translocation (56).

Besides a DNA-binding forkhead domain, the transcription factors harbor a transactivation domain, a classical nuclear localization signal (NLS) and a nuclear export signal (NES). Furthermore, they all contain multiple conserved PKB phosphorylation motifs equivalent to Thr28, Ser183 and Ser258 in FOXO4 (see Table 3). Recently, a new mammalian FOXO member has been cloned and designated FOXO6 (67). FOXO6 contains the first two conserved PKB phosphorylation motifs but lacks the third one.

In *C. elegans* it has been demonstrated that PKB mediates the insulin-induced inhibition of DAF16, however no phosphorylation assays have been performed (126). In mammalian cells the FOXO proteins are phosphorylated by PKB within the predicted PKB motifs, and this leads to nuclear export and inactivation of the transcription factors. (15, 88, 145). In non-stimulated conditions where PKB is inactive, FOXO factors interact with the promoter sequences in DNA or shuttle between the nucleus and cytoplasm in an importin and exportin-dependent mechanism. More specifically, exportin Crm1 is involved in this as leptomycin B inhibits FOXO export and its binding is regulated by the small GTPase Ran (14). The importin involved in the shuttling of FOXO proteins is not known yet. The balance between PKB and FOXO activity in cells determines its fate in terms of cellular processes (Figure 4).

Table 3: Conservation of PKB phosphorylation motifs in the *C. elegans* DAF16 and its human orthologues.

Transcription factor	Motif 1	Motif 2	Motif 3
DAF16	RDRCN**T**^{54}	RTRER**S**^{240}	RPRTQ**S**^{314}
FOXO1/FKHR	RPRSC**T**^{24}	RRRAV**S**^{256}	RSRTN**S**^{319}
FOXO3a/FKHR-L1	RPRSC**T**^{32}	RPRAA**S**^{253}	RPRTS**S**^{315}
FOXO4/AFX	RPRSC**T**^{28}	RRRAA**S**^{193}	RPRSS**S**^{258}
FOXO6	RPRSC**T**^{26}	RRRAV**S**^{184}	

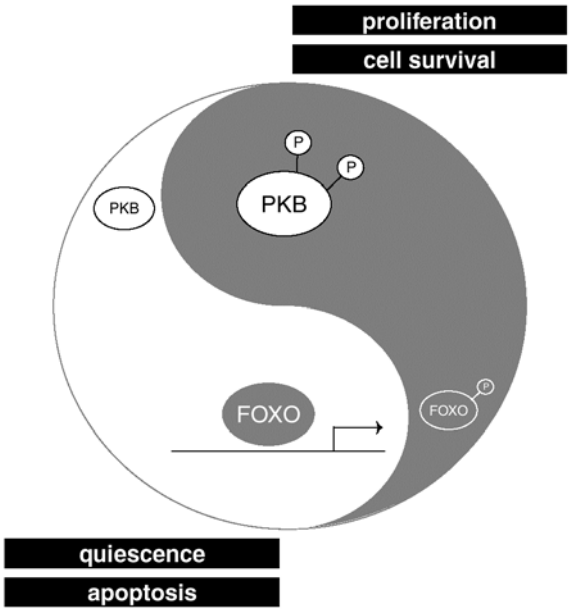

Figure 4: Yin-Yang: a balance between PKB and FOXO activity.
The cellular state of active PKB and inactive FOXO is characterized by proliferation and cellular survival, whereas inactive PKB and active FOXO lead to quiescence and apoptosis.

Upon PKB activation, PKB translocates to the nucleus where it can phosphorylate nuclear targets. Phosphorylation leads to an exclusion of the FOXO proteins from the nucleus, likely via binding to the family of 14-3-3 scaffolding protein (15, 146). This protein can enter the nucleus freely in its unbound state. When bound to FOXO, 14-3-3 masks the NLS of FOXO and once translocated to the cytoplasm it can sequester the transcription factor in that compartment. In addition, other kinases can phosphorylate FOXO factors as well. Serum- and glucocorticoid-inducible kinase (SGK) can phosphorylate PKB motif 1 and 3 (16). Similar to PKB, SGK is activated by PDK1 and therefore insulin/PI3K can stimulate phosphorylation of FOXO factors through several mechanisms. Ser329 within FOXO1 can also be phosphorylated both *in vitro* and *in vivo*. This is carried out by dual-specificity tyrosine-phosphorylated and –regulated kinase 1a (DYRK1a) (184). Phosphorylation of this residue affects nuclear localization and activity of the factor but the relevance of this phosphorylation remains unsolved yet since DYRK1a appears to be constitutively active. More recently, it has been shown that also IKK can phosphorylate FOXO3a leading to an inhibition via proteolytic degradation (61). At the carboxyterminus, FOXO can be phosphorylated in a Ras/Ral-dependent fashion (88). This occurs through kinases of the JNK family.

The FOXO factors can regulate expression of a variety of genes that are depicted in Table 4 and described in more detail below.

FOXO proteins can regulate a variety of cellular processes, and through the ability to regulate the transcriptional activity of these factors, PKB is able to control cell signaling and processes on more levels than solely by direct phosphorylation and subsequent regulation of proteins. One process regulated by FOXO factors is apoptosis or programmed cell death (Figure 5), which can be induced through two mechanisms. One is activated through Fas receptor activation, whereas the other is activated by release of Cytochrome c from mitochondria. Both pathways lead to activation of pro-apoptotic proteases called caspases. **FasL** is the ligand for the Fas receptor. Activation of this receptor by binding of the ligand stimulates apoptosis by the activation of caspase 8, and subsequently caspase 3, via FADD (Fas-associating death domain). FOXO3a can bind the promoter of FasL via three putative binding sequences thereby leading to enhanced transcription(15). FOXO3a also increases expression of another ligand for an apoptosis-triggering receptor, namely tumor necrosis factor (TNF)-related apoptosis-inducing ligand (**TRAIL**) (110). TRAIL can bind the receptor DR4/5 thereby activating it and subsequently stimulating caspase activity. Upon TNF binding to the TNF receptor TNF receptor-associated death domain (**TRADD**) will also interact with the receptor. This protein is upregulated on a transcriptional

Table 4: Target genes of DAF16, dFOXO and FOXO factors. Depicted are the described transcriptional target genes of FOXO factors and its homologues, whether FOXO positively (+) or negatively (-) regulates transcription, and in which processes the target gene products are involved.

Target gene	FOXO	-/+	Process	Reference
4EBP1	dFOXO	+	Cell growth	(71, 139)
Atrogin	3a	+	Muscle atrophy	(152)
Bcl6	4	+	Apoptosis	(169)
Bim	3a	+	Apoptosis	(36)
BTG1	3a	+	Cell cycle/differentiation	(6)
Catalase	3a	+	Oxidative stress	(118)
Collagenase	3a	+	Extracellular matrix	(104)
Cyclin B	3a	+	Cell cycle	(3)
Cyclin D1	1	-	Cell cycle	(155)
FasL	3a	+	Apoptosis	(15)
G6Pase	1	+	Metabolism	(115)
GADD45	3a, 4	+	Cell cycle/DNA repair	(48, 173)
HIF1	4	-	Angiogenesis/metabolism	(170)
HMGCS2	3a	+	Metabolism	(114)
IGFBP1	1	+	Metabolism	(41)
InsR	dFOXO	+	Insulin signaling	(139)
LPL	1	+	Lipid metabolism	(73)
MnSOD	3a	+	Oxidative stress	(87)
OLD1	DAF16	+	Longevity	(113)
p130	3a	+	Cell cycle/quiescence	(89)
p27Kip1	3a, 4	+	Cell cycle	(37, 106)
PDK4	1	+	Metabolism	(49)
PEPCK	4	+	Metabolism	(186)
PGC1	1	+	Hepatic metabolism	(28)
Plk	3a	+	Cell cycle	(3)
Scl1	DAF16	+	Longevity, stress resistance	(123)
SCP	3a	+	Stress resistance	(30)
TRADD	1	+	Apoptosis	(149)
TRAIL	3a	+	Apoptosis	(110)

level by FOXO1 (149). Together with FADD, TRADD can lead to caspase 8 activation and thereby induction of apoptosis. Next to apoptosis-inducing receptors, cells can undergo apoptosis via an intracellular pathway i.e. the mitochondrial leakage of Cytochrome c that leads to apoptosome formation, a complex of Apaf1 and caspase 9, and subsequent apoptosis. The integrity of the mitochondrial outer membrane is highly regulated by proteins such as BAD and BAX. These proteins in turn are under control of the anti-apoptotic proteins BclXL, Bcl2 and other Bcl2-like proteins such as Bim, which is a pro-apoptotic regulator. FOXO4 can bind and activate the promoter of the transcriptional repressor **Bcl6** (169). This upregulation of Bcl6 expression leads to a subsequent transcriptional downregulation of BclXL thereby inducing Cytochrome c release from the mitochondria. The Bcl2-like protein **Bim** is also under direct transcriptional control of FOXO3a (36) (53). In the haematopoietic BaF3 pre-B cell line upregulation of Bim expression by FOXO factors was found to be responsible for a Fas receptor independent apoptosis. Stahl and colleagues showed that this increase in Bim expression is also inhibited in T cells by IL2 (163). Depending on cell-type and stimulus, FOXO factors can induce apoptosis either via a (death) receptor-dependent mechanism or via the intracellular mitochondria-dependent mechanism.

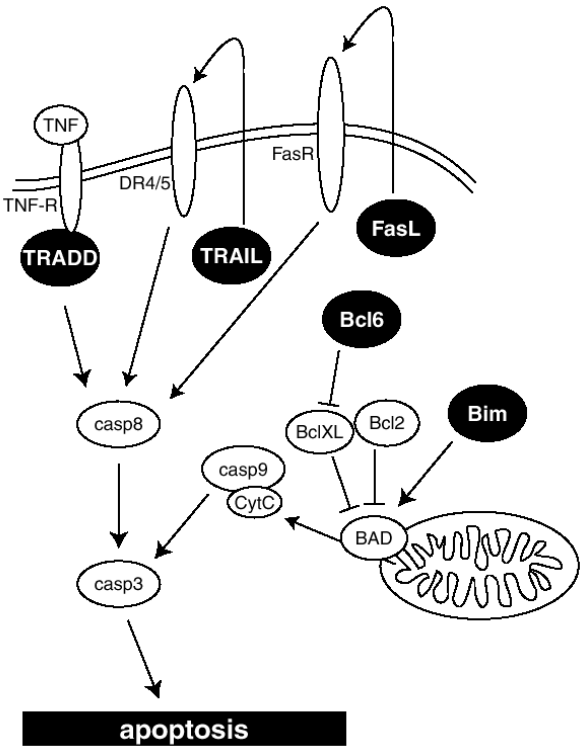

Figure 5: FOXO factors induce apoptosis.
Through transcriptional upregulation of the proteins represented by the filled ovals FOXO factors can induce activation of caspases leading to a subsequent cell death. CytoC = Cytochrome c

The physiological outcome of FOXO activation is not the same in every cell type, probably due to effects of co-factors or specific manners by which FOXOs are activated. Another process that is regulated by FOXO is cell cycle regulation. Whereas PKB can trigger cells to go into cell cycle, FOXO factors do the opposite by making cells go into cell cycle arrest or keeping them quiescent (Figure 6).

One of the genes identified in this process is the cell cycle inhibitor **p27Kip1**. Medema and colleagues showed that FOXO1, 3a and 4 are all capable of inducing cell cycle arrest in the G1 phase, and that FOXO4 is able to increase p27Kip1 levels by increasing transcription (106). FOXO3a is also able to elevate p27Kip1 expression and this can be regulated by haematopoietic cytokines (37). Besides regulating p27Kip1 levels via transcriptional regulation, FOXO factors can also enhance the stability and thereby its activity (116). Next to regulating p27Kip1 expression, FOXO proteins can induce a G1-arrest by downregulating **cyclin D** levels, particularly D1 and D2 cyclins (155). The FOXO factors do however not seem to bind directly to the promoters of the cyclin genes but act for instance through upregulating Bcl6 levels, or indirect binding to the promoters through complex formation with nuclear receptors. The reduction in cyclin D levels subsequently leads to a decrease in cyclin D/CDK4 activity. Furthermore, FOXO activity can lead to regulated exit from the M phase (3). PI3K/PKB activity is normally attenuated in G2 and M phase, and sustained PI3K/PKB activation impairs termination of the cell cycle. The absence of PKB-mediated inactivation of FOXO factors results in binding of FOXO3a to sequences in the Polo-like kinase (**Plk**) and **cyclin B** promoters thereby enhancing transcription of these genes (3). Quiescence is the cell state in which cells are not in cell cycle but they can re-enter the cycle upon the correct

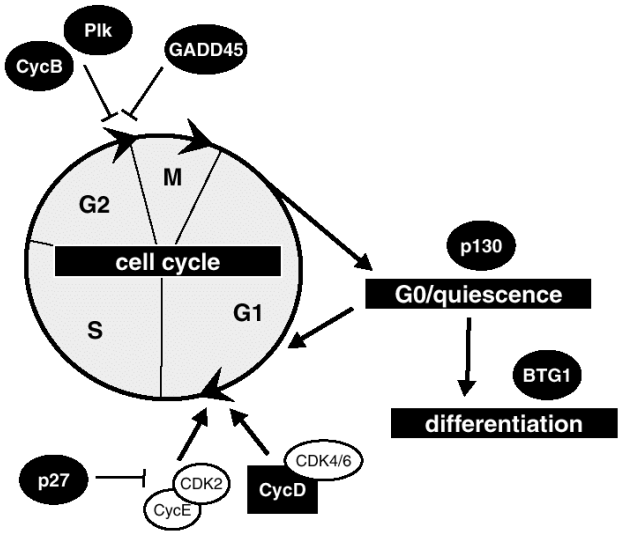

Figure 6: FOXO induce cell cycle arrest.
Through transcriptional regulation of the proteins represented in black (increased expression: filled ovals; decreased expression: filled square) FOXO factors are able to arrest cells in their G1 or G2 phase, or lead cells out of the cell cycle into a state of quiescence or subsequent differentiation.

stimuli. This is in contrast to senescence, which is a non-reversible state. Although there is no absolute consensus yet about markers of the quiescent phenotype, it is always accompanied by increased **p130**/pRb2 levels. In the absence of PKB activity in G1 phase FOXO3a upregulates p130 expression thereby leading cells out of the cell cycle into a quiescent state (89). From a quiescent state cells can subsequently undergo differentiation. A novel identified gene specific for the differentiation process of erythropoiesis that is regulated by FOXO is B cell translocation gene 1 (**BTG1**) (6). Expression of BTG1 inhibits cell growth and via regulation of protein arginine methyl transferase activity it leads to a subsequent differentiation.

FOXO factors are also involved in the response to and protection against (oxidative) stress. In *C. elegans* Dauer formation is dependent on oxidative stress and controlled by DAF16 (59). In mammalian cells FOXO factors can protect cells from oxidative stress by inducing transcription of the genes encoding the proteins manganese super-oxide dismutase (**MnSOD**) (87) and **catalase** (118). MnSOD is a mitochondrial enzyme that converts $O_2^{\bullet -}$ into H_2O_2 whereas the peroxisome-specific catalase converts the H_2O_2 into H_2O and O_2. Whether these two proteins cooperate to allow cells to eliminate toxic radicals or they serve independent functions is unclear. Another protein involved in stress response that is regulated by FOXO is **GADD45** (173). This protein stimulates repair of damaged DNA and thereby controls the transition of G2 to M phase in the cell cycle. FOXO3a activation increases GADD45 expression thereby increasing the resistance to DNA-damaging stress (173). The same effect can be observed with FOXO4 (48).

When cells are faced with caloric restriction, the expression levels of FOXO proteins are increased (51). Together with the absence of PI3K/PKB signaling, this leads to an increased total amount of active FOXO factors in the nucleus (64). Cells that undergo caloric restriction show a shift in their metabolism, from glucose metabolism to fat/fatty acid metabolism. FOXO factors are critical in regulating expression of various proteins that are involved in these processes (Figure 7).

During energy deprivation, FOXO1 increases pyruvate dehydrogenase kinase 4 (**PDK4**) expression (49). This enzyme inactivates pyruvate dehydrogenase thereby inhibiting the conversion of pyruvate into acetyl CoA that is necessary for the Krebs cycle. FOXO4 also upregulates expression of phosphoenolpyruvate carboxykinase (**PEPCK**) (186). PEPCK decarboxylates oxaloacetate to phosphoenolpyruvate (PEP) in a GTP-dependent manner. Subsequently, PEP can be converted to pyruvate but during caloric restriction it is used for gluconeogenesis. The final step of gluconeogenesis is the dephosphorylation of glucose-6-phosphate by the enzyme glucose-6-phosphatase (**G6Pase**). Insulin negatively regulates G6Pase expression via FOXO1 (115), via binding to the first two of three insulin response elements in its promoter (177). Instead of generating energy from glucose, it derives from fat and fatty acids. This process is enhanced by FOXO factors. Plasma triglycerides are converted into fatty acids in part by the enzyme lipoprotein lipase (**LPL**),

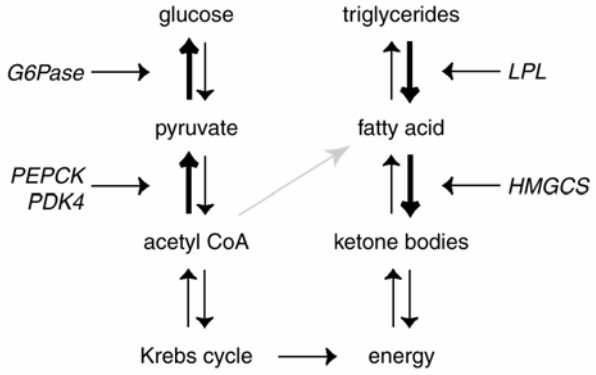

Figure 7: FOXO factors regulate metabolism.
Through transcriptional regulation of the indicated proteins FOXO factors can shift the cellular metabolism towards gluconeogenesis and fatty acid metabolism.

and expression of LPL is increased by FOXO1 (73). Fatty acids are further processed into ketone bodies through β oxidation and ketogenesis. FOXO3a increases expression of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (**HMGCS2**), an enzyme involved in generation of these ketone bodies (114). Hepatic gene expression is regulated in part by peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1 (**PGC1**). FOXO1 increases PGC1 expression by direct binding to its promoter (28). Furthermore, PGC1 regulates gene expression of for instance PEPCK and G6P through HNF4 and FOXO1 (140, 148). Both HNF4 and FOXO1 can bind to PGC1 and can interact with each other as well (58). It is possible that some of the effects on metabolism observed with FOXO might be directed through regulation of PGC1 and/or other factors.

Recent literature shows that besides directly binding to promoter sequences FOXO factors can also modulate gene expression through interacting with other transcription factors. FOXO1 is able to bind sequences in the IGF-binding protein 1 (**IGFBP1**) promoter (41) and stimulates transcription from this promoter. Foucher and colleagues find an interaction of FOXO1 and the homeobox protein Hoxa5 leading to a synergistic transcriptional activation of IGFBP1 (46). In addition, FOXOs can interact with CREB-binding protein (CBP) and steroid receptor coactivator although these interactions have only been shown *in vitro* (117). Nuclear hormone receptors such as the estrogen receptor (ER) and androgen receptor (AR) can also bind to FOXO factors. Binding of FOXO1 with the AR is dependent on androgens and this interaction inhibits the capacity of FOXO to bind DNA subsequently stimulating cell survival (97). The interaction of FOXO1 and the ER is inhibited by estrogen whereas binding of FOXO3a and FOXO4 with the ER is not (157). In this complex, FOXO activity is inhibited whereas the activity of ER-mediated transcription is enhanced. However, others have reported that the estrogen-induced FOXO1-ER complex represses ER-mediated transactivation (189). For the process of Dauer formation in *C. elegans*, DAF16 works synergistically with proteins from the DAF7 pathway, which is the homologue of the TGFβ pathway in mammals (121). In mammalian cells the PI3K/PKB pathway can inhibit TGFβ/Smad-induced apoptosis (23). These observations suggest that FOXO proteins can cooperate with Smad transcription factors in regulating cellular processes. This was confirmed recently by showing a direct interaction between FOXO and Smad3 (158). The Smad3/Smad4 complex can in cooperation with FOXO lead to p21Cip1 gene expression and this can be blocked by the transcription factor FOXG1. STAT3-dependent expression is also regulated by FOXO factors. FOXO1 can interact with STAT3 thereby enhancing STAT3-mediated expression (90). The PI3K/PKB pathway inhibits via FOXO1 IL6-induced and STAT3-mediated gene expression.

Scope of the thesis

Extracellular stimuli can activate different intracellular pathways that in turn can signal in parallel or crosstalk between one another. One of the major kinases involved in growth factor signaling is phosphoinositide-3-kinase (PI3K). This lipid kinase can recruit protein kinase B (PKB) to the plasma membrane where it is phosphorylated by PDK1 and a yet unidentified kinase. Upon activation, PKB in turn can phosphorylate multiple proteins in a so-called PKB motif (R-X-R-X-X-S/T) thereby regulating their activity. These PKB substrates regulate proliferation, cell survival, protein translation and other processes such as transcription. Transcription factors phosphorylated by PKB are members of the Forkhead box O (FOXO). Upon

phosphorylation these factors are excluded from the nucleus and subsequently inactivated.

In **Chapter 2**, the identification of a novel interaction partner for PKB is described. In a binding screen, the plakin family member periplakin was found to interact with PKB. The interacting regions have been narrowed down to nine amino acids within the PH domain of PKB, and the carboxyterminus of periplakin. Periplakin is present in distinct cellular compartments such as nucleus, plasma membrane, mitochondria and the intermediate filament network through direct binding to vimentin. Binding of PKB to periplakin does not affect PKB activation but can inhibit PKB-mediated phosphorylation and subsequent inactivation of FOXO transcription factors.

Chapter 3 describes an *in silico* approach to identify novel substrates for PKB and target genes for FOXO factors. To identify downstream targets of PKB, proteins containing the consensus PKB motif (RXRXXS/T) were selected. This selection was narrowed down on the basis of their known function, and evolutionary conservation of the motifs was assessed. Two potential PKB substrates selected for further analysis are Pit-1 and Prop-1. To identify novel FOXO target genes, microarray analysis was performed using a cell line containing an inducible active FOXO3a. Results from this array were compared with other relevant microarrays and putative target genes were checked for the presence of FOXO binding elements in the promoter region.

In **Chapter 4**, the analysis of Pit-1 and Prop-1 as putative PKB substrates is described. Pit-1 and Prop-1 are pituirary-specific homeodomain transcription factors involved in the production of growth hormone and other pituitary-specific hormones. Both proteins contain PKB consensus motifs and are indeed phosphorylated *in vitro* by PKB. *In vivo* phosphorylation however is not observed neither are any changes in localization or transcriptional activity.

Chapter 5 deals with the validation of a novel FOXO target gene encoding for caveolin-1. Caveolin-1 expression is induced upon FOXO activation and downregulated by insulin-dependent PI3K/PKB signaling on both mRNA and protein levels. Regulation of caveolin-1 transcription occurs by direct binding of FOXO to binding elements in the caveolin-1 promoter region. The regulation of caveolin-1 expression results in functional attenuation of EGF signaling.

The consequences of the described findings are discussed in **Chapter 6**.

References

1. **Altiok, S., D. Batt, N. Altiok, A. Papautsky, J. Downward, T. M. Roberts, and H. Avraham.** 1999. Heregulin induces phosphorylation of BRCA1 through phosphatidylinositol 3-Kinase/AKT in breast cancer cells. J Biol Chem **274:**32274-8.
2. **Altomare, D. A., K. Guo, J. Q. Cheng, G. Sonoda, K. Walsh, and J. R. Testa.** 1995. Cloning, chromosomal localization and expression analysis of the mouse Akt2 oncogene. Oncogene **11:**1055-60.
3. **Alvarez, B., A. C. Martinez, B. M. Burgering, and A. C. Carrera.** 2001. Forkhead transcription factors contribute to execution of the mitotic programme in mammals. Nature **413:**744-7.
4. **Asada, M., T. Yamada, H. Ichijo, D. Delia, K. Miyazono, K. Fukumuro, and S. Mizutani.** 1999. Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. Embo J **18:**1223-34.
5. **Ashcroft, M., R. L. Ludwig, D. B. Woods, T. D. Copeland, H. O. Weber, E. J. MacRae, and K. H. Vousden.** 2002. Phosphorylation of HDM2 by Akt. Oncogene **21:**1955-62.
6. **Bakker, W. J., M. Blazquez-Domingo, A. Kolbus, J. Besooyen, P. Steinlein, H. Beug, P. J. Coffer, B. Lowenberg, M. Von Lindern, and T. B. Van Dijk.** 2004. FoxO3a regulates erythroid differentiation and induces BTG1, an activator of protein arginine methyl transferase 1. J Cell Biol **164:**175-84.
7. **Basso, A. D., D. B. Solit, G. Chiosis, B. Giri, P. Tsichlis, and N. Rosen.** 2002. Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. J Biol Chem **277:**39858-66.

8. **Basso, A. D., D. B. Solit, P. N. Munster, and N. Rosen.** 2002. Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. Oncogene **21:**1159-66.
9. **Basu, S., N. F. Totty, M. S. Irwin, M. Sudol, and J. Downward.** 2003. Akt phosphorylates the Yes-associated protein, YAP, to induce interaction with 14-3-3 and attenuation of p73-mediated apoptosis. Mol Cell **11:**11-23.
10. **Bellacosa, A., J. R. Testa, S. P. Staal, and P. N. Tsichlis.** 1991. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. Science **254:**274-7.
11. **Bertrand, L., D. R. Alessi, J. Deprez, M. Deak, E. Viaene, M. H. Rider, and L. Hue.** 1999. Heart 6-phosphofructo-2-kinase activation by insulin results from Ser-466 and Ser-483 phosphorylation and requires 3-phosphoinositide-dependent kinase-1, but not protein kinase B. J Biol Chem **274:**30927-33.
12. **Berwick, D. C., I. Hers, K. J. Heesom, S. K. Moule, and J. M. Tavare.** 2002. The identification of ATP-citrate lyase as a protein kinase B (Akt) substrate in primary adipocytes. J Biol Chem **277:**33895-900.
13. **Borkhardt, A., R. Repp, O. A. Haas, T. Leis, J. Harbott, J. Kreuder, J. Hammermann, T. Henn, and F. Lampert.** 1997. Cloning and characterization of AFX, the gene that fuses to MLL in acute leukemias with a t(X;11)(q13;q23). Oncogene **14:**195-202.
14. **Brownawell, A. M., G. J. Kops, I. G. Macara, and B. M. Burgering.** 2001. Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. Mol Cell Biol **21:**3534-46.
15. **Brunet, A., A. Bonni, M. J. Zigmond, M. Z. Lin, P. Juo, L. S. Hu, M. J. Anderson, K. C. Arden, J. Blenis, and M. E. Greenberg.** 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell **96:**857-68.
16. **Brunet, A., J. Park, H. Tran, L. S. Hu, B. A. Hemmings, and M. E. Greenberg.** 2001. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). Mol Cell Biol **21:**952-65.
17. **Burd, C. G., and S. D. Emr.** 1998. Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. Mol Cell **2:**157-62.
18. **Burgering, B. M., and P. J. Coffer.** 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature **376:**599-602.
19. **Cardone, M. H., N. Roy, H. R. Stennicke, G. S. Salvesen, T. F. Franke, E. Stanbridge, S. Frisch, and J. C. Reed.** 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science **282:**1318-21.
20. **Cenni, V., A. Sirri, M. Riccio, G. Lattanzi, S. Santi, A. de Pol, N. M. Maraldi, and S. Marmiroli.** 2003. Targeting of the Akt/PKB kinase to the actin skeleton. Cell Mol Life Sci **60:**2710-20.
21. **Chen, H. K., P. Fernandez-Funez, S. F. Acevedo, Y. C. Lam, M. D. Kaytor, M. H. Fernandez, A. Aitken, E. M. Skoulakis, H. T. Orr, J. Botas, and H. Y. Zoghbi.** 2003. Interaction of Akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1. Cell **113:**457-68.
22. **Chen, Q., D. W. Powell, M. J. Rane, S. Singh, W. Butt, J. B. Klein, and K. R. McLeish.** 2003. Akt Phosphorylates p47(phox) and Mediates Respiratory Burst Activity in Human Neutrophils. J Immunol **170:**5302-8.
23. **Chen, R. H., Y. H. Su, R. L. Chuang, and T. Y. Chang.** 1998. Suppression of transforming growth factor-beta-induced apoptosis through a phosphatidylinositol 3-kinase/Akt-dependent pathway. Oncogene **17:**1959-68.
24. **Ching, T. T., H. P. Lin, C. C. Yang, M. Oliveira, P. J. Lu, and C. S. Chen.** 2001. Specific binding of the C-terminal Src homology 2 domain of the p85alpha subunit of phosphoinositide 3-kinase to phosphatidylinositol 3,4,5-trisphosphate. Localization and engineering of the phosphoinositide-binding motif. J Biol Chem **276:**43932-8.
25. **Coffer, P. J., and J. R. Woodgett.** 1991. Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. Eur J Biochem **201:**475-81.
26. **Conery, A. R., Y. Cao, E. A. Thompson, C. M. Townsend, Jr., T. C. Ko, and K. Luo.** 2004. Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. Nat Cell Biol **6:**366-72.
27. **Cross, D. A., D. R. Alessi, P. Cohen, M. Andjelkovich, and B. A. Hemmings.** 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature **378:**785-9.
28. **Daitoku, H., K. Yamagata, H. Matsuzaki, M. Hatta, and A. Fukamizu.** 2003. Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. Diabetes **52:**642-9.
29. **Dan, H. C., M. Sun, S. Kaneko, R. I. Feldman, S. V. Nicosia, H. G. Wang, B. K. Tsang, and J. Q. Cheng.** 2004. Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP). J Biol Chem **279:**5405-12.

30. **Dansen, T. B., G. J. Kops, S. Denis, N. Jelluma, R. J. Wanders, J. L. Bos, B. M. Burgering, and K. W. Wirtz.** 2004. Regulation of sterol carrier protein gene expression by the Forkhead transcription factor FOXO3a. J Lipid Res **45:**81-8. Epub 2003 Oct 16.
31. **Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M. E. Greenberg.** 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell **91:**231-41.
32. **Davis, R. J., C. M. D'Cruz, M. A. Lovell, J. A. Biegel, and F. G. Barr.** 1994. Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. Cancer Res **54:**2869-72.
33. **Delcommenne, M., C. Tan, V. Gray, L. Rue, J. Woodgett, and S. Dedhar.** 1998. Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc Natl Acad Sci U S A **95:**11211-6.
34. **Deprez, J., D. Vertommen, D. R. Alessi, L. Hue, and M. H. Rider.** 1997. Phosphorylation and activation of heart 6-phosphofructo-2-kinase by protein kinase B and other protein kinases of the insulin signaling cascades. J Biol Chem **272:**17269-75.
35. **Diehl, J. A., M. Cheng, M. F. Roussel, and C. J. Sherr.** 1998. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev **12:**3499-511.
36. **Dijkers, P. F., R. H. Medema, J. W. Lammers, L. Koenderman, and P. J. Coffer.** 2000. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr Biol **10:**1201-4.
37. **Dijkers, P. F., R. H. Medema, C. Pals, L. Banerji, N. S. Thomas, E. W. Lam, B. M. Burgering, J. A. Raaijmakers, J. W. Lammers, L. Koenderman, and P. J. Coffer.** 2000. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol Cell Biol **20:**9138-48.
38. **Dimmeler, S., I. Fleming, B. Fisslthaler, C. Hermann, R. Busse, and A. M. Zeiher.** 1999. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature **399:**601-5.
39. **Downward, J.** 1999. How BAD phosphorylation is good for survival. Nat Cell Biol **1:**E33-5.
40. **Du, K., S. Herzig, R. N. Kulkarni, and M. Montminy.** 2003. TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. Science **300:**1574-7.
41. **Durham, S. K., A. Suwanichkul, A. O. Scheimann, D. Yee, J. G. Jackson, F. G. Barr, and D. R. Powell.** 1999. FKHR binds the insulin response element in the insulin-like growth factor binding protein-1 promoter. Endocrinology **140:**3140-6.
42. **Dutil, E. M., A. Toker, and A. C. Newton.** 1998. Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). Curr Biol **8:**1366-75.
43. **Feng, J., R. Tamaskovic, Z. Yang, D. P. Brazil, A. Merlo, D. Hess, and B. A. Hemmings.** 2004. Stabilization of Mdm2 via decreased ubiquitination is mediated by protein kinase B/Akt-dependent phosphorylation. J Biol Chem **28:**28.
44. **Figueroa, C., S. Tarras, J. Taylor, and A. B. Vojtek.** 2003. Akt2 negatively regulates assembly of the POSH-MLK-JNK signaling complex. J Biol Chem **278:**47922-7.
45. **Fontana, J., D. Fulton, Y. Chen, T. A. Fairchild, T. J. McCabe, N. Fujita, T. Tsuruo, and W. C. Sessa.** 2002. Domain mapping studies reveal that the M domain of hsp90 serves as a molecular scaffold to regulate Akt-dependent phosphorylation of endothelial nitric oxide synthase and NO release. Circ Res **90:**866-73.
46. **Foucher, I., M. Volovitch, M. Frain, J. J. Kim, J. C. Souberbielle, L. Gan, T. G. Unterman, A. Prochiantz, and A. Trembleau.** 2002. Hoxa5 overexpression correlates with IGFBP1 upregulation and postnatal dwarfism: evidence for an interaction between Hoxa5 and Forkhead box transcription factors. Development **129:**4065-74.
47. **Fukumoto, S., C. M. Hsieh, K. Maemura, M. D. Layne, S. F. Yet, K. H. Lee, T. Matsui, A. Rosenzweig, W. G. Taylor, J. S. Rubin, M. A. Perrella, and M. E. Lee.** 2001. Akt participation in the Wnt signaling pathway through Dishevelled. J Biol Chem **276:**17479-83.
48. **Furukawa-Hibi, Y., K. Yoshida-Araki, T. Ohta, K. Ikeda, and N. Motoyama.** 2002. FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress. J Biol Chem **277:**26729-32.
49. **Furuyama, T., K. Kitayama, H. Yamashita, and N. Mori.** 2003. Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscles during energy deprivation. Biochem J **23**.
50. **Furuyama, T., T. Nakazawa, I. Nakano, and N. Mori.** 2000. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J **349:**629-34.
51. **Furuyama, T., H. Yamashita, K. Kitayama, Y. Higami, I. Shimokawa, and N. Mori.** 2002. Effects of aging and caloric restriction on the gene expression of Foxo1, 3, and 4 (FKHR, FKHRL1,

and AFX) in the rat skeletal muscles. Microsc Res Tech **59:**331-4.

52. **Galili, N., R. J. Davis, W. J. Fredericks, S. Mukhopadhyay, F. J. Rauscher, 3rd, B. S. Emanuel, G. Rovera, and F. G. Barr.** 1993. Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat Genet **5:**230-5.
53. **Gilley, J., P. J. Coffer, and J. Ham.** 2003. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. J Cell Biol **162:**613-22.
54. **Hayakawa, J., M. Ohmichi, H. Kurachi, Y. Kanda, K. Hisamoto, Y. Nishio, K. Adachi, K. Tasaka, T. Kanzaki, and Y. Murata.** 2000. Inhibition of BAD phosphorylation either at serine 112 via extracellular signal-regulated protein kinase cascade or at serine 136 via Akt cascade sensitizes human ovarian cancer cells to cisplatin. Cancer Res **60:**5988-94.
55. **Hill, M. M., J. Feng, and B. A. Hemmings.** 2002. Identification of a plasma membrane Raft-associated PKB Ser473 kinase activity that is distinct from ILK and PDK1. Curr Biol **12:**1251-5.
56. **Hillion, J., M. Le Coniat, P. Jonveaux, R. Berger, and O. A. Bernard.** 1997. AF6q21, a novel partner of the MLL gene in t(6;11)(q21;q23), defines a forkhead transcriptional factor subfamily. Blood **90:**3714-9.
57. **Hirata, Y., K. Kiuchi, H. C. Chen, J. Milbrandt, and G. Guroff.** 1993. The phosphorylation and DNA binding of the DNA-binding domain of the orphan nuclear receptor NGFI-B. J Biol Chem **268:**24808-12.
58. **Hirota, K., H. Daitoku, H. Matsuzaki, N. Araya, K. Yamagata, S. Asada, T. Sugaya, and A. Fukamizu.** 2003. Hepatocyte nuclear factor-4 is a novel downstream target of insulin via FKHR as a signal-regulated transcriptional inhibitor. J Biol Chem **278:**13056-60.
59. **Honda, Y., and S. Honda.** 2002. Oxidative stress and life span determination in the nematode Caenorhabditis elegans. Ann N Y Acad Sci **959:**466-74.
60. **Hoyal, C. R., A. Gutierrez, B. M. Young, S. D. Catz, J. H. Lin, P. N. Tsichlis, and B. M. Babior.** 2003. Modulation of p47PHOX activity by site-specific phosphorylation: Akt-dependent activation of the NADPH oxidase. Proc Natl Acad Sci U S A **100:**5130-5.
61. **Hu, M. C., D. F. Lee, W. Xia, L. S. Golfman, F. Ou-Yang, J. Y. Yang, Y. Zou, S. Bao, N. Hanada, H. Saso, R. Kobayashi, and M. C. Hung.** 2004. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell **117:**225-37.
62. **Huang, S., L. Shu, M. B. Dilling, J. Easton, F. C. Harwood, H. Ichijo, and P. J. Houghton.** 2003. Sustained activation of the JNK cascade and rapamycin-induced apoptosis are suppressed by p53/p21(Cip1). Mol Cell **11:**1491-501.
63. **Humbert, S., E. A. Bryson, F. P. Cordelieres, N. C. Connors, S. R. Datta, S. Finkbeiner, M. E. Greenberg, and F. Saudou.** 2002. The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves Huntingtin phosphorylation by Akt. Dev Cell **2:**831-7.
64. **Imae, M., Z. Fu, A. Yoshida, T. Noguchi, and H. Kato.** 2003. Nutritional and hormonal factors control the gene expression of FoxOs, the mammalian homologues of DAF-16. J Mol Endocrinol **30:**253-62.
65. **Ingley, E., and B. A. Hemmings.** 2000. PKB/Akt interacts with inosine-5' monophosphate dehydrogenase through its pleckstrin homology domain. FEBS Lett **478:**253-9.
66. **Inoki, K., Y. Li, T. Zhu, J. Wu, and K. L. Guan.** 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol **4:**648-57.
67. **Jacobs, F. M., L. P. Van Der Heide, P. J. Wijchers, J. P. Burbach, M. F. Hoekman, and M. P. Smidt.** 2003. FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. J Biol Chem **11:**11.
68. **Jahn, T., P. Seipel, S. Urschel, C. Peschel, and J. Duyster.** 2002. Role for the adaptor protein Grb10 in the activation of Akt. Mol Cell Biol **22:**979-91.
69. **Jefferson, A. B., A. Klippel, and L. T. Williams.** 1998. Inhibition of mSOS-activity by binding of phosphatidylinositol 4,5-P2 to the mSOS pleckstrin homology domain. Oncogene **16:**2303-10.
70. **Jones, P. F., T. Jakubowicz, F. J. Pitossi, F. Maurer, and B. A. Hemmings.** 1991. Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. Proc Natl Acad Sci U S A **88:**4171-5.
71. **Junger, M. A., F. Rintelen, H. Stocker, J. D. Wasserman, M. Vegh, T. Radimerski, M. E. Greenberg, and E. Hafen.** 2003. The Drosophila Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol **2:**20.
72. **Kaestner, K. H., W. Knochel, and D. E. Martinez.** 2000. Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev **14:**142-6.
73. **Kamei, Y., J. Mizukami, S. Miura, M. Suzuki, N. Takahashi, T. Kawada, T. Taniguchi, and O. Ezaki.** 2003. A forkhead transcription factor FKHR up-regulates lipoprotein lipase expression in skeletal muscle. FEBS Lett **536:**232-6.

74. **Kane, L. P., M. N. Mollenauer, Z. Xu, C. W. Turck, and A. Weiss.** 2002. Akt-dependent phosphorylation specifically regulates Cot induction of NF-kappa B-dependent transcription. Mol Cell Biol **22:**5962-74.
75. **Kang, S. S., T. Kwon, D. Y. Kwon, and S. I. Do.** 1999. Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. J Biol Chem **274:**13085-90.
76. **Kim, A. H., G. Khursigara, X. Sun, T. F. Franke, and M. V. Chao.** 2001. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. Mol Cell Biol **21:**893-901.
77. **Kim, A. H., H. Yano, H. Cho, D. Meyer, B. Monks, B. Margolis, M. J. Birnbaum, and M. V. Chao.** 2002. Akt1 regulates a JNK scaffold during excitotoxic apoptosis. Neuron **35:**697-709.
78. **King, F. W., J. Skeen, N. Hay, and E. Shtivelman.** 2004. Inhibition of Chk1 by Activated PKB/Akt. Cell Cycle **3:**634-7.
79. **Kitamura, T., Y. Kitamura, S. Kuroda, Y. Hino, M. Ando, K. Kotani, H. Konishi, H. Matsuzaki, U. Kikkawa, W. Ogawa, and M. Kasuga.** 1999. Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3B by the serine-threonine kinase Akt. Mol Cell Biol **19:**6286-96.
80. **Klarlund, J. K., L. E. Rameh, L. C. Cantley, J. M. Buxton, J. J. Holik, C. Sakelis, V. Patki, S. Corvera, and M. P. Czech.** 1998. Regulation of GRP1-catalyzed ADP ribosylation factor guanine nucleotide exchange by phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem **273:**1859-62.
81. **Kohn, A. D., S. A. Summers, M. J. Birnbaum, and R. A. Roth.** 1996. Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. J Biol Chem **271:**31372-8.
82. **Konishi, H., S. Kuroda, and U. Kikkawa.** 1994. The pleckstrin homology domain of RAC protein kinase associates with the regulatory domain of protein kinase C zeta. Biochem Biophys Res Commun **205:**1770-5.
83. **Konishi, H., S. Kuroda, M. Tanaka, H. Matsuzaki, Y. Ono, K. Kameyama, T. Haga, and U. Kikkawa.** 1995. Molecular cloning and characterization of a new member of the RAC protein kinase family: association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and beta gamma subunits of G proteins. Biochem Biophys Res Commun **216:**526-34.
84. **Konishi, H., H. Matsuzaki, M. Tanaka, Y. Takemura, S. Kuroda, Y. Ono, and U. Kikkawa.** 1997. Activation of protein kinase B (Akt/RAC-protein kinase) by cellular stress and its association with heat shock protein Hsp27. FEBS Lett **410:**493-8.
85. **Konishi, H., T. Shinomura, S. Kuroda, Y. Ono, and U. Kikkawa.** 1994. Molecular cloning of rat RAC protein kinase alpha and beta and their association with protein kinase C zeta. Biochem Biophys Res Commun **205:**817-25.
86. **Koo, S. H., H. Satoh, S. Herzig, C. H. Lee, S. Hedrick, R. Kulkarni, R. M. Evans, J. Olefsky, and M. Montminy.** 2004. PGC-1 promotes insulin resistance in liver through PPAR-alpha-dependent induction of TRB-3. Nat Med **10:**530-4.
87. **Kops, G. J., T. B. Dansen, P. E. Polderman, I. Saarloos, K. W. Wirtz, P. J. Coffer, T. T. Huang, J. L. Bos, R. H. Medema, and B. M. Burgering.** 2002. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature **419:**316-21.
88. **Kops, G. J., N. D. de Ruiter, A. M. De Vries-Smits, D. R. Powell, J. L. Bos, and B. M. Burgering.** 1999. Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature **398:**630-4.
89. **Kops, G. J., R. H. Medema, J. Glassford, M. A. Essers, P. F. Dijkers, P. J. Coffer, E. W. Lam, and B. M. Burgering.** 2002. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol Cell Biol **22:**2025-36.
90. **Kortylewski, M., F. Feld, K. D. Kruger, G. Bahrenberg, R. A. Roth, H. G. Joost, P. C. Heinrich, I. Behrmann, and A. Barthel.** 2003. Akt modulates STAT3-mediated gene expression through a FKHR (FOXO1a)-dependent mechanism. J Biol Chem **278:**5242-9.
91. **Kovacina, K. S., G. Y. Park, S. S. Bae, A. W. Guzzetta, E. Schaefer, M. J. Birnbaum, and R. A. Roth.** 2003. Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. J Biol Chem **278:**10189-94.
92. **Koyasu, S.** 2003. The role of PI3K in immune cells. Nat Immunol **4:**313-9.
93. **Kwon, T., D. Y. Kwon, J. Chun, J. H. Kim, and S. S. Kang.** 2000. Akt protein kinase inhibits Rac1-GTP binding through phosphorylation at serine 71 of Rac1. J Biol Chem **275:**423-8.
94. **Laine, J., G. Kunstle, T. Obata, M. Sha, and M. Noguchi.** 2000. The protooncogene TCL1 is an Akt kinase coactivator. Mol Cell **6:**395-407.
95. **Lee, M. J., S. Thangada, J. H. Paik, G. P. Sapkota, N. Ancellin, S. S. Chae, M. Wu, M. Morales-Ruiz, W. C. Sessa, D. R. Alessi, and T. Hla.** 2001. Akt-mediated phosphorylation of the G

protein-coupled receptor EDG-1 is required for endothelial cell chemotaxis. Mol Cell **8:**693-704.

96. **Li, J., C. Yen, D. Liaw, K. Podsypanina, S. Bose, S. I. Wang, J. Puc, C. Miliaresis, L. Rodgers, R. McCombie, S. H. Bigner, B. C. Giovanella, M. Ittmann, B. Tycko, H. Hibshoosh, M. H. Wigler, and R. Parsons.** 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science **275:**1943-7.
97. **Li, P., H. Lee, S. Guo, T. G. Unterman, G. Jenster, and W. Bai.** 2003. AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR. Mol Cell Biol **23:**104-18.
98. **Lin, H. K., L. Wang, Y. C. Hu, S. Altuwaijri, and C. Chang.** 2002. Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. Embo J **21:**4037-48.
99. **Lin, H. K., S. Yeh, H. Y. Kang, and C. Chang.** 2001. Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. Proc Natl Acad Sci U S A **98:**7200-5.
100. **Lindvall, J., and T. C. Islam.** 2002. Interaction of Btk and Akt in B cell signaling. Biochem Biophys Res Commun **293:**1319-26.
101. **Lynch, D. K., and R. J. Daly.** 2002. PKB-mediated negative feedback tightly regulates mitogenic signalling via Gab2. Embo J **21:**72-82.
102. **Magun, R., B. M. Burgering, P. J. Coffer, D. Pardasani, Y. Lin, J. Chabot, and A. Sorisky.** 1996. Expression of a constitutively activated form of protein kinase B (c-Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation. Endocrinology **137:**3590-3.
103. **Maira, S. M., I. Galetic, D. P. Brazil, S. Kaech, E. Ingley, M. Thelen, and B. A. Hemmings.** 2001. Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. Science **294:**374-80.
104. **Mawal-Dewan, M., A. Lorenzini, L. Frisoni, H. Zhang, V. J. Cristofalo, and C. Sell.** 2002. Regulation of collagenase expression during replicative senescence in human fibroblasts by Akt-forkhead signaling. J Biol Chem **277:**7857-64.
105. **Mayo, L. D., and D. B. Donner.** 2001. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A **98:**11598-603.
106. **Medema, R. H., G. J. Kops, J. L. Bos, and B. M. Burgering.** 2000. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature **404:**782-7.
107. **Michaud, N. R., J. R. Fabian, K. D. Mathes, and D. K. Morrison.** 1995. 14-3-3 is not essential for Raf-1 function: identification of Raf-1 proteins that are biologically activated in a 14-3-3- and Ras-independent manner. Mol Cell Biol **15:**3390-7.
108. **Mitsuuchi, Y., S. W. Johnson, G. Sonoda, S. Tanno, E. A. Golemis, and J. R. Testa.** 1999. Identification of a chromosome 3p14.3-21.1 gene, APPL, encoding an adaptor molecule that interacts with the oncoprotein-serine/threonine kinase AKT2. Oncogene **18:**4891-8.
109. **Miyoshi, J., T. Higashi, H. Mukai, T. Ohuchi, and T. Kakunaga.** 1991. Structure and transforming potential of the human cot oncogene encoding a putative protein kinase. Mol Cell Biol **11:**4088-96.
110. **Modur, V., R. Nagarajan, B. M. Evers, and J. Milbrandt.** 2002. FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. J Biol Chem **277:**47928-37.
111. **Morrison, D. K., and R. E. Cutler.** 1997. The complexity of Raf-1 regulation. Curr Opin Cell Biol **9:**174-9.
112. **Muise-Helmericks, R. C., H. L. Grimes, A. Bellacosa, S. E. Malstrom, P. N. Tsichlis, and N. Rosen.** 1998. Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. J Biol Chem **273:**29864-72.
113. **Murakami, S., and T. E. Johnson.** 2001. The OLD-1 positive regulator of longevity and stress resistance is under DAF-16 regulation in Caenorhabditis elegans. Curr Biol **11:**1517-23.
114. **Nadal, A., P. F. Marrero, and D. Haro.** 2002. Down-regulation of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by insulin: the role of the forkhead transcription factor FKHRL1. Biochem J **366:**289-97.
115. **Nakae, J., T. Kitamura, D. L. Silver, and D. Accili.** 2001. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. J Clin Invest **108:**1359-67.
116. **Nakamura, N., S. Ramaswamy, F. Vazquez, S. Signoretti, M. Loda, and W. R. Sellers.** 2000. Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. Mol Cell Biol **20:**8969-82.
117. **Nasrin, N., S. Ogg, C. M. Cahill, W. Biggs, S. Nui, J. Dore, D. Calvo, Y. Shi, G. Ruvkun, and M. C. Alexander-Bridges.** 2000. DAF-16 recruits the CREB-binding protein coactivator complex to the insulin-like growth factor binding protein 1 promoter in HepG2 cells. Proc Natl Acad Sci U S A **97:**10412-7.

118. **Nemoto, S., and T. Finkel.** 2002. Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. Science **295:**2450-2.
119. **Nicholson, K. M., and N. G. Anderson.** 2002. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal **14:**381-95.
120. **Odorizzi, G., M. Babst, and S. D. Emr.** 1998. Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. Cell **95:**847-58.
121. **Ogg, S., S. Paradis, S. Gottlieb, G. I. Patterson, L. Lee, H. A. Tissenbaum, and G. Ruvkun.** 1997. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature **389:**994-9.
122. **Ogg, S., and G. Ruvkun.** 1998. The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. Mol Cell **2:**887-93.
123. **Ookuma, S., M. Fukuda, and E. Nishida.** 2003. Identification of a DAF-16 Transcriptional Target Gene, scl-1, that Regulates Longevity and Stress Resistance in Caenorhabditis elegans. Curr Biol **13:**427-31.
124. **Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer, and D. B. Donner.** 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature **401:**82-5.
125. **Paradis, S., M. Ailion, A. Toker, J. H. Thomas, and G. Ruvkun.** 1999. A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans. Genes Dev **13:**1438-52.
126. **Paradis, S., and G. Ruvkun.** 1998. Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes Dev **12:**2488-98.
127. **Paramio, J. M., M. L. Casanova, C. Segrelles, S. Mittnacht, E. B. Lane, and J. L. Jorcano.** 1999. Modulation of cell proliferation by cytokeratins K10 and K16. Mol Cell Biol **19:**3086-94.
128. **Paramio, J. M., C. Segrelles, S. Ruiz, and J. L. Jorcano.** 2001. Inhibition of protein kinase B (PKB) and PKCzeta mediates keratin K10-induced cell cycle arrest. Mol Cell Biol **21:**7449-59.
129. **Parekh, D. B., W. Ziegler, and P. J. Parker.** 2000. Multiple pathways control protein kinase C phosphorylation. Embo J **19:**496-503.
130. **Parry, P., Y. Wei, and G. Evans.** 1994. Cloning and characterization of the t(X;11) breakpoint from a leukemic cell line identify a new member of the forkhead gene family. Genes Chromosomes Cancer **11:**79-84.
131. **Partovian, C., and M. Simons.** 2004. Regulation of protein kinase B/Akt activity and Ser(473) phosphorylation by protein kinase Calpha in endothelial cells. Cell Signal **16:**951-7.
132. **Paz, K., Y. F. Liu, H. Shorer, R. Hemi, D. LeRoith, M. Quan, H. Kanety, R. Seger, and Y. Zick.** 1999. Phosphorylation of insulin receptor substrate-1 (IRS-1) by protein kinase B positively regulates IRS-1 function. J Biol Chem **274:**28816-22.
133. **Pekarsky, Y., C. Hallas, A. Palamarchuk, A. Koval, F. Bullrich, Y. Hirata, R. Bichi, J. Letofsky, and C. M. Croce.** 2001. Akt phosphorylates and regulates the orphan nuclear receptor Nur77. Proc Natl Acad Sci U S A **98:**3690-4.
134. **Pekarsky, Y., A. Koval, C. Hallas, R. Bichi, M. Tresini, S. Malstrom, G. Russo, P. Tsichlis, and C. M. Croce.** 2000. Tcl1 enhances Akt kinase activity and mediates its nuclear translocation. Proc Natl Acad Sci U S A **97:**3028-33.
135. **Pierce, M. W., J. L. Palmer, H. T. Keutmann, T. A. Hall, and J. Avruch.** 1982. The insulin-directed phosphorylation site on ATP-citrate lyase is identical with the site phosphorylated by the cAMP-dependent protein kinase in vitro. J Biol Chem **257:**10681-6.
136. **Piper, R. C., A. A. Cooper, H. Yang, and T. H. Stevens.** 1995. VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in Saccharomyces cerevisiae. J Cell Biol **131:**603-17.
137. **Potter, C. J., L. G. Pedraza, and T. Xu.** 2002. Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol **4:**658-65.
138. **Powell, D. W., M. J. Rane, Q. Chen, S. Singh, and K. R. McLeish.** 2002. Identification of 14-3-3zeta as a protein kinase B/Akt substrate. J Biol Chem **277:**21639-42.
139. **Puig, O., M. T. Marr, M. L. Ruhf, and R. Tjian.** 2003. Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes Dev **17:**2006-20.
140. **Puigserver, P., J. Rhee, J. Donovan, C. J. Walkey, J. C. Yoon, F. Oriente, Y. Kitamura, J. Altomonte, H. Dong, D. Accili, and B. M. Spiegelman.** 2003. Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. Nature **423:**550-5.
141. **Rameh, L. E., A. Arvidsson, K. L. Carraway, 3rd, A. D. Couvillon, G. Rathbun, A. Crompton, B. VanRenterghem, M. P. Czech, K. S. Ravichandran, S. J. Burakoff, D. S. Wang, C. S. Chen, and L. C. Cantley.** 1997. A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. J Biol Chem **272:**22059-66.

142. **Rameh, L. E., C. S. Chen, and L. C. Cantley.** 1995. Phosphatidylinositol (3,4,5)P3 interacts with SH2 domains and modulates PI 3-kinase association with tyrosine-phosphorylated proteins. Cell **83:**821-30.
143. **Ravichandran, L. V., H. Chen, Y. Li, and M. J. Quon.** 2001. Phosphorylation of PTP1B at Ser(50) by Akt impairs its ability to dephosphorylate the insulin receptor. Mol Endocrinol **15:**1768-80.
144. **Remy, I., A. Montmarquette, and S. W. Michnick.** 2004. PKB/Akt modulates TGF-beta signalling through a direct interaction with Smad3. Nat Cell Biol **6:**358-65.
145. **Rena, G., S. Guo, S. C. Cichy, T. G. Unterman, and P. Cohen.** 1999. Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. J Biol Chem **274:**17179-83.
146. **Rena, G., A. R. Prescott, S. Guo, P. Cohen, and T. G. Unterman.** 2001. Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targetting. Biochem J **354:**605-12.
147. **Reusch, H. P., S. Zimmermann, M. Schaefer, M. Paul, and K. Moelling.** 2001. Regulation of Raf by Akt controls growth and differentiation in vascular smooth muscle cells. J Biol Chem **276:**33630-7.
148. **Rhee, J., Y. Inoue, J. C. Yoon, P. Puigserver, M. Fan, F. J. Gonzalez, and B. M. Spiegelman.** 2003. Regulation of hepatic fasting response by PPARgamma coactivator-1alpha (PGC-1): requirement for hepatocyte nuclear factor 4alpha in gluconeogenesis. Proc Natl Acad Sci U S A **100:**4012-7.
149. **Rokudai, S., N. Fujita, O. Kitahara, Y. Nakamura, and T. Tsuruo.** 2002. Involvement of FKHR-dependent TRADD expression in chemotherapeutic drug-induced apoptosis. Mol Cell Biol **22:**8695-708.
150. **Rong, S. B., Y. Hu, I. Enyedy, G. Powis, E. J. Meuillet, X. Wu, R. Wang, S. Wang, and A. P. Kozikowski.** 2001. Molecular modeling studies of the Akt PH domain and its interaction with phosphoinositides. J Med Chem **44:**898-908.
151. **Salmeron, A., T. B. Ahmad, G. W. Carlile, D. Pappin, R. P. Narsimhan, and S. C. Ley.** 1996. Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. Embo J **15:**817-26.
152. **Sandri, M., C. Sandri, A. Gilbert, C. Skurk, E. Calabria, A. Picard, K. Walsh, S. Schiaffino, S. H. Lecker, and A. L. Goldberg.** 2004. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell **117:**399-412.
153. **Santos, M., J. M. Paramio, A. Bravo, A. Ramirez, and J. L. Jorcano.** 2002. The expression of keratin k10 in the basal layer of the epidermis inhibits cell proliferation and prevents skin tumorigenesis. J Biol Chem **277:**19122-30.
154. **Sato, S., N. Fujita, and T. Tsuruo.** 2000. Modulation of Akt kinase activity by binding to Hsp90. Proc Natl Acad Sci U S A **97:**10832-7.
155. **Schmidt, M., S. Fernandez de Mattos, A. van der Horst, R. Klompmaker, G. J. Kops, E. W. Lam, B. M. Burgering, and R. H. Medema.** 2002. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol **22:**7842-52.
156. **Schurmann, A., A. F. Mooney, L. C. Sanders, M. A. Sells, H. G. Wang, J. C. Reed, and G. M. Bokoch.** 2000. p21-activated kinase 1 phosphorylates the death agonist bad and protects cells from apoptosis. Mol Cell Biol **20:**453-61.
157. **Schuur, E. R., A. V. Loktev, M. Sharma, Z. Sun, R. A. Roth, and R. J. Weigel.** 2001. Ligand-dependent interaction of estrogen receptor-alpha with members of the forkhead transcription factor family. J Biol Chem **276:**33554-60.
158. **Seoane, J., H. V. Le, L. Shen, S. A. Anderson, and J. Massague.** 2004. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell **117:**211-23.
159. **Shapiro, D. N., J. E. Sublett, B. Li, J. R. Downing, and C. W. Naeve.** 1993. Fusion of PAX3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyosarcoma. Cancer Res **53:**5108-12.
160. **Shin, I., F. M. Yakes, F. Rojo, N. Y. Shin, A. V. Bakin, J. Baselga, and C. L. Arteaga.** 2002. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat Med **8:**1145-52.
161. **Simonsen, A., R. Lippe, S. Christoforidis, J. M. Gaullier, A. Brech, J. Callaghan, B. H. Toh, C. Murphy, M. Zerial, and H. Stenmark.** 1998. EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature **394:**494-8.
162. **Staal, S. P.** 1987. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A **84:**5034-7.
163. **Stahl, M., P. F. Dijkers, G. J. Kops, S. M. Lens, P. J. Coffer, B. M. Burgering, and R. H. Medema.** 2002. The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. J Immunol **168:**5024-31.
164. **Sun, L., L. Liu, X. J. Yang, and Z. Wu.** 2004. Akt binds prohibitin 2 and relieves its repression of MyoD and muscle differentiation. J Cell Sci **1**.

165. **Surdo, P. L., M. J. Bottomley, A. Arcaro, G. Siegal, G. Panayotou, A. Sankar, P. R. Gaffney, A. M. Riley, B. V. Potter, M. D. Waterfield, and P. C. Driscoll.** 1999. Structural and biochemical evaluation of the interaction of the phosphatidylinositol 3-kinase p85alpha Src homology 2 domains with phosphoinositides and inositol polyphosphates. J Biol Chem **274:**15678-85.
166. **Sussman, J., D. Stokoe, N. Ossina, and E. Shtivelman.** 2001. Protein kinase B phosphorylates AHNAK and regulates its subcellular localization. J Cell Biol **154:**1019-30.
167. **Suzuki, A., J. L. de la Pompa, V. Stambolic, A. J. Elia, T. Sasaki, I. del Barco Barrantes, A. Ho, A. Wakeham, A. Itie, W. Khoo, M. Fukumoto, and T. W. Mak.** 1998. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr Biol **8:**1169-78.
168. **Tanaka, M., H. Konishi, K. Touhara, F. Sakane, M. Hirata, Y. Ono, and U. Kikkawa.** 1999. Identification of myosin II as a binding protein to the PH domain of protein kinase B. Biochem Biophys Res Commun **255:**169-74.
169. **Tang, T. T., D. Dowbenko, A. Jackson, L. Toney, D. A. Lewin, A. L. Dent, and L. A. Lasky.** 2002. The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. J Biol Chem **277:**14255-65.
170. **Tang, T. T., and L. A. Lasky.** 2003. The Forkhead Transcription Factor FOXO4 Induces the Down-regulation of Hypoxia-inducible Factor 1{alpha} by a von Hippel-Lindau Protein-independent Mechanism. J Biol Chem **278:**30125-30135.
171. **Tang, Y., H. Zhou, A. Chen, R. N. Pittman, and J. Field.** 2000. The Akt proto-oncogene links Ras to Pak and cell survival signals. J Biol Chem **275:**9106-9.
172. **Toker, A., and A. C. Newton.** 2000. Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. J Biol Chem **275:**8271-4.
173. **Tran, H., A. Brunet, J. M. Grenier, S. R. Datta, A. J. Fornace, Jr., P. S. DiStefano, L. W. Chiang, and M. E. Greenberg.** 2002. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science **296:**530-4.
174. **Tsukazaki, T., T. A. Chiang, A. F. Davison, L. Attisano, and J. L. Wrana.** 1998. SARA, a FYVE domain protein that recruits Smad2 to the TGFbeta receptor. Cell **95:**779-91.
175. **van den Heuvel, A. P., A. M. de Vries-Smits, P. C. van Weeren, P. F. Dijkers, K. M. de Bruyn, J. A. Riedl, and B. M. Burgering.** 2002. Binding of protein kinase B to the plakin family member periplakin. J Cell Sci **115:**3957-66.
176. **van Weeren, P. C., K. M. de Bruyn, A. M. de Vries-Smits, J. van Lint, and B. M. Burgering.** 1998. Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. J Biol Chem **273:**13150-6.
177. **Vander Kooi, B. T., R. S. Streeper, C. A. Svitek, J. K. Oeser, D. R. Powell, and R. M. O'Brien.** 2003. The three insulin response sequences in the glucose-6-phosphatase catalytic subunit gene promoter are functionally distinct. J Biol Chem **278:**11782-93.
178. **Venkateswarlu, K., P. B. Oatey, J. M. Tavare, and P. J. Cullen.** 1998. Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. Curr Biol **8:**463-6.
179. **Virgilio, L., M. G. Narducci, M. Isobe, L. G. Billips, M. D. Cooper, C. M. Croce, and G. Russo.** 1994. Identification of the TCL1 gene involved in T-cell malignancies. Proc Natl Acad Sci U S A **91:**12530-4.
180. **Wang, Q., L. Liu, L. Pei, W. Ju, G. Ahmadian, J. Lu, Y. Wang, F. Liu, and Y. T. Wang.** 2003. Control of synaptic strength, a novel function of akt. Neuron **38:**915-28.
181. **Wen, Y., M. C. Hu, K. Makino, B. Spohn, G. Bartholomeusz, D. H. Yan, and M. C. Hung.** 2000. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. Cancer Res **60:**6841-5.
182. **Wolfrum, C., D. Besser, E. Luca, and M. Stoffel.** 2003. Insulin regulates the activity of forkhead transcription factor Hnf-3beta/Foxa-2 by Akt-mediated phosphorylation and nuclear/cytosolic localization. Proc Natl Acad Sci U S A **100:**11624-9.
183. **Woodcock, J. M., J. Murphy, F. C. Stomski, M. C. Berndt, and A. F. Lopez.** 2003. The dimeric versus monomeric status of 14-3-3zeta is controlled by phosphorylation of Ser58 at the dimer interface. J Biol Chem **15:**15.
184. **Woods, Y. L., G. Rena, N. Morrice, A. Barthel, W. Becker, S. Guo, T. G. Unterman, and P. Cohen.** 2001. The kinase DYRK1A phosphorylates the transcription factor FKHR at Ser329 in vitro, a novel in vivo phosphorylation site. Biochem J **355:**597-607.
185. **Yang, L., H. K. Lin, S. Altuwaijri, S. Xie, L. Wang, and C. Chang.** 2003. APPL suppresses androgen receptor transactivation via potentiating Akt activity. J Biol Chem **278:**16820-7.
186. **Yang, Z., J. Whelan, R. Babb, and B. R. Bowen.** 2002. An mRNA splice variant of the AFX gene

with altered transcriptional activity. J Biol Chem **277:**8068-75.

187. **Yu, K. T., W. B. Benjamin, S. Ramakrishna, N. Khalaf, and M. P. Czech.** 1990. An insulin-sensitive cytosolic protein kinase accounts for the regulation of ATP citrate-lyase phosphorylation. Biochem J **268:**539-45.
188. **Yue, Y., J. Lypowy, N. Hedhli, and M. Abdellatif.** 2004. Ras GTPase-activating protein binds to Akt and is required for its activation. J Biol Chem **279:**12883-9.
189. **Zhao, H. H., R. E. Herrera, E. Coronado-Heinsohn, M. C. Yang, J. H. Ludes-Meyers, K. J. Seybold-Tilson, Z. Nawaz, D. Yee, F. G. Barr, S. G. Diab, P. H. Brown, S. A. Fuqua, and C. K. Osborne.** 2001. Forkhead homologue in rhabdomyosarcoma functions as a bifunctional nuclear receptor-interacting protein with both coactivator and corepressor functions. J Biol Chem **276:**27907-12.
190. **Zhou, B. P., Y. Liao, W. Xia, B. Spohn, M. H. Lee, and M. C. Hung.** 2001. Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat Cell Biol **3:**245-52.
191. **Zhou, B. P., Y. Liao, W. Xia, Y. Zou, B. Spohn, and M. C. Hung.** 2001. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol **3:**973-82.
192. **Zimmermann, S., and K. Moelling.** 1999. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science **286:**1741-4.

Chapter

2

Binding of protein kinase B

to the plakin family member periplakin

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The serine/threonine kinase protein kinase B (PKB/c-Akt) acts downstream of the lipid kinase phosphoinositide 3-kinase (PI-3K) and functions as an essential mediator in many growth factor-induced cellular responses like cell cycle regulation, cell survival and transcriptional regulation. PI-3K activation generates 3' phosphorylated phosphoinositide lipids (PtdIns3Ps) and PKB activation requires PtdIns3P-dependent membrane translocation and phosphorylation by upstream kinases. However PKB activation and function is also regulated by interaction with other proteins. Here we show binding of PKB to periplakin, a member of the plakin family of cytolinker proteins. Interaction between PKB and periplakin was mapped to part of the pleckstrin homology (PH) domain of PKB, most likely not involved in lipid binding, and indeed binding to periplakin did not affect PKB activation. We therefore investigated the possibility that periplakin may act as a scaffold or localization signal for PKB. In cells endogenous periplakin localizes to different cellular compartments, including plasma membrane, intermediate filament structures, nucleus and mitochondria. Overexpression of the c-terminal part of periplakin, encompassing the PKB binding region, results in predominant intermediate filament localization and little nuclear staining. This also resulted in inhibition of nuclear PKB signalling as indicated by inhibition of PKB-dependent Forkhead transcription factor regulation. These results suggest a possible role for periplakin as localization signal in PKB-mediated signalling.

Introduction

Protein kinase B (PKB, also known as c-akt) is a pleckstrin-homology (PH) domain containing serine/threonine kinase. In mammalian cells PKB is activated upon treatment of cells with a wide variety of extracellular growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF) and insulin (for review see (14)). Activation of the cognate receptor for these factors results in the recruitment to the plasma membrane and activation of phosphoinositide 3'-kinase (PI-3K). Active PI-3K produces 3' phosphorylated inositol (PtdIns3P) lipids that act as second messengers to recruit amongst others, PH-domain containing proteins to the plasma membrane. Upon translocation to the cell membrane PKB becomes phosphorylated on two residues essential for activation, Thr308 and Ser473. Besides translocation and phosphorylation, PKB activity can also be regulated by interaction with other proteins. For example it has been suggested that aPKCs can bind to PKB and by phosphorylation of an unidentified residue can negatively regulate PKB activity (21). More recently a novel small protein named CTMP has been shown to interact with the c-terminal region of PKB and binding of CTMP results in inhibition of PKB activity (29).

Once activated, PKB can phosphorylate a range of proteins on either serine or threonine residues contained within a RxRxxS/T motif. One of the first identified substrates for PKB is glycogen synthase kinase-3 (GSK3) (15). PKB phosphorylates Ser 9 of GSK3b and Ser 21 of GSK3a both in vitro (15) and in vivo (46), and this results in inactivation of GSK3 thereby regulating glucose utilization and activation of glycogen synthesis (39).

PKB can also regulate transcription factor activity. Upon activation PKB has been shown to translocate to the nucleus (3, 31), where it can phosphorylate transcription factors such as the FOXO members of the Forkhead family (reviewed in (28)). This leads to nuclear export and inactivation of these FOXO transcription

factors. Regulation of FOXO transcriptional activity has been implicated in cell cycle and cell death control by PKB. However, besides FOXO regulation many other mechanisms have been described for PKB-mediated protection from apoptosis. For example direct phosphorylation of proteins like the pro-apoptotic Bcl-2 family member BAD (17), or caspase-9 (13). Irrespective of the multitude of potential protein targets for PKB it is clear that PKB can protect from mitochondrial-dependent apoptosis (27). Thus PKB activity can maintain mitochondrial membrane stability and prevent cytochrome-C leakage under conditions of stress (25, 36).

Periplakin belongs to the plakin family of cytolinker proteins that also includes desmoplakin, envoplakin, plectin and bullous pemphigoid antigen 1 (BPAG1) (38). It has an approximate molecular mass of 195 kDa, contains a central rod dimerisation domain and its carboxy-terminal region is involved in intermediate filament binding whereas its amino-terminus has been shown to interact with cortical actin (20). Periplakin is expressed in epithelial cells (2), where it is found to be, together with envoplakin, the precursor of the epidermal cornified envelope (20). Furthermore periplakin has been shown to be a target antigen in paraneoplastic pemphigus (18), and in keratinocytes it localizes at desmosomes and the interdesmosomal plasma membrane (40). Although these observations suggest a specified role of plakin family members in epidermal cornification there is substantial evidence that within other cell types plakins may be involved in a variety of cellular processes. For example plectin, the best-studied member of the plakin family, has been suggested to play a role in reorganization of microfilaments in apoptosis when it is cleaved by caspase 8 (42). A further role in this is suggested by the observation that in muscle cells it is found associated with mitochondria and intermediate filaments (37). Also in endothelial cells plectin localizes to focal contacts and here a role in adhesion is suggested (24).

In order to investigate PKB regulation through binding to other proteins in more detail we performed a yeast-two-hybrid screen with a part of the PH domain of PKB. We found that periplakin is an interaction partner of PKB and determined the binding side within PKB. Furthermore we also show binding of periplakin to the intermediate filament protein vimentin. Besides to intermediate filament structures, periplakin localizes to the cell membrane, nucleus and mitochondria. Localization to these cellular compartments is in part influenced by the presence or absence of vimentin. These and other observations suggest that periplakin may act as localization signal for PKB signalling.

Materials and methods

Plasmids

The yeast-two-hybrid bait pPC97PKB was constructed by cloning a 534 nt PvuII-SmaI fragment of bovine PKBa (11), into SmaI digested pPC97. The c-terminal fragment of periplakin (pPC86-c-ppl) interacting with this bait contained the last 1357 nt of the mRNA (including 529 nt 3' UTR). Myc-c-ppl was constructed by subcloning the insert (SmaI-NotI) from pPC86-c-ppl into Myc9.1 (F.Zwartkruis unpublished). GST-c-ppl was created by cloning the insert (EcoRI-NotI) of pPC86-c-ppl into EcoRI-NotI digested pRP261 (pGEX-3X derivative (41)). A bait of the c-terminal part of periplakin (pPC97-c-ppl) was constructed by inserting the SmaI-NotI fragment of pPC86-c-ppl into SmaI-NotI digested pPC97. The vimentin expressing clones interacting with pPC97-c-ppl all contained vimentin, missing the first N-terminal 10 aa (pPC86-vim). HA-vimentin was constructed by cloning the insert (SalI-NotI) from pPC86-vim into SalI-NotI digested pMT2-HA (11). HA-PKB and HA-PKBdead have been described (11). Deletion mutants of HA-PKB were created as follows; Δ30-130 and Δ30-214 were created by isolation of partially digested MscI fragments of HA-PKB and religation. Δ130-311 was created by isolation of a partially digested AvaI fragment of HA-PKB and religation. Δ311-507 was created by digestion of HA-PKB with XmaI and BclI, blunt ending with T4-DNApolymerase and religation. Δ9 was created by digestion of HA-PKB with SacII and PstI followed by blunt ending by S1 nuclease and religation. Δ9/+9GLY was made by inserting a SacII-PstI oligo encoding 9 glycine residues in SacII/PstI digested HA-PKB. All

mutants were verified by sequencing. The following constructs that were used have been described: HA-MAPK and HA-p70s6k (11), HA-FOXO4 (30) HA-full length periplakin (20). All tagged constructs contain the tag at the 5' position.

Antibodies
Anti-periplakin c-terminal peptide antibody was raised by immunizing two rabbits with a peptide encoding the last twelve amino acids of periplakin (IQELAVLVSGQK) coupled to KLH (7445 and 7446). This peptide is fully conserved between human and mouse periplakin. The anti-GST-c-ppl sera were raised by immunizing two rabbits with purified GST-c-ppl (5117 and 5118). Anti-PKB (5179 and 5178) (11); anti-MAPK (124, (19)); anti-HA (12CA5, (11)) and anti-myc (9E10, (46)) were described previously. Anti-cytochrome c was kindly provided by F.J.T. Zwartkruis. Anti-vimentin was purchased from Oncogene Science, anti-cox4 from Molecular Probes. MAB1273 recognizing a 65 kDa mitochondrial protein was obtained from Chemicon International Inc., anti-pSer193 FOXO4 from Cell signaling, and anti-actin from Santa Cruz.

Cell culture
Rat-1, A14 and Cos-7 cells were grown in Dulbecco's modified Eagle's medium. 293T (human embryonic kidney 293 cells immortalised with SV40 large T antigen), MCF-7 and MCF-7/FR cells (MCF-7 cells stably expressing the Fas receptor) were grown in RPMI. Both media were supplemented with 10% fetal calf serum (Bio-Whittaker, Belgium), 1% Pen/Strep (Bio-Whittaker, Belgium) and 2 mM L-Gln (Bio-Whittaker, Belgium). Treatment of cells was for 10 minutes with 1 µM insulin after overnight starvation in medium without serum unless indicated otherwise. Cells were transfected using the $Ca(PO_4)_2$ procedure, except MCF-7 cells which were transfected with Fugene6 (Roche).

Yeast-two-hybrid screen
Yeast-two-hybrid screens were performed as described previously (48). An oligo-dT primed 13.5 day mouse embryo cDNA library cloned into the pPC86 yeast-two-hybrid vector was used in all cases. To diminish background 50 mM 3-amino-triazol was included in the screen and clones were picked after 3 and 4 days.
GST pull down assay
For purification of GST-c-ppl, protein expression was induced in DH5α using 100 nM isopropyl-1-thio-β-D-galactopyranoside for 20 hours at room temperature. Bacteria were collected and lysed in ice-cold phosphate-buffered saline containing 1% Triton X-100 and protease inhibitors. The lysates were sonicated three times for 20 seconds at 60 Hz (UP200S GmbH) and centrifuged at 10,000xg for 20 minutes to remove insoluble material. GST-c-ppl was purified from the cleared lysate by batchwise incubation with glutathione-agarose beads (Sigma), and after washing the protein was eluted from the beads in buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 10% glycerol, and 10 mM glutathione. The eluted protein was dialysed for 20 hours in the same buffer without glutathione.
GST or GST-c-ppl attached to glutathione sepharose were incubated for 1 hour with extracts of A14 cells transiently transfected with either wild type or kinase-dead HA-tagged PKB and washed four times in solubilisation buffer. Subsequently, bound protein was removed by elution with 40 mM reduced glutathione. Samples were analysed for the presences of PKB by western blotting using the anti-HA antibody.

Immunoprecipitations
Non-confluent cells were lysed in 0.5 ml RIPA buffer (20 mM Tris pH 8.0, 1% Triton X-100, 0.5% Na-DOC, 0.1% SDS, 10 mM EDTA, 150 mM NaCl, 1 mM NaF, 1 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin and 2 µg/ml aprotinin) except for the endogenous Ppl-PKB and vimentin co-immunoprecipitations (1% Triton X-100, 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mg/ml trypsin inhibitor, 1 µg/ml leupeptin and 2 µg/ml aprotinin). Lysates were incubated for 2 hours at 4°C with 10 µl antibody and 100 µl pre-washed prot-A beads. The immunoprecipitations were washed 4 times with the used lysis buffer before taken up in Leammli buffer.

Immunoblot analysis
Protein samples in Laemmli buffer were separated by SDS-PAGE on 6% (periplakin) or 10% (others) gels and transferred to PVDF membrane (NEN). Western blots were blocked overnight at 4°C in phosphate-buffered saline (PBS) containing 2% non-fat dry milk (Protifar, Nutricia) and 0.5% bovine serum albumin (Sigma). The western blots were then incubated for 2 hours with the indicated primary antibodies in PBS containing 0.1% Tween 20 using the dilutions recommended by the manufacturers or 1:8000 for the self-generated antibodies. After washing four times 5 minutes with PBS/0.1% Tween-20 blots were incubated with secondary antibodies anti-mouse HRP and anti-rabbit HRP (1:10000) for 1 hour at 4°C. Blots were washed again four times 5 minutes with PBS/0.1% Tween-20 and analysed with chemiluminescence (ECL (NEN)).

Immunofluorescence staining
Cells on cover slips were fixed in 4% paraformaldehyde for 30 minutes at 4ºC and then permeabilised with 0.1% Triton X-100 in the presence of 0.5% BSA for 30 minutes at 4ºC. Before fixation cells were incubated with 100 nM Mitotracker (Molecular Probes) for 30 minutes. Cells were incubated with primary antibodies for 2 hours at 4ºC, washed in PBS with 0.1% Triton X-100 and

0.01% BSA, and then incubated further for 1 hour with the appropriate conjugated secondary antibody. After further washing coverslips were mounted in Immu-mount (Shandon) and examined using a 63x planapo objective on a Leitz DMIRB fluorescence microscope (Leica, Voorburg, the Netherlands) interfaced with a Leica TCS4D confocal laser-scanning microscope (Leica, Heidelberg, Germany). Digital images were recorded using Leica TCS NT version 1.6.587.
As primary antibodies the anti-vimentin (Oncogene), anti-periplakin anti-serum 5117 (3rd boost) or non-immune antiserum were used; FITC conjugated anti-rabbit, anti-mouse or anti-goat antibodies were used as a secondary antibody.

Vimentin extraction
A14 cells were lysed with 0.5% Triton X-100 in CSK-buffer (10 mM Pipes pH 6.8, 250 mM sucrose, 3 mM $MgCl_2$, 150 mM KCl, 1 mM EGTA, 1 mM PMSF) for 10 minutes (fraction 1: total cells). Lysates were centrifuged at 14,000xg for 10 minutes (supernatant, fraction 2: membrane proteins, cytosol, tubulin). The pellet was further extracted with 0.3 M KI in CSK-buffer for 5 min and centrifuged at 14,000xg for 30 minutes (pellet, fraction 4: nuclei, intermediate filaments). Subsequently fraction 3 (actin) was isolated by dialyzing the supernatant against CSK-buffer for 16 hours and centrifuging at 14,000xg for 30 min. The pellet was resuspended in 0.3 M KI in CSK-buffer and incubated for 10 minutes after which the same dialysis was performed.

Percoll gradient-based cell fractionation
MCF-7, Rat-1 and Cos-7 cells were fractionated using a percoll gradient-based assay as described (4).

Affinity purification of mitochondria
Mitochondria from MCF-7 and Rat-1 cells were isolated as described (26).

Results

PKB interacts with periplakin

To search for PKB interacting proteins we performed a yeast two-hybrid screen using a C-terminal part of the PH domain of bovine PKB as bait, since it has been shown that the N-terminal part is predominantly involved in the interaction with PtdIns3P lipids (22). The bovine bait has 98% similarity and 96% identity with human PKB. From a screen of 10^6 transformants, positive clones were isolated and from these we identified the homologue of a recently cloned protein named periplakin as a potential PKB binding protein. We determined the full-length sequence of this clone and sequence comparison indicates that this protein (deposited accession number AF126834) is indeed the mouse homologue of human periplakin (Fig. 1A), which has 97% similarity and 88% identity with human periplakin. To further investigate binding of PKB to periplakin we performed a number of experiments. First, the C-terminal domain of periplakin was fused to GST (GST-c-ppl) and lysates of cells expressing transiently transfected HA-tagged wild type or kinase-dead PKB were incubated with GST-c-ppl, or GST alone as a negative control. Both HA-PKB and HA-PKBdead were found to bind to GST-c-ppl (Fig. 1B). Second, the C-terminal domain of periplakin was fused to a N-terminal myc tag (myc-c-ppl) and myc-c-ppl was co-transfected in A14 cells with either HA- PKB or HA-p70S6K as a control. myc-c-ppl was found to coprecipitate with HA-PKB and not with HA-p70S6K (Fig. 1C). Finally to determine association between endogenous PKB and periplakin we first developed two different polyclonal antisera against periplakin. One polyclonal was directed against GST-c-ppl and the other against a C-terminal peptide of periplakin coupled to KLH. Both types of antiserum recognized GST-c-ppl (Fig. 2A) immunoprecipitated transfected myc-c-ppl (Fig. 2B) and specifically recognized a protein with the expected approximate molecular weight of periplakin (~200 kD) in MCF-7 cells (Fig. 2C). Thus we conclude that these antisera indeed recognize endogenous periplakin. To determine what cell line(s) was suitable for analysing binding between endogenous PKB and periplakin, we analysed several cell lines for periplakin expression and observed highest expression in cells of (neuro)epithelial origin (Fig. 2D and data not shown), in keeping with the expression observed for several other members of the plakin family. Therefore we analysed in MCF-7 cells whether endogenous interaction between periplakin and PKB occurs

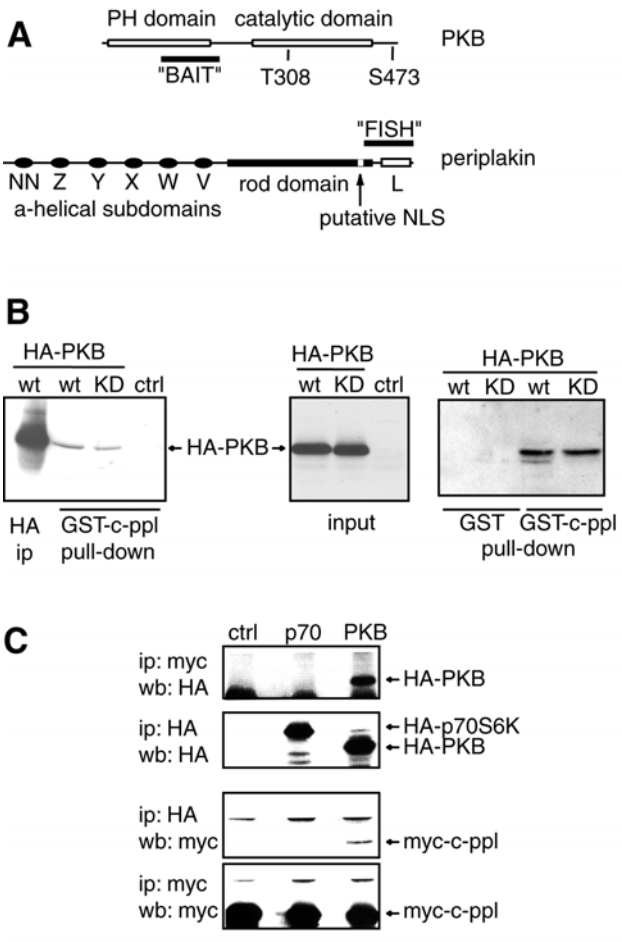

Figure 1: PKB binds periplakin.
(A) Schematic representation of the yeast-two-hybrid fragments of PKB (fish) and periplakin (bait). Periplakin consists of 6 α-helical subdomains (aa 16-920), a rod domain (aa 945-1554) and a linker domain (L; aa 1556-1756). (B) GST pull down assay with GST-c-ppl. A14 cells were transiently transfected either wild type or kinase-dead HA-tagged PKB. Lysates were incubated for 1 hour with GST-c-ppl. Samples were analysed for the presences of PKB by western blotting using the anti-HA antibody. A direct immunoprecipitation of HA-PKB using anti-HA was used as a control. The experiment was also performed with either GST or GST-c-ppl (right panel). (C) COS-7 cells were transiently co-transfected with myc-tagged c-ppl and either HA-tagged PKB or p70S6K. Co-immunoprecipitations were performed with anti-HA and anti-myc antibodies and immunocomplexes were resolved with SDS-PAGE and immunoblotted with anti-myc and anti-HA antibodies respectively.

and we could indeed observe co-immunoprecipitation between endogenous PKB and periplakin, after immunoprecipitating for PKB and analyzing for periplakin (Fig. 2E). Thus from these results we conclude that PKB and periplakin can indeed interact in vivo in cells. Binding of periplakin to PKB can influence PKB function in several ways. To examine the consequence of periplakin binding on PKB activation, myc-c-ppl and HA-PKB were co-expressed in A14 cells. A14 cells are NIH3T3 cells overexpressing the human insulin receptor and treatment of these cells with insulin leads to a rapid and strong increase in PKB activity (11). However, overexpression of myc-c-ppl did not affect activation of HA-PKB by insulin (Fig. 2F). This strongly suggests that binding of periplakin does not interfere in growth factor-induced activation of PKB.

Binding interface of PKB with periplakin

Having established that PKB and periplakin interact, but that this interaction does not affect activation of PKB by growth factors, we next wanted to investigate in more detail the binding of PKB to periplakin. Therefore we analysed binding of a series of HA-PKB mutants to periplakin. HA-PKB mutants were co-transfected with myc-c-ppl, immunoprecipitated and binding was determined by immunoblotting. In keeping with the performed yeast-two-hybrid, in which part of the PH domain was used as a bait, the binding site within full length PKB could be defined to a short stretch of nine amino acids within the N-terminal part of the PH domain (Fig. 3A,B), as deletion mutants encompassing this region (Δ30-130 and Δ30-214), and the mutant Δ9 no longer did bind to myc-c-ppl. The results obtained by immunoblot analysis, were confirmed by analyzing binding in the yeast-two-hybrid system. The structure of PH domains present within a variety of different proteins has been determined and consists of seven beta-sheets followed by one alpha-helix. The presence of the beta-sheets and alpha-helix in the PH domain of PKB is indicated in

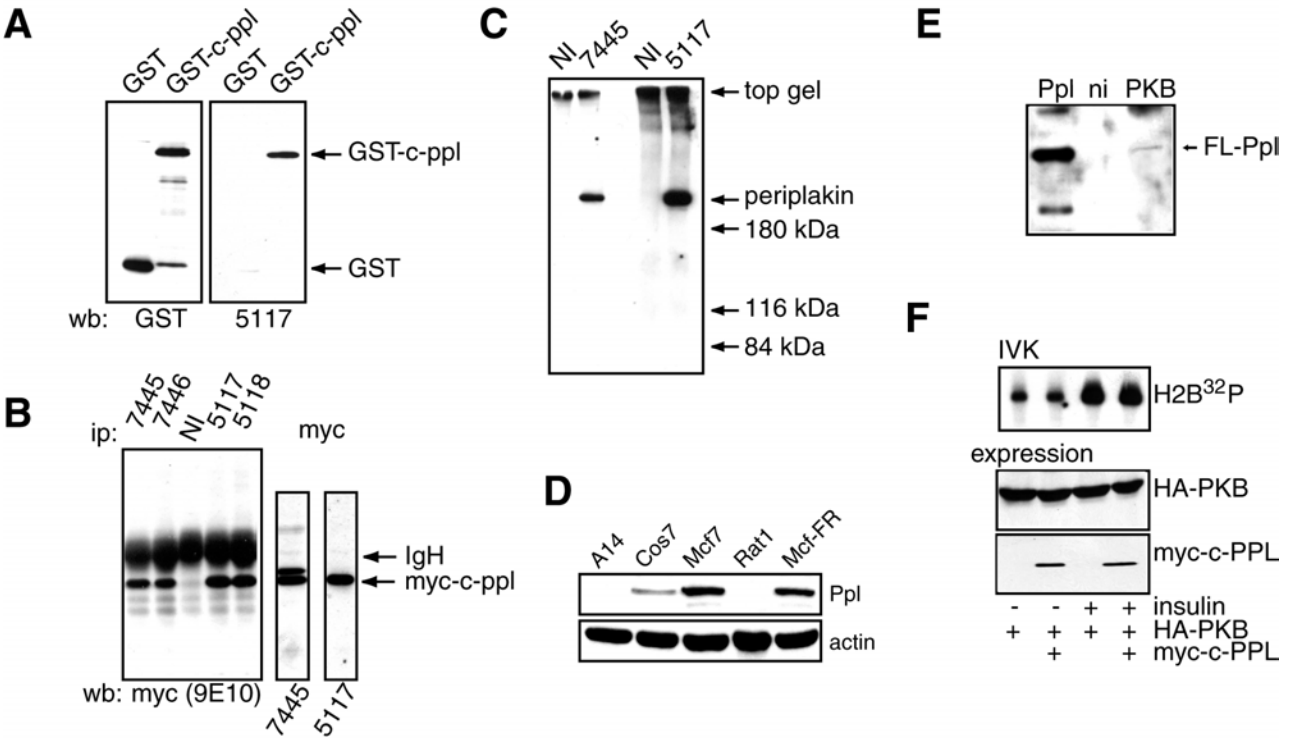

Figure 2: Co-immunoprecipitation of endogenous PKB and periplakin.
(A) Characterization of generated anti-periplakin antibodies. Purified GST and GST-c-ppl were separated by SDS-PAGE and immunoblotted with anti-GST or anti-GST-c-ppl (5117). (B) Cell lysates of COS-7 cells transfected with myc-c-ppl were either immunoprecipitated with anti-GST-c-ppl (5117 and 5118), anti-periplakin peptide serum (7445 and 7446; left panel), or anti-myc (9E10; right two panels). Immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-myc (9E10; right panel), anti-periplakin peptide serum (7445) and anti-GST-c-ppl (5117; left two panels). (C). MCF-7 cells were lysed and periplakin was immunoprecipitated with anti-GST-c-ppl or anti-peptide serum. (NI) is an immunoprecipitation with non-immune serum as a control. Immunoprecipitates were separated by SDS-PAGE and immunoblotted for the presence of periplakin with 5117. (D) Epithelial cells express periplakin. Various cell lines were lysed and analyzed by western blotting using the anti-periplakin 5117 antibody. (E) Periplakin interacts with PKB endogenously. Periplakin and PKB were immunoprecipitated from MCF-7 cells using the anti-periplakin 5117 antibody and anti-PKB antibody respectively, and the immune complex was resolved by SDS-PAGE and immunoblotted with the anti-periplakin 5117 antibody. Non-immune anti-serum was used as a negative control. (F) c-ppl expression does not affect insulin-induced PKB activation. A14 cells were transfected with HA-PKB either in the presence or absence of myc-c-ppl. After 36 hours cells were either treated with insulin for 7 minutes or left untreated. HA-PKB was immunoprecipitated and analysed for kinase activity as described (11).

figure3C (adapted from (22)) and shows that the Δ9 mutant lacks the last part of the 5th flexible loop and most of the 6th beta sheet. Therefore, this deletion is expected to have an effect on the 3D structure of the PH domain. To restore at least the spacing of the amino acid residues within the pH domain, we added back to the Δ9 mutant a stretch of 9 glycine residues. However this did not restore binding to myc-c-ppl. To see whether any distortion of the PH domain structure would result in loss of myc-c-ppl binding we also analysed binding of myc-c-ppl to the W99A mutant of HA-PKB. In this mutant the residue (W99) conserved in all known PH domains was mutated and this is likely to result in a structural change of the PH domain, as W99A is catalytically inactive (data not shown). Yet myc-c-ppl still did bind to this mutant, suggesting that the loss of myc-c-ppl binding to the Δ9 mutant is not necessarily due to a

conformational change of the PH domain, but that myc-c-ppl probably binds to the 5th flexible loop.

Periplakin binds the intermediate filament vimentin

As periplakin expression does not interfere in growth factor-induced PKB activation, we started to address the possibility that binding between periplakin and PKB may serve as a localization signal for PKB. Therefore, we first studied the cellular localization of periplakin in detail. All members of the plakin family have been shown to bind cytoskeletal proteins. Thus, to investigate to what cytoskeletal proteins periplakin binds we performed a yeast-two-hybrid analysis with the C-terminal part of periplakin on a 13.5 day mouse embryo cDNA library. This way we identified the intermediate filament protein vimentin as a potential binding partner of periplakin. To further establish periplakin binding to vimentin we co-expressed myc-tagged c-ppl with HA-vimentin in Cos-7 cells and observed co-immuno precipitation when precipitating myc-c-ppl in a stringent buffer and analysing by immunoblotting for the presence of vimentin (Fig. 4A). For reasons that are not entirely clear to us we could only very faintly observe co-precipitation when precipitating vimentin and analysing for the presence of myc-c-ppl. Therefore, we also analysed co-localization by immunofluorescence in Rat-1 cells. In these experiments a clear co-localization is observed between myc-c-ppl and the endogenous vimentin network (Fig. 4B). A fractionation on

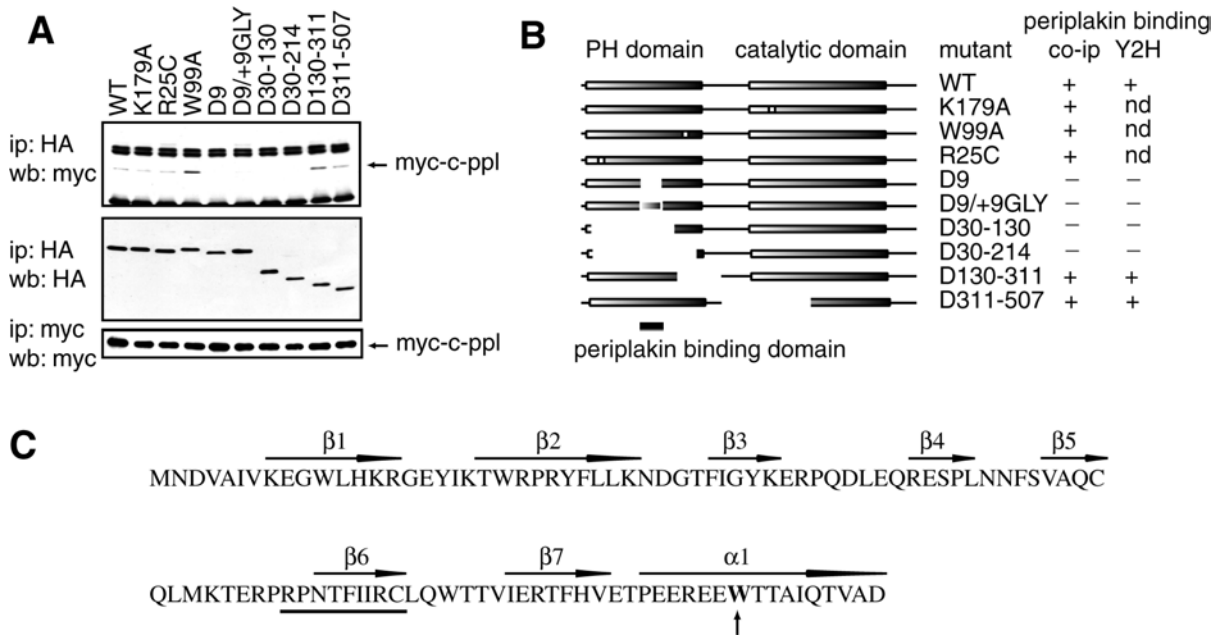

Figure 3: Determination of the binding site of PKB to periplakin.
(A) COS-7 cells were transiently transfected with myc-tagged c-ppl in combination with HA-tagged PKB constructs as indicated. HA-PKB was immunoprecipitated with anti-HA and analysed by western blotting for myc-c-ppl binding by immunoblotting with anti-myc (9E10) (upper panel) Expression of HA-PKB constructs and myc-c-ppl was checked by immunoblotting with anti-HA and anti-myc respectively (B) Schematic representation of the outcome of the interaction studies by co-immunoprecipitation and yeast-two-hybrid analysis. nd stands for not done. (C) Primary structure of the PH domain of PKB. The positions of the b-sheets and a-helix are indicated by arrows on top. The 9 amino acid deletion that results in loss of periplakin binding is indicated by a black line below the sequence. The conserved W99 residue is depicted in bold and indicated by an arrow. Location of the b-sheets was shown as determined (22).

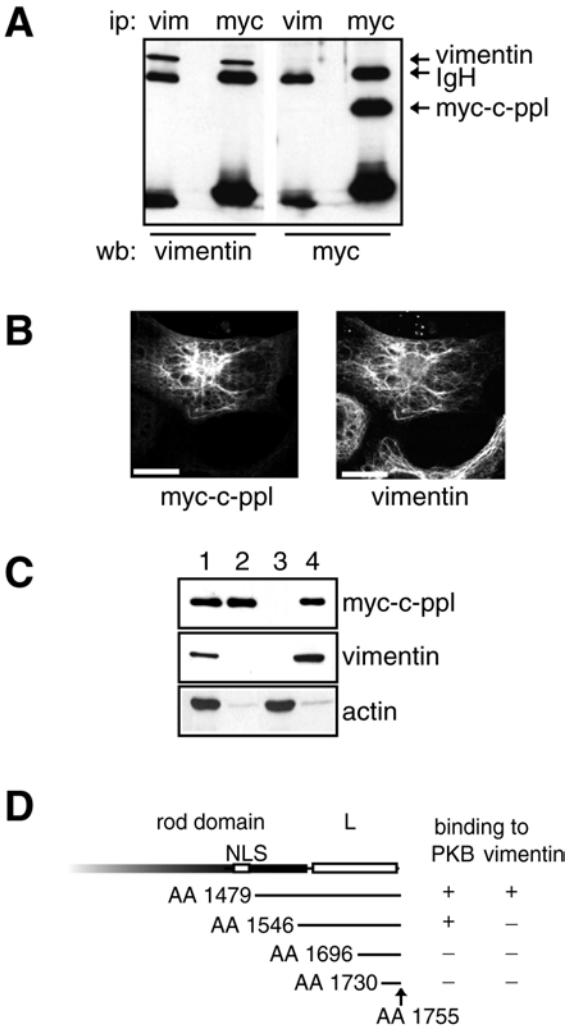

Figure 4: Periplakin binds to the intermediate filament vimentin.
(A) Periplakin interacts with vimentin. Cos-7 cells were transiently transfected with myc-tagged c-ppl and co-immunoprecipitations were performed in PKB buffer using anti-myc and anti-vimentin antibodies. Samples were analysed by western blotting using either the anti-myc (anti-myc 9E10) or anti-vimentin (anti-vimentin Oncogene Science) antibody. (B) Periplakin colocalises with vimentin. Rat-1 cells were transiently transfected with myc-tagged c-ppl and immunostained for myc-c-ppl and endogenous vimentin. Bars, 10 μm. (C) Myc-c-ppl is present in vimentin fractions. A14 cells were transiently transfected with myc-tagged c-ppl and fractionated using a KI-protocol. The fractions were immunostained for vimentin, actin and myc-c-ppl. Fraction 1: total; 2: cytosolic and membrane; 3: actin; 4: vimentin. (D) Comparison of C-terminal deletion mutants and their binding to PKB and vimentin. The C-terminal part of periplakin is shown. Binding was determined by co-immunoprecipitation and yeast-two-hybrid analysis.

A14 cells transiently transfected with myc-c-ppl was performed in which the vimentin network was solubilised and purified. Fractions were analysed for actin, vimentin and myc-c-ppl that was found to be present in the vimentin fraction (Fig. 4C). Thus from these experiments we conclude that periplakin binds both PKB and vimentin through its C-terminus and is colocalised with vimentin. To determine whether PKB and vimentin bind to different or overlapping sites within the C-terminal part of periplakin we co-expressed both in cells, and in the yeast-two-hybrid system, deletion mutants of the C-terminal part of periplakin together with either vimentin or PKB. However, in both cases we were unable to identify within periplakin a small peptide sequence responsible for vimentin or PKB binding. Although some difference was observed between PKB and vimentin binding (Fig. 4D) we could not establish whether PKB and vimentin bind different parts of periplakin, or whether binding of PKB and vimentin is mutually exclusive.

Periplakin localizes to different cellular compartments

In tumor cell development, loss of epithelial morphology and acquisition of mesenchymal characteristics is often correlated with increased expression of vimentin (16). As PKB function is also implicated in tumor development, we were interested to know where periplakin would localize in cells that lack vimentin expression and if differential localization, due to presence or absence of vimentin, may influence PKB function. In contrast to many other breast cancer cell lines, the breast epithelial carcinoma cell line MCF-7 expresses little or no vimentin (43, 45), yet high levels of periplakin (Fig. 2) and therefore this cell line was used for confocal microscopy using the periplakin antibodies developed. Endogenous periplakin

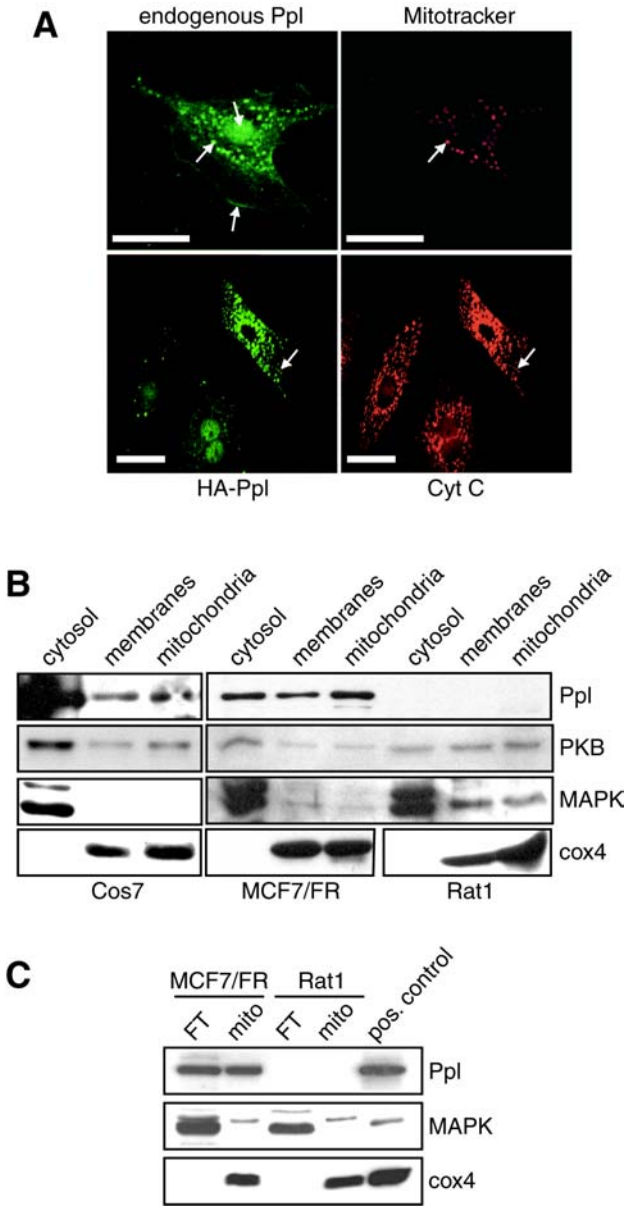

Figure 5: Periplakin colocalizes with mitochondria.
(A) Confocal immunostaining for periplakin. MCF-7 cells were pre-incubated with Mitotracker and co-immunostained for periplakin using the anti-periplakin 5117 antibody (upper panel). Similar staining was observed with the other antibodies (5118, 7445 and 7446) and with affinity-purified 5117. No staining was observed using pre-immune serum from the rabbits (5117 and 5118) used for immunization with GST-c-ppl. MCF-7 cells were transiently transfected with HA-Ppl and immunostained for HA-Ppl using the HA antibody and for cytochrome c (lower panel). Bars, 10 μm. (B/C) Periplakin is found in mitochondrial fractions. (B) Cos-7, MCF-7/FR and Rat-1 cells were fractionated using a Percoll gradient and the fractions were analysed by western blotting using anti-periplakin 5117, anti-PKB, anti-cox4 and anti-MAPK antibodies. (C) MCF-7/FR and Rat-1 cells were fractionated using the mitochondria affinity purification protocol. Fractions were analysed by immunoblotting, using the same antibodies as in (B).

was shown to be co-localizing with the cell membrane, nucleus and mitochondria. The latter was demonstrated by co-staining with a mitochondrial marker (Mitotracker). Also transfected HA-tagged periplakin was shown to be co-localizing with a mitochondrial marker (cytochrome c) in MCF-7 cells (Fig. 5A). Mitochondrial localization of periplakin was confirmed by biochemical fractionation methods. First, from MCF-7, Cos-7 and Rat-1 cells various cellular fractions including a mitochondrial fraction were isolated by fractionation on a Percoll gradient (Fig. 5B). In a second approach we used sorting by magnetic beads (MACS) with a ferro-conjugated antibody (MAB1273, see materials and methods) that recognizes a 65-Kd mitochondrial membrane protein (26). This method allows rapid single step purification of organelles (Fig. 5C). Fractionation was monitored in both cases by using COX4 as mitochondrial marker and MAPK as non-mitochondrial marker. Both approaches revealed the presence of periplakin in the mitochondrial fraction, confirming the immunofluorescent data. Interestingly, also in Cos-7 cells we could demonstrate mitochondrial localization of periplakin, albeit that the fraction of periplakin localized to mitochondria appeared less compared to MCF-7. This observation suggests that in vimentin containing cells such as COS-7, periplakin is bound to vimentin and may function to recruit mitochondria to intermediate filament structures, whereas in non-vimentin containing cells owing to the absence of clear filamentous staining periplakin localizes apparently more clearly to mitochondria.

Periplakin expression can affect PKB-signalling by sequestration

Since periplakin binds to PKB, but does not interfere with activation of PKB and localizes to specific cellular compartments we wanted to test whether periplakin expression may affect the ability of PKB to generate specific signalling outputs. Recently, we and others have shown that phosphorylation and inactivation of FOXO transcription factors by PKB occurs within the nucleus (9, 10). Consequently, inhibition of PKB nuclear transport should result in loss of FOXO regulation by PKB. To test this possibility we made use of the observation that in vimentin expressing cells ectopically expressed myc-c-ppl localizes to intermediate filament structures, whereas little or no expression in the nucleus is observed (Fig. 4B). As expected and reported previously, co-expression of PKB resulted in inhibition of FOXO4 (AFX) dependent transcription (Fig. 6A). Interestingly both full length and myc-c-ppl expression enhanced transcription by FOXO4. That this is likely to be due to sequestration of PKB within the cytosol is indicated by a lack of effect of myc-c-ppl expression on insulin-induced PKB activity (Fig. 2F) and a decrease of phosphorylation of the PKB site S193 of FOXO4 (Fig. 6B). Thus these results suggest that periplakin can indeed act as a scaffold, as it can modulate PKB-dependent signalling outputs without interfering with PKB activation itself.

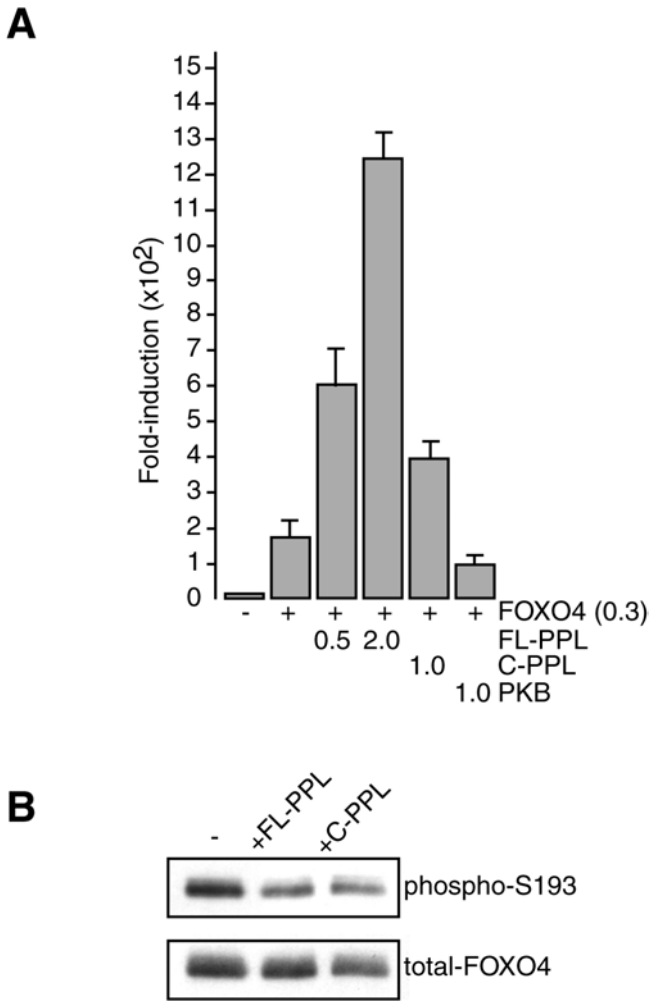

Figure 6: Periplakin expression modulates PKB-dependent FOXO4 regulation.
(A) 293T cells were transfected with a TK-renilla-luciferase (internal control) and 6xDBE luciferase reporter construct (30), in the absence or presence of FOXO4. Different amounts (μg) of full-length periplakin, myc-c-ppl, or HA-PKB were cotransfected with FOXO4. 36 hours after transfection lysates were prepared and luciferase counts were measured and normalized against renilla luciferase counts. 6xDBE activity in the absence of FOXO4 was set at 1. Results represent the averages of three independent experiments. (B) Periplakin expression reduces PKB dependent FOXO4 phosphorylation. 293T cells were transfected with the 0.5 μg full-length periplakin or 1.0 μg c-ppl, and 1 μg HA-FOXO4. HA-FOXO4 was immunoprecipitated and the level of PKB dependent phosphorylation was determined by immunoblotting using a phospho-specific antibody against pSer193.

Discussion

PKB is a major player in various essential growth factor regulated cellular processes (6, 8), and tumorigenesis (28, 44). To better understand how PKB exerts its function in these processes we performed a yeast-two-hybrid screen to find PKB interacting proteins. Here we demonstrate that PKB binds to periplakin, a member of the plakin family, and we provide evidence that periplakin may function as a

localization signal for PKB to its correct cellular compartments. PKB binding to periplakin could be demonstrated by yeast-two-hybrid analysis as well as co-immunoprecipitations of ectopically and endogenously expressed proteins. The binding region within PKB that is responsible for the interaction with periplakin was narrowed down to a small region in the C-terminal part of the PH domain (Fig. 3B). Alignment of the PH domain of PKB with PH domains of which the 3D structure has been resolved indicates that binding to periplakin is most likely mediated by the 5th flexible loop of the PH domain. (Fig. 3C). Although it remains possible that binding to the Δ9 mutant is lost due to general distortion of the 3D structure of the PH domain, we consider this unlikely for several reasons. First, restoring at least the spacing by replacing the deleted amino acids by glycine residues (Δ9-gly9) does not restore binding, Second, as one can still argue that also Δ9-gly9 is no longer properly folded, the presence of the binding site within a flexible loop would at least suggest that the primary sequence rather than its folding determines binding. In keeping with the latter conclusion would be the observations that the yeast-two-hybrid interaction was isolated with a truncated PH domain construct and that myc-c-ppl still binds to the W99A mutant. With respect to this mutant it should then be noted that this tryptophane residue is likely to be an essential structural determinant for PH domains, since between all known PH domains this residue is the only one that is actually conserved (32). Indeed the W99A mutation results in a catalytically inactive mutant (not shown) and on SDS-PAGE this mutant shows aberrant migration behavior suggesting that this mutation distorts PH domain structure but does not affect binding to myc-c-ppl.

Finally, as the residues involved in binding PtdIns3P lipids are primarily located within the first half of the PH domain, binding of periplakin to the 5th flexible loop would be expected to have little effect on PtdIns3P binding. Although not measured directly, the fact that we do not observe inhibition of insulin-induced activation of HA-PKB when co-expressing myc-c-ppl (Fig. 2F) corroborates this suggestion.

Plakin family members have been shown to bind or colocalize with intermediate filaments (38) and indeed we also found in our yeast-two-hybrid screen with c-ppl binding to the intermediate filament vimentin. This interaction was confirmed by co-immunoprecipitation and co-localization studies. The binding to and colocalisation with vimentin in fibroblasts is in agreement with the observations of DiColandrea et al., who also observe periplakin colocalisation with vimentin in Cos-7 cells. In addition they observe in keratinocytes that full-length and the c-terminal part of periplakin partially colocalise with keratin filaments (38). Thus, it appears that depending on the cell type periplakin may localize to different intermediate filament networks. The function of intermediate filaments is considered to be a scaffolding one which structures the cytoplasm and resists extracellular stresses (23). The function of the binding of periplakin to vimentin is unclear and needs to be further investigated, but we hypothesize that periplakin through binding to vimentin correctly localizes PKB and other proteins within the cell. Recently it was reported that PKB could interact with another intermediate filament, keratin K-10, and that K-10 binding results in PKB inhibition and keratin-induced cell cycle arrest (35). As this study did not identify the nature of the interaction between K-10 and PKB, our results indicate the possibility that periplakin functions to bridge this interaction. Also our observation that periplakin expression enhances FOXO4 transcriptional activity is consistent with the role of intermediate filaments in inducing cell cycle arrest as proposed by Paramio et al. (35). Previously we have shown that FOXO transcription factors can cause cell cycle arrest

in a p27kip-dependent manner (30). Since activated PKB translocates to the nucleus where it inactivates FOXO transcription factors, sequestering PKB within the cytosolic compartment through binding to periplakin/intermediate filaments would enhance FOXO transcriptional activity and consequently stimulate cell cycle arrest.

In many cell types and under many conditions PKB signaling has been shown crucial to provide cellular protection against apoptosis. Apoptosis is often initiated by, or requires mitochondrial damage and leakage of cytochrome-C (1). Recent reports have shown that PKB signalling can maintain mitochondrial membrane stability under conditions of stress (25, 27, 36). Therefore, the clear colocalisation of periplakin with mitochondria combined with its interaction with PKB suggests a possible role for periplakin in mediating PKB dependent protection. In keeping with such a model are reports that show that vimentin is a substrate for caspase-9 and is cleaved at an early stage within the apoptosis process (12, 33) and that caspase resistant vimentin delays apoptosis (5). Also plectin is a substrate for caspase-8, and again is cleaved early in apoptosis (42). This caspase cleavage site of plectin is conserved in periplakin. However attractive, we have not been able to obtain clear evidence to support a role for periplakin in PKB mediated apoptosis protection. This could be due to for instance redundancy in apoptosis protection signalling. Many cell types do not or only very little, express periplakin and still depend on PKB-mediated protection. Interestingly in this respect, plectin, a family member of periplakin, has been shown to associate with mitochondria as well (37). It is possible that plectin and periplakin may act redundant in PKB-dependent protection since plectin has been shown to have a ubiquitous expression pattern (47). Also our observation that PKB localization to mitochondria as determined by biochemical fractionations appears independent of periplakin and/or vimentin expression indicates either redundant or alternative means of PKB localization to this compartment. However, these biochemical fractionations do not exclude the possibility that the presence of PKB is due to contamination of this fraction. Unfortunately, with respect to PKB localization we could not complement the biochemical fractionation data with immunofluorescence studies, as the quality of all different PKB antibodies we tested thus far precluded this.

Periplakin may in addition, to acting as a localization signal, also function as a shuttle for delivery of PKB to the various cellular compartments. This possibility derives primarily from the observation that periplakin localizes to all sites where PKB is either observed or suspected to localize. Periplakin localizes to the plasma membrane and PKB becomes activated at the plasma membrane by PI-3 kinase-mediated production of phosphoinositol lipids (7, 11). Following activation, PKB should be able to translocate to other cellular compartments like the nucleus and the vicinity of mitochondria, since substrates for PKB present within these cellular domains have been identified. For example a fraction of PKB has been shown to translocate to the nucleus (3, 9). Within the nucleus PKB is thought to phosphorylate substrates such as the FOXO family of transcription factors. We observe nuclear localization of periplakin and in addition periplakin as well as other members of the plakin family contain a putative bipartite nuclear localization (NLS). As other reports suggested that this NLS sequence in other plakins (e.g. plectin (34)) is also a part of the intermediate filament-binding region, it could be that (induced) loss of intermediate filament binding unmasks the NLS and results in nuclear translocation. These possibilities are currently under investigation. Finally, although our experiments only start to delineate such a possibility it is interesting to note that such a function for periplakin and possibly plakins in

general is much alike the function of other large cytolinkers such as APC.

In conclusion, this study shows binding of periplakin to PKB and vimentin. The differential cellular localization of periplakin and its ability to affect PKB signalling when targeted to a specific localization suggest a role in determining cell type specific signalling by the PI3K/PKB pathway.

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References

1. **Adrain, C., and S. J. Martin.** 2001. The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. Trends Biochem Sci **26:**390-7.
2. **Aho, S., W. H. McLean, K. Li, and J. Uitto.** 1998. cDNA cloning, mRNA expression, and chromosomal mapping of human and mouse periplakin genes. Genomics **48:**242-7.
3. **Andjelkovic, M., D. R. Alessi, R. Meier, A. Fernandez, N. J. Lamb, M. Frech, P. Cron, P. Cohen, J. M. Lucocq, and B. A. Hemmings.** 1997. Role of translocation in the activation and function of protein kinase B. J Biol Chem **272:**31515-24.
4. **Baumann, N. A., J. Vidugiriene, C. E. Machamer, and A. K. Menon.** 2000. Cell surface display and intracellular trafficking of free glycosylphosphatidylinositols in mammalian cells. J Biol Chem **275:**7378-89.
5. **Belichenko, I., N. Morishima, and D. Separovic.** 2001. Caspase-resistant vimentin suppresses apoptosis after photodynamic treatment with a silicon phthalocyanine in Jurkat cells. Arch Biochem Biophys **390:**57-63.
6. **Bevan, P.** 2001. Insulin signalling. J Cell Sci **114:**1429-30.
7. **Bos, J. L.** 1995. A target for phosphoinositide 3-kinase: Akt/PKB. Trends Biochem Sci **20:**441-2.
8. **Brazil, D. P., and B. A. Hemmings.** 2001. Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem Sci **26:**657-64.
9. **Brownawell, A. M., G. J. Kops, I. G. Macara, and B. M. Burgering.** 2001. Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. Mol Cell Biol **21:**3534-46.
10. **Brunet, A., F. Kanai, J. Stehn, J. Xu, D. Sarbassova, J. V. Frangioni, S. N. Dalal, J. A. DeCaprio, M. E. Greenberg, and M. B. Yaffe.** 2002. 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. J Cell Biol **156:**817-28.
11. **Burgering, B. M., and P. J. Coffer.** 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature **376:**599-602.
12. **Byun, Y., F. Chen, R. Chang, M. Trivedi, K. J. Green, and V. L. Cryns.** 2001. Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis. Cell Death Differ **8:**443-50.
13. **Cardone, M. H., N. Roy, H. R. Stennicke, G. S. Salvesen, T. F. Franke, E. Stanbridge, S. Frisch, and J. C. Reed.** 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science **282:**1318-21.
14. **Coffer, P. J., J. Jin, and J. R. Woodgett.** 1998. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. Biochem J **335:**1-13.
15. **Cross, D. A., D. R. Alessi, P. Cohen, M. Andjelkovich, and B. A. Hemmings.** 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature **378:**785-9.
16. **Dandachi, N., C. Hauser-Kronberger, E. More, B. Wiesener, G. W. Hacker, O. Dietze, and G. Wirl.** 2001. Co-expression of tenascin-C and vimentin in human breast cancer cells indicates phenotypic transdifferentiation during tumour progression: correlation with histopathological parameters, hormone receptors, and oncoproteins. J Pathol **193:**181-9.
17. **Datta, S. R., H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, and M. E. Greenberg.** 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell **91:**231-41.
18. **de Bruin, A., E. Muller, M. Wyder, G. J. Anhalt, P. Lemmens, and M. M. Suter.** 1999. Periplakin and envoplakin are target antigens in canine and human paraneoplastic pemphigus. J Am Acad Dermatol **40:**682-5.
19. **de Vries-Smits, A. M., B. M. Burgering, S. J. Leevers, C. J. Marshall, and J. L. Bos.** 1992. Involvement of p21ras in activation of extracellular signal-regulated kinase 2. Nature **357:**602-4.
20. **DiColandrea, T., T. Karashima, A. Maatta, and F. M. Watt.** 2000. Subcellular distribution of envoplakin and periplakin: insights into their role as precursors of the epidermal cornified envelope. J Cell Biol **151:**573-86.
21. **Doornbos, R. P., M. Theelen, P. C. van der Hoeven, W. J. van Blitterswijk, A. J. Verkleij,**

and P. M. van Bergen en Henegouwen. 1999. Protein kinase Czeta is a negative regulator of protein kinase B activity. J Biol Chem **274:**8589-96.

22. **Ferguson, K. M., J. M. Kavran, V. G. Sankaran, E. Fournier, S. J. Isakoff, E. Y. Skolnik, and M. A. Lemmon.** 2000. Structural basis for discrimination of 3-phosphoinositides by pleckstrin homology domains. Mol Cell **6:**373-84.
23. **Fuchs, E., and D. W. Cleveland.** 1998. A structural scaffolding of intermediate filaments in health and disease. Science **279:**514-9.
24. **Gonzales, M., B. Weksler, D. Tsuruta, R. D. Goldman, K. J. Yoon, S. B. Hopkinson, F. W. Flitney, and J. C. Jones.** 2001. Structure and function of a vimentin-associated matrix adhesion in endothelial cells. Mol Biol Cell **12:**85-100.
25. **Gottlob, K., N. Majewski, S. Kennedy, E. Kandel, R. B. Robey, and N. Hay.** 2001. Inhibition of early apoptotic events by Akt/PKB is dependent on the first committed step of glycolysis and mitochondrial hexokinase. Genes Dev **15:**1406-18.
26. **Herrnstadt, C., W. Clevenger, S. S. Ghosh, C. Anderson, E. Fahy, S. Miller, N. Howell, and R. E. Davis.** 1999. A novel mitochondrial DNA-like sequence in the human nuclear genome. Genomics **60:**67-77.
27. **Kennedy, S. G., E. S. Kandel, T. K. Cross, and N. Hay.** 1999. Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. Mol Cell Biol **19:**5800-10.
28. **Kops, G. J., and B. M. Burgering.** 1999. Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. J Mol Med **77:**656-65.
29. **Maira, S. M., I. Galetic, D. P. Brazil, S. Kaech, E. Ingley, M. Thelen, and B. A. Hemmings.** 2001. Carboxyl-terminal modulator protein (CTMP), a negative regulator of PKB/Akt and v-Akt at the plasma membrane. Science **294:**374-80.
30. **Medema, R. H., G. J. Kops, J. L. Bos, and B. M. Burgering.** 2000. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature **404:**782-7.
31. **Meier, R., D. R. Alessi, P. Cron, M. Andjelkovic, and B. A. Hemmings.** 1997. Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bbeta. J Biol Chem **272:**30491-7.
32. **Musacchio, A., T. Gibson, P. Rice, J. Thompson, and M. Saraste.** 1993. The PH domain: a common piece in the structural patchwork of signalling proteins. Trends Biochem Sci **18:**343-8.
33. **Nakanishi, K., M. Maruyama, T. Shibata, and N. Morishima.** 2001. Identification of a caspase-9 substrate and detection of its cleavage in programmed cell death during mouse development. J Biol Chem **276:**41237-44.
34. **Nikolic, B., E. Mac Nulty, B. Mir, and G. Wiche.** 1996. Basic amino acid residue cluster within nuclear targeting sequence motif is essential for cytoplasmic plectin-vimentin network junctions. J Cell Biol **134:**1455-67.
35. **Paramio, J. M., C. Segrelles, S. Ruiz, and J. L. Jorcano.** 2001. Inhibition of protein kinase b (pkb) and pkczeta mediates keratin k10-induced cell cycle arrest. Mol Cell Biol **21:**7449-59.
36. **Plas, D. R., S. Talapatra, A. L. Edinger, J. C. Rathmell, and C. B. Thompson.** 2001. Akt and Bcl-xL promote growth factor-independent survival through distinct effects on mitochondrial physiology. J Biol Chem **276:**12041-8.
37. **Reipert, S., F. Steinbock, I. Fischer, R. E. Bittner, A. Zeold, and G. Wiche.** 1999. Association of mitochondria with plectin and desmin intermediate filaments in striated muscle. Exp Cell Res **252:**479-91.
38. **Ruhrberg, C., M. A. Hajibagheri, D. A. Parry, and F. M. Watt.** 1997. Periplakin, a novel component of cornified envelopes and desmosomes that belongs to the plakin family and forms complexes with envoplakin. J Cell Biol **139:**1835-49.
39. **Rylatt, D. B., A. Aitken, T. Bilham, G. D. Condon, N. Embi, and P. Cohen.** 1980. Glycogen synthase from rabbit skeletal muscle. Amino acid sequence at the sites phosphorylated by glycogen synthase kinase-3, and extension of the N-terminal sequence containing the site phosphorylated by phosphorylase kinase. Eur J Biochem **107:**529-37.
40. **Simon, M., and H. Green.** 1984. Participation of membrane-associated proteins in the formation of the cross-linked envelope of the keratinocyte. Cell **36:**827-34.
41. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene **67:**31-40.
42. **Stegh, A. H., H. Herrmann, S. Lampel, D. Weisenberger, K. Andra, M. Seper, G. Wiche, P. H. Krammer, and M. E. Peter.** 2000. Identification of the cytolinker plectin as a major early in vivo substrate for caspase 8 during CD95- and tumor necrosis factor receptor-mediated apoptosis. Mol Cell Biol **20:**5665-79.
43. **Stover, D. M., I. Carey, R. J. Garzon, and Z. E. Zehner.** 1994. A negative regulatory factor is missing in a human metastatic breast cancer cell line. Cancer Res **54:**3092-5.
44. **Tsatsanis, C., and D. A. Spandidos.** 2000. The role of oncogenic kinases in human cancer (Review). Int J Mol Med **5:**583-90.
45. **van de Klundert, F. A., G. J. van Eldik, F. R. Pieper, H. J. Jansen, and H. Bloemendal.** 1992.

Identification of two silencers flanking an AP-1 enhancer in the vimentin promoter. Gene **122:**337-43.

46. **van Weeren, P. C., K. M. de Bruyn, A. M. de Vries-Smits, J. van Lint, and B. M. Burgering.** 1998. Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. J Biol Chem **273:**13150-6.
47. **Wiche, G., R. Krepler, U. Artlieb, R. Pytela, and H. Denk.** 1983. Occurrence and immunolocalization of plectin in tissues. J Cell Biol **97:**887-901.
48. **Wolthuis, R. M., B. Bauer, L. J. van't Veer, A. M. de Vries-Smits, R. H. Cool, M. Spaargaren, A. Wittinghofer, B. M. Burgering, and J. L. Bos.** 1996. RalGDS-like factor (Rlf) is a novel Ras and Rap 1A-associating protein. Oncogene **13:**353-62.

Chapter

3

In silico studies for the discovery of novel PKB substrates and FOXO target genes

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The phosphatidylinositol-3-OH kinase (PI(3)K)/protein kinase B (PKB)/Forkhead box O (FOXO) pathway can regulate the activity of numerous proteins. PKB does so by phosphorylating substrates on Ser/Thr residues within the PKB consensus motif (R-X-R-X-X-S/T-X). FOXO transcription factors are inactivated by PKB via phosphorylation and subsequent nuclear exclusion. The FOXO family members, so far consisting of FOXO1, FOXO3a, FOXO 4 and FOXO6, share a highly homologous 'winged-helix' DNA binding domain that can bind specific DNA sequences (i.e. 5'-TTGTTTAC-3'). An *in silico* approach was taken to discover novel PKB substrates and FOXO target genes. PKB substrates were selected based on evolutionary conservation of PKB motifs in combination with the known function of the substrates. To identify novel FOXO targets microarrays were performed with the DL23 cell line that contains an inducible active FOXO3a, and analyzed in combination with other arrays and the presence of consensus binding elements in promoter sequences.

Discovery of novel PKB substrates using *in silico* analysis

PKB belongs to the family of Ser/Thr kinases, and thus it can phosphorylate its substrates on serine and threonine residues that are located within a so-called PKB motif. The minimum peptide motif that is required for efficient phosphorylation by PKB has been defined as Arginine-X-Arginine-X-X-Serine/Threonine-X (R-X-R-X-X-S/T-X) in which the X stands for any amino acid. This motif was developed based on the efficiency of PKB to phosphorylate small peptides that were related to the sequence surrounding the phosphorylation site on glycogen synthase kinase 3 (GSK3)(20). Originally, the consensus motif contained a bulky hydrophobic residue directly on the carboxyterminal side next to the Ser or Thr residue such as Phe or Leu residues (4). However, the currently described PKB substrates do not show this hydrophobic residue in the motif whatsoever, and therefore it might be considered non-essential for phosphorylation by PKB. Many described substrates have only been validated as being a true PKB substrate through in vitro kinase assays. Phosphorylated PKB substrate antibodies are often used but it should be considered that these antibodies might also recognize other kinase substrates that are phosphorylated within a similar motif. The Arg residue at position –3 from the Ser/Thr within the described substrates can be considered as conserved since it is present in all substrates, whereas Arg residue on position –5 from the Ser/Thr is absent in some substrates (see Table 2 of Chapter 1).

Throughout evolution the basic layout of signaling pathways appear conserved, especially the insulin/PI3K/PKB pathway. This suggests that kinase recognition motifs within substrates are also conserved. This conservation between species not only comprises conservation of important motifs between *Homo sapiens* and *Mus musculus* or *Rattus norvegicus* but also with lower eukaryotic species such as *Xenopus laevis*, *Drosophila melanogaster* and *Caenorhabditis elegans*. When the presence of PKB motif(s) in proteins between higher and lower eukaryotic species is conserved, it suggests that the protein containing the sequence is likely to be a genuine PKB substrate. For example, the FOXO transcription factors are highly conserved between worm and human with respect to their phosphorylation motifs. However, this does not hold true for all described PKB substrates. For instance, the PKB motif in caspase 9 is not conserved. Cardone and colleagues described the phosphorylation by PKB of Ser196 within the caspase domain of human caspase 9 (13). The relevance of this finding is controversial since the PKB motif is not present in relatively

related species such as *Mus musculus* and *Canis familiaris* (26). Cardone and colleagues raise the possibility that caspase 9 phosphorylation occurs in more complex long-lived organisms since there is a need for a proper response to human-specific cellular disfunctioning. The fact that these differences between higher and lower organisms are not reflected in cell culture systems, where effects are similar in both human and for instance mouse cell lines, argues against this hypothesis. Also Ser157 of human $p27^{Kip1}$, which is phosphorylated by PKB, is not conserved in mouse $p27^{Kip1}$. However, negative regulation of $p27^{Kip1}$ by PKB can also occur through inhibition of FOXO-mediated $p27^{Kip1}$ transcription, thereby circumventing the lack of conservation of the PKB motif.

In order to find novel substrates of PKB a BLAST search was performed with R-X-R-X-X-S-X and R-X-R-X-X-T-X as peptides using Swiss Prot (ExPASy). This search resulted in approximately 1500 annotated human proteins carrying one or more motifs. From this list a subgroup of proteins was selected based on the known or suggested functions and the physiological roles of these proteins (Table 1). For that reason, transcription factors were selected since so far only members of the FOXO family have been well characterized as being PKB substrates that directly regulate transcription. Although FOXO transcription factors do seem to have distinct functions since specific FOXO knockouts give rise to different phenotypes (31), they are considered to have overlapping if not identical target genes. We assume that besides FOXO regulation, PKB can regulate transcriptional regulation through additional pathways. Proteins that are (possibly) linked to longevity were also selected, due to the relatively unknown mechanisms involved. The conservation of the putative PKB motifs in the human sequences were compared to homologues found in lower species using the alignment program Clustal X (Figure 1). Functional protein domains such as DNA binding motifs are, in general, more conserved between species. This could explain the conservation in several PKB motifs when they are located inside such a conserved domain. One could suggest then that the protein is not a valid substrate based on conservation of the PKB motif. However, it might be expected that post-translational modifications such as phosphorylation exert a greater effect when occurring in functional domains.

Table 1: Selected possible PKB substrates.
Shown are the human amino acid sequences, whether these are evolutionary conserved and whether the proteins are transcription factors (TF). K indicates a lysine residue instead of an arginine residue in lower organisms.

Name	Motif(s)		Conservation	TF	Validated target
BF1	RRRSTT^{267}		yes	yes	
Brn5	RKRRTS^{240}		yes	yes	
c-Fos	RRRELT^{162}		yes	yes	
FOXO4	RRRAAS^{193}	RPRSSS^{258}	yes	yes	(43)
Fra2	RRRELT^{149}		yes	yes	
Mdm2	RRRAIS^{166}	RKRHKS^{186}	yes	no	(48, 86)
Mef2a	RNRQVT^{20}		yes	yes	
Pit1	RKRRTT^{220}		K	yes	
Prop1	RRRHRT^{74}		yes	yes	
Rsk1	RDRVRT^{120}		K	no	
TSC2	RCRSIS^{981}	RPRGYT^{1462}	yes	no	(34, 58)
Tub	RKRKKS^{307}		yes	yes	
Tulp1	RKRKRS^{345}		yes	yes	

BF1	human	RRRSTT
	mouse	RRRSTT
	xenopus	RRRSTT
Brn5	human	RKRRTS
	mouse	RKRRTS
	zebrafish	RKRRTS
	elegans	RKRRTS
c-Fos	human	RRRELT
	mouse	RRRELT
	xenopus	RRRELT
FOXO4	human	RRRAAS RPRSSS
	mouse	RRRAAS RPRSSS
	droso	RRRAAS RQRASS
	elegans	RERSNT RPRTQS
Fra2	human	RRRELT
	mouse	RRRELT
	xenopus	RRRELT
Mdm2	human	RRRAIS RKRHKS
	mouse	RRRSIS RKRRRS
	xenopus	SQRKSS RKRHKS
Mef2a	human	RNRQVT
	droso	RNRQVT
Pit1	human	RKRRTT
	rat	RKRRTT
	chick	RKRRTT
	droso	RKKRTS
Prop1	human	RRRHRT
	mouse	RRRHRT
	droso	RRRSRT
	elegans	RRRTRT
Rsk1	human	RDRVRT
	mouse	RKRVRS
	xenopus	RKRVRT
	droso	KDRVRS
TSC2	human	RCRSIS RPRGYT
	mouse	RCRSIS RPRGYT
	droso	RKRSTS RGRSKT
Tub	human	RKRKKS
	mouse	RKRKKS
	droso	RKRKKS
	elegans	RKRKKS
Tulp1	human	RKRKRS
	mouse	RKRKKS
	chickpea	RNRESS

Figure 1: Alignments of the conserved PKB motifs in selected possible substrates.

Analysis of the PKB motifs within the selected proteins revealed a strong preference for Arg and Lys residues on the open positions in the motif, thereby forming the following motif: R-K/R-R-K/R-K/R-S/T. This is in contrast with the previously reported PKB substrates, described in Chapter 1, where no preference for Arg or Lys residues is observed. This motif strongly resembles a classical monopartite nuclear localization signal (NLS), namely a stretch of 4 to 6 K/R residues (17). The fact that transcription factors and other proteins such as Mdm2 were identified, that need to be nuclear in order to exert their function, suggests that the PKB motif in the selected substrates also contains a NLS that upon phosphorylation might become masked or at least modulated in its nuclear localization efficiency.

Pit1 is a member of the POU family of transcription factors, and **Prop1** is a paired-like homeodomain transcription factor. Both are specifically expressed in the pituitary, where they are responsible for growth hormone production (reviewed in (57)). Mice deficient for Pit1 or Prop1 show an increase in lifespan through the lack of growth hormone secretion (9, 23). Analysis of these two proteins as putative substrates of PKB is described in Chapter 4.

c-Fos and **Fra2** (Fos-related antigen-2) belong to the fos family of leucine-zipper containing transcriptional regulators (reviewed in (74)). The family also includes the proto-oncogene v-Fos and Fra1. They can dimerize with members of the Jun family to form the complex AP-1. AP-1 regulates processes such as cell proliferation and differentiation, and it is implied in transformation.

Mef2a (myocyte enhancer factor 2a) belongs to the MADS gene family (named for the yeast mating type-specific transcription factor MCM1, the plant plant homeotic genes 'agamous' and 'deficiens' and the human serum response factor SRF), a family that also includes several homeotic gene products and other transcription factors, all of which share a conserved DNA-binding domain. The PKB consensus motif is present within this domain. These proteins are involved in the process of myoblast differentiation and mef2a is also involved in for instance GLUT4 transcription (51). It has been shown that PI3K signaling is important for the transcriptional activation of Mef2a in myoblast differentiation (69).

Brn5 is a member of the POU family of transcription factors and is specifically expressed in the neocortex (5). Its expression is induced by neuregulin, and is an early event in the terminal differentiation of neurons (21, 82). The perfectly conserved PKB motif is located within the POU domain which is involved in DNA binding. Phosphorylation of Brn5 by PKB is a putative mechanism for regulating its DNA binding affinity.

Tubby is an autosomal recessive syndrome characterized by maturity-onset obesity, insulin resistance, infertility and progressive cochlear and retinal degeneration. The loss-of-function mutation in tubby is within the gene coding for **Tub** (66). Tub is a relatively uncharacterized protein that has a role in regulating insulin and G-protein signaling (39, 62). It is tyrosine-phosphorylated in response to insulin and is subsequently capable of binding to SH2 domain-containing proteins. The N-terminal part of the Tub protein is responsible for plasma membrane localization, whereas the conserved C-terminal tubby domain that contains the PKB motif directs the protein to the nucleus where it can bind PI(4,5)P2. Nuclear translocation of Tub is induced by $G\alpha_q$ activation via a PLCβ-dependent pathway where it can bind DNA via its C-terminal domain. Another family member of the Tubby family is **Tulp1** (Tubby-like protein 1) that is, like its other family members, expressed primarily in neuronal tissues. Tulp1-deficient mice show a mild phenotype of retinal degradation (32).

Brain factor (**BF1**) is a member of the forkhead family (in the current nomenclature known as FOXG1 (38)). FOXG1 can interact with FOXO factors, thereby antagonizing cellular effects regulated through binding of FOXO factors to Smad proteins (63). Involvement of PKB in FOXG1 activity could thus provide an additional mechanism through which the convergence of FOXO and Smad signaling is regulated.

Mdm2 was originally identified as an amplified gene in spontaneously transformed cells, which encodes an E3 ubiquitin ligase. Recently it has been described that PKB can bind and phosphorylate Mdm2 on Ser166 and Ser186, which leads to a nuclear localization (48, 86). Subsequently Mdm2 interacts with p53 and p300 thereby inducing p53 ubiquitination and degradation. Nuclear localization of Mdm2 can suppress the dominant-negative PKB-mediated sensitization of etoposide-induced apoptosis (86). However, Mayo and colleagues show that ectopic expression of dominant-negative PKB inhibits nuclear localization of Mdm2 thereby stabilizing p53 and increasing its transcriptional activity (48).

Tuberous Sclerosis Complex 2 (**TSC2**) can form a complex with TSC1 thereby inhibiting protein synthesis and subsequent cell growth by keeping mammalian target of rapamycin (mTOR) in an inactive state. Upon phosphorylation of TSC2 by PKB on conserved residues Ser981 and Thr1462 TSC2 is no longer able to interact with TSC1. This enables mTOR to induce S6 kinase activation and 4E-BP1 inhibition, and subsequently stimulate translation (34, 58).

Rsk1 stands for p90 ribosomal S6 kinase. It is a serine/threonine kinase that is

phosphorylated within the C-terminus by extracellular signal-related kinases 1/2 upon mitogen stimulation (reviewed in (25)). Phosphorylated Rsk1 is translocated to the plasma membrane where PDK1 can phosphorylate Rsk1 on Ser239 (in avian)(human: Ser253) (61). Upon activation Rsk1 can regulate transcription through association and phosphorylation of transcriptional regulators including c-Fos, the estrogen receptor and CBP (16, 36, 54). The PKB phosphorylation motif within the N-terminus of Rsk1 is conserved except for the motif in *Drosophila melanogaster*. In this case the closely resembling basic lysine residue replaces the first arginine residue.

Discovery of FOXO target genes using *in silico* analysis

FOXO transcription factors are members of the family of winged-helix Forkhead transcription factors. The subfamily of FOXO factors contains FOXO1, FOXO3a, FOXO4 (in the old nomenclature known as FKHR, FKHRL1 and AFX respectively) and FOXO6. FOXO factors regulate the transcription of multiple target genes that are important for a variety of cellular processes including proliferation and apoptosis (see Chapter 1 for described target genes). FOXO shuttles between the cytosolic and nuclear compartments. In the nucleus FOXO factors can regulate transcription by either direct binding to DNA sequences in promoter regions or through interacting with other transcription factors. Phosphorylation of FOXO factors by PKB on evolutionary conserved serine and threonine residues leads to a nuclear exclusion and subsequent inactivation (reviewed in (10)). The elements within promoter regions of target genes to which FOXO proteins can bind (DBEs) have been identified and conform to the general consensus namely 5'-TTGTTTAC-3' (28). It is known that FOXOs can also bind to related nucleotide motifs i.e. IRS motifs (5'-TGTTTT-3'), but it is not known if co-factors are needed for the interactions of FOXO factors with these sequences.

To identify novel putative target genes of FOXO factors, an *in silico* approach was undertaken. Microarrays, used or reported, were utilized to identify FOXO target genes or genes involved in physiological processes in which FOXO factors have been implicated, such as diabetes, caloric restriction and aging. The microarray analysis was combined with *in silico* finding of DBEs in promoter regions.

Microarray analysis was performed using the DL23 cell line. This cell line is derived from the DLD1 human colon carcinoma cell line and contains an inducible active FOXO3a system (44). The cells express a fusion of HA-tagged FOXO3a-A3 (mutated in the PKB motifs and thereby constitutively active) with a modified form of the estrogen receptor hormone-binding

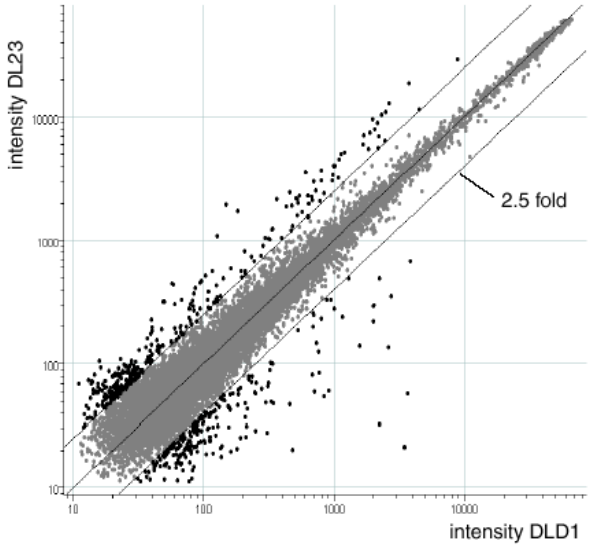

Figure 2: Scatter plot of the merged dye swap experiments.
The black points correspond to genes that were induced >2.5 fold.

domain that can be activated upon treatment with the estrogen analogue 4-hydroxy-tamoxifen (4-OHT). In the arrays gene expression in DL23 cells was compared to gene expression in DLD1 cells, both treated for 24 hours with 4-OHT. The arrays have been performed as described (76). Figure 2 shows a scatter plot of the merged dye swap experiment, and in Table 2 a summary of genes of which the expression changed more than 2.5 fold is depicted, since this leaves a practicable amount of genes regulated.

Table 2: FOXO3a-induced upregulated (A) and downregulated (B) genes.
Depicted is a summary of genes changing >2.5 fold in the two independent dye swap experiments and having a background corrected intensity >10.
A:

Gene ID	Description	Process/function	Up	Swissprot
		transcription		
COPEB	core promoter element binding protein	general transcription	3.7	Q99612
TAF11	TAF11 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 28kDa	general transcription	5.0	Q15544
GTF2I	general transcription factor II, i	general transcription	4.0	P78347
FOXP1	forkhead box P1	transcription	4.8	Q9H334
TP73	tumor protein p73	transcription	4.4	O15350
BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51)	transcription	7.9	P41182
KLF5	Kruppel-like factor 5 (intestinal)	transcription	5.3	Q13887
OTX2	orthodenticle homolog 2 (Drosophila)	transcription	2.7	P32243
PITX1	paired-like homeodomain transcription factor 1	transcription	2.9	P78337
GTF2IRD1	GTF2I repeat domain containing 1	transcription	3.9	Q9UHL9
BGLAP	bone gamma-carboxyglutamate (gla) protein (osteocalcin)	transcription, cell adhesion	5.6	P02818
		signal transduction		
ESR1	estrogen receptor 1	receptor signaling	4.9	P03372
CCR3	chemokine (C-C motif) receptor 3	receptor signaling, chemotaxis	3.0	P51677
CALCR	calcitonin receptor	receptor signaling, ossification	4.1	P30988
INSR	insulin receptor	receptor signaling	3.1	P06213
TACR1	tachykinin receptor 1	receptor signaling	3.6	P25103
ROR1	receptor tyrosine kinase-like orphan receptor 1	receptor signaling	5.0	Q01973
GPR49	G protein-coupled receptor 49	GPCR signaling	4.0	O75473
GPR52	G protein-coupled receptor 52	GPCR signaling	4.8	Q9Y2T5
RGS4	regulator of G-protein signalling 4	GPCR signaling	9.4	P49798
GNG11	guanine nucleotide binding protein (G protein), gamma 11	GPCR signaling	4.0	P50152
GNA12	guanine nucleotide binding protein (G protein) alpha 12	GPCR signaling	7.1	Q03113
PLCG2	phospholipase C, gamma 2 (phosphatidylinositol-specific)	signal transduction	7.4	P16885
PLD1	phospholipase D1, phophatidylcholine-specific	signal transduction	6.1	Q13393
ARHGAP1	Rho GTPase activating protein 1	signal transduction	2.9	Q07960
PDE7B	phosphodiesterase 7B	signal transduction	4.2	Q9NP56
MPP2	membrane protein, palmitoylated 2 (MAGUK p55 subfamily member 2)	signal transduction	4.2	Q14168
		transport		
APPBP2	amyloid beta precursor protein (cytoplasmic tail)-binding protein 2	intracellular protein transport	5.3	Q92624

continued

Gene ID	Description	Process/function	Up	Swissprot
RSC1A1	regulatory solute carrier protein, family 1, member 1	protein transport	3.5	Q92681
PACSIN3	protein kinase C and casein kinase substrate in neurons 3	endocytosis	3.6	Q9UKS6
ITM2B	integral membrane protein 2B	neurogenesis	3.4	Q9Y287
NDUFS4	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase)	electron transport	3.0	O43181
CYP3A5	cytochrome P450, subfamily IIIA (niphedipine oxidase)	electron transport	4.7	O00768
CYP27A1	cytochrome P450, subfamily XXVIIA	electron transport	4.5	Q02318
GLRB	glycine receptor, beta	ion transport	2.8	P48167
		metabolism		
PEPD	peptidase D	amino acid metabolism	3.6	P12955
GDA	guanine deaminase	nucleic acid metabolism	4.1	Q9Y2T3
ABCA1	ATP-binding cassette, sub-family A (ABC1), member 1	metabolism, transport	3.5	O95477
HADHB	hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase, beta subunit	fatty acid metabolism	3.9	P55084
SORL1	sortilin-related receptor, L(DLR class) A repeats-containing	lipid metabolism and transport	2.7	Q92673
HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	metabolism	5.2	P15428
		stress response		
SOD2	superoxide dismutase 2, mitochondrial	response to oxidative stress	3.3	P04179
CAT	catalase	response to oxidative stress	3.2	P04040
SEPP1	selenoprotein P, plasma, 1	response to radical stress	3.7	P49908
		miscellanious		
CAV1	caveolin 1, caveolae protein, 22kDa	caveolae	2.6	Q03135
RNF122	ring finger protein 122		4.5	Q9H9V4
FNBP2	formin binding protein 2		3.7	O75044
DAF	decay accelerating factor for complement (CD55, Cromer blood group system)	immune response	8.2	P08174
LY6G6D	lymphocyte antigen 6 complex, locus G6D	membrane protein	2.9	Q9NZJ1
M11S1	membrane component, chromosome 11, surface marker 1	membrane protein	4.1	Q14444
RNUT1	RNA, U transporter 1	RNA cap binding	3.6	O95149
APOBEC2	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 2	mRNA processing	3.8	Q9Y235
CLU	clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, apolipoprotein J)	apoptosis	3.6	P10909
NTF6A	neurotrophin 6, alpha	neuron differentiation	3.8	AAB22779
NTF6A	neurotrophin 6, alpha pseudogene	neuron differentiation	3.5	P34132
RFPL2	ret finger protein-like 2	protein binding	3.2	O75678
S100P	S100 calcium binding protein P	protein binding	3.2	P25815
MYBPC2	myosin binding protein C, fast type	muscle contraction, cell adhesion	3.9	Q14324
PPL	periplakin	structural constituent of cytoskeleton	6.7	O60437
PCDHA13	protocadherin alpha 13	cell adhesion	4.1	Q9Y5I0
CHODL	chondrolectin	cell adhesion	4.4	Q9H9P2
MACF1	microtubule-actin crosslinking factor 1	cell cycle, actin binding	2.6	Q9UPN3
CDC25B	cell division cycle 25B	cell cycle	4.2	P30305
PTPN14	protein tyrosine phosphatase, non-receptor type 14	phosphatase activity	3.0	Q15678
CYR61	cysteine-rich, angiogenic inducer, 61	cell growth, cell adhesion	3.4	O00622

B:

Gene ID	Description	Process/function	Down	Swissprot
		transcription		
MYF6	myogenic factor 6 (herculin)	differentiation, transcription	3.0	P23409
ASCL2	achaete-scute complex-like 2 (Drosophila)	differentiation, transcription	3.3	Q99929
TAF5	TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor	general transcription	3.2	Q15542
		signal transduction		
CAMK1	calcium/calmodulin-dependent protein kinase I	signal transduction	4.2	Q14012
PREX1	phosphatidylinositol 3,4,5-triphosphate-dependent RAC exchanger 1 protein	Rho signal transduction	3.4	Q8TCU6
EPHA8	EphA8	ephrin signal transduction	3.3	P29322
PLEKHC1	pleckstrin homology domain containing, family C (with FERM domain) member 1	cell growth	3.1	Q14840
		transport		
OCA2	oculocutaneous albinism II (pink-eye dilution homolog, mouse)	eye pigment biosynthesis, transport	3.2	Q04671
NAPA	N-ethylmaleimide-sensitive factor attachment protein, alpha	intracellular protein transport	4.1	P54920
SLC25A10	solute carrier family 25 (mitochondrial carrier; dicarboxylate transporter), member 10	(mitochondrial) transport	4.5	Q9UBX3
SLC28A1	solute carrier family 28 (sodium-coupled nucleoside transporter), member 1	transport	5.4	O04204
TRPA1	transient receptor potential cation channel, subfamily A, member 1	cation transport	4.2	O75762
COX7C	cytochrome c oxidase subunit VIIc	electron transport	3.7	P15954
		metabolism		
PECI	peroxisomal D3,D2-enoyl-CoA isomerase	fatty acid metabolism	2.9	O75521
ASAH2	N-acylsphingosine amidohydrolase (non-lysosomal ceramidase) 2	metabolism	3.5	Q9NR71
GALNT8	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 8 (GalNAc-T8)	metabolism	6.0	Q9NY28
SQLE	squalene epoxidase	sterol biosynthesis	2.6	Q14534
ADPRHL2	ADP-ribosylhydrolase like 2	hydrolase activity	4.4	Q9BY47
VARS2L	valyl-tRNA synthetase 2-like	valyl-tRNA aminoacylation	3.7	Q96Q02
		stress reponse		
MSH5	mutS homolog 5 (E. coli)	DNA damage response	4.4	O43196
		miscellanious		
CRIP1	cysteine-rich protein 1 (intestinal)		2.6	AAH02738
ZNF318	zinc finger protein 318	RNA binding	5.4	Q9UNU8
THOC4	THO complex 4	nucleic acid binding	12.5	AAH52302
CPB2	carboxypeptidase B2 (plasma, carboxypeptidase U)	proteolysis	4.2	Q15114

Other microarrays that were used for the *in silico* analysis include additional arrays performed with DL23 cells, by A. Schulze (personal communication), from hereon referred to as the Schulze arrays. In our array 19K human oligochips were used whereas the Schulze arrays were performed using Human cDNA microarrays (Sanger human 10K). In addition, the Schulze arrays include both 6 and 24 hours time points of 4-OHT stimulation. The other microarrays have been previously described. FOXO3a and FOXO1 dependent gene expression has been studied using adenoviral overexpression of active

FOXO3a or FOXO1 in LAPC4 prostate cancer cells (50). In addition, the effect of FOXO1 on gene transcription in endothelial cells has been investigated by viral infection of active FOXO1 into HUVECs (22). Different time points after introduction of FOXO1 in these cells were studied, as well as the effect of active PKB on gene transcription in HUVECs. Other array experiments in which the effect of FOXO1 was studied were performed in PTEN-deficient 786-O renal carcinoma cells (60). These cells were virally infected with either active FOXO1, with or without a mutation in the third helix loop causing loss of DNA binding of FOXO1 to the classical insulin response element (IRS). Differential expression induced by these constructs allowed the authors to distinguish between classical and non-classical FOXO target genes, although it must be noted that differences in induced expression could also be caused by differences in expression levels of the different FOXO constructs. In addition, no controls for the effects of adenoviral infection were performed.

In addition, microarrays were analyzed that were performed to assess gene transcription induced by physiological phenomena such as diabetes, aging and caloric restriction (35, 46, 47, 73, 78, 79, 83). Diabetes arises when insulin signaling is constitutively inhibited, thus causing a continuous relatively high level of FOXO activity by removal of the PKB-mediated inhibition. Caloric restriction also can be considered a physiological phenomenon for FOXO activity since it has been shown that caloric restricted rodents show higher levels of FOXO1, FOXO3a and FOXO4 compared to their control littermates (29). Importantly, in the process of caloric restriction insulin/PKB signaling is downregulated causing relatively high levels of active FOXO factors (33). The latter is important since increased expression of FOXO factors alone is likely not to be sufficient to cause a relatively higher FOXO activity (37). Caloric restriction leads to an increase in lifespan, preventing the process of aging. At the level of gene expression it has been shown that caloric restriction can revert, in part, the age-induced gene expression (35, 46, 47, 79).

Combining the results from the DL23 microarray experiments with previously described FOXO microarrays and relevant more physiological arrays, and with the absence or presence of FOXO DNA binding elements in promoter sequences resulted in a list of putative novel FOXO targets (Table 3), of which most targets have been described in more detail below.

Some of the selected target genes that are depicted in Table 3 have been previously identified as valid FOXO target genes, thereby validating the chosen approach. FOXO factors can protect cells from oxidative stress by inducing transcription of manganese super-oxide dismutase (**MnSOD**) (42), and **catalase** (56). MnSOD, also known as SOD2, is a mitochondrial enzyme that converts $O_2\bullet^-$ into H_2O_2 whereas the peroxisome-specific catalase converts H_2O_2 into H_2O and O_2. Whether these two proteins cooperate to quench toxic radicals, or they serve independent functions, being localized in different cellular compartments, is currently unclear. When cells undergo caloric restriction, genes involved in the response to oxidative stress are transcriptionally activated. Both MnSOD and catalase are examples of this (64, 79). Another gene implied to be involved in response to oxidant stress is selenoprotein P (**SEPP1**). SEPP1 is a selenium-rich extracellular protein suggested to be involved in free radical scavenging (11). It has been shown to be downregulated in mouse and human tumors (12). It has been shown that TGFβ signaling through Smad3/4 can lead to SEPP1 expression but whether crosstalk between FOXO and Smad signaling is involved in this is not known (53). SEPP1 was found upregulated in the DL23 arrays and it contains two consensus binding elements only 79 and 315 base pairs upstream of the ATG of the SEPP1

Table 3: Potential FOXO target genes.
Depicted are potential FOXO target genes, the results of the promoter analysis for putative FOXO binding elements (DBEs)(p: perfect, ip: imperfect (one point mutation)), DL23 array and other relevant microarrays ((S): Schulze array); and in which processes the target genes are involved.

Target	DBE	DL23	Other arrays	Function
Bcl6	1 p, 7 ip	Up	Up (S)(22, 70)	Apoptosis
Catalase	1 p, 5 ip	Up	Up (56), CR up (79)	Oxidant response
Caveolin-1	1 p, 3 ip	Up	Up (S), CR up (19), tumor down (81)	Caveolae
Clusterin	1 p, 3 ip	Up	Up (S)(22), diabetes up (73), age down/CR up (35, 46)	Glycoprotein
DAF	6 ip	Up	Up (S)(50, 60)	Immune response
Estrogen R	7 ip	Up		Transcription, synergy with insulin/IGF
FOXP1	1 p, 11 ip	Up	Up (60)	Transcription
GADD45	1 p, 2 ip	-	Down (S), up (71), aging up/CR down (47, 78)	G2/M arrest
Grb7	1 p, 4 ip	-	Up (S), breast cancer up (40)	Cell migration
IGFBP6	6 ip	-	Up (S), diabetes up (83), lung adeno(carcino)ma down (84), HNSCC up (2)	Ins/IGF signaling
InsR	2 p, 3 ip	Up	Up (59)	Insulin signaling
KLF5	5 ip	Up	Up (S)(22)	Transcription
MMP7	8 ip	-	Up (22, 60)	Matrix remodeling
MMP10	1 p, 7 ip	-	PKB down/FOXO up (22)	Matrix remodeling
MnSOD	1 p, 7 ip	Up	Up (42), CR up (64, 79)	Oxidant response
Periplakin	1 ip	Up		Binds PKB ((77) + chapter 2) and cytoskeletal binding
PLD1	10 ip	Up	CR up (46), senescence down (85)	Signaling
PP2A	3 ip	-	Up (S), age down/CR up (47)	Phosphatase, signaling
Rhodanese	1 p, 2 ip	-	Up (S), aging down/CR up (47, 78)	Sulfur metabolism
S100P	3 ip	Up	Up (S), senescence down (75)	Protein binding
SEPP1	2 p, 4 ip	Up	Up (22, 71), tumor down (3),	Oxidant response
TP73	7 ip	Up	Tumor up (68)	Transcription

gene. Importantly, Tran and colleagues observed SEPP1 transcriptional upregulation by FOXO3a (71). The authors performed microarrays with Rat1 cells expressing an inducible FOXO3a-A3, and identified **GADD45** as being upregulated (27, 71). This protein is involved in DNA damage response, leading to a G2/M cell cycle arrest. This arrest can be induced by oxidative stress and is mediated by FOXO (27). In contrast, the microarrays performed by Schulze show a clear decrease in GADD45 expression. Besides that, it has been shown that aging leads to an increase in GADD45 expression that can be reverted by caloric restriction (47, 78). A possible explanation could be that regulation of GADD45 expression by FOXO is cell specific.

Another previously identified FOXO target is **Bcl6** (22, 70). In part through regulation of Bcl6 expression FOXO factors are able to trigger apoptosis. An additional FOXO target is the **insulin receptor**, albeit in *D. melanogaster* (59). This target gene, dInR, was identified after performing microarray analysis in Drosophila S2 cells with dFOXO. From this it can be suggested that via transcriptional upregulation of dInR dFOXO can provide a negative feedback loop on its own activity. It has been

shown that the insulin/IGF1 signaling can synergize with estrogen signaling (reviewed in (45)). Estrogen can lead to proliferation via binding to the nuclear estrogen receptor and subsequent transcriptional regulation. The **estrogen receptor** was found to be regulated by FOXO in the DL23 microarrays thereby providing a possible upregulation of synergistic signaling pathways. Expression of the estrogen receptor is associated with expression of the forkhead transcription factor **FOXP1** and this leads to an improved survival in human cancer (24). Interestingly, FOXP1 expression has been found both upregulated and downregulated in tumors, in stomach tumors and colon tumors respectively, suggesting that FOXP1 has an important function in normal cellular behavior (7). Similar to the estrogen receptor, FOXP1 is also found upregulated by the DL23 arrays. Besides that, its expression was found regulated in the microarrays performed with FOXO1 in renal carcinoma cells, where it should be noted that expression is thought to be regulated by FOXO through a classical IRS (60). In combination with the presence of FOXO DBE sequences in the promoter region, FOXP1 is clearly a potential FOXO target.

Caveolin-1 is the main constituent of microdomains within the plasma membrane, called caveolae. In these domains caveolin-1 can interact with and modulate the activity of various signaling molecules. Differential expression of caveolin-1 has been implied in tumorigenesis, but it is still unclear what the exact role of caveolin-1 expression is in the process of tumor formation and/or metastasis. Caveolin-1 expression was found increased in both the DL23 as well as the Schulze arrays and analysis of the promoter region revealed one consensus FOXO DNA binding sequence. In rats, caveolin-1 expression was upregulated upon fasting (19). The role of caveolin-1 in being a putative FOXO target is described in Chapter 5 of this thesis.

Rhodanese, also known as thiosulfate sulfur transferase (TST), was found to be upregulated by FOXO in the Schulze arrays. It is a mitochondrial matrix enzyme that is proposed to play roles in sulfur metabolism and cyanide detoxification (80). The presence of a consensus DBE in its promoter in combination with its upregulation in the Schulze microarrays predicts rhodanese to be a valid target gene for FOXO signaling. Also regulation of expression by aging and caloric restriction indicates a possible role for FOXO in this (47, 78). In keeping with this hypothesis, rhodanese expression is negatively regulated by diet-induced obesity (52).

DAF stands for decay accelerating factor and is also known as CD55. It is an extracellular glycoprotein that can block complement deposition during inflammation (1, 41), and a very potential FOXO target gene product. Besides the DL23 arrays, DAF was also found upregulated in a variety of other FOXO arrays such as FOXO1 in renal carcinoma (60), and FOXO3a in prostate carcinoma cells (50). Although it is suggested that FOXO1 would lead to an increase of DAF expression through binding to a classical IRS, still a clear increase in expression was found when using the FOXO1 mutant that is not able to bind classical IRS motifs (60), implying that it is regulated by FOXO through non-classical IRS. Another glycoprotein is **clusterin**, also known as apolipoprotein J and SP-40,40. It can have both pro-apoptotic and anti-apoptotic effects, although no mechanisms are known, and it also has been shown to be transcriptionally upregulated during tumorigenesis (reviewed in (72)). Clusterin was found upregulated in both the DL23 arrays, Schulze arrays and in endothelial cells (22). In addition, clusterin expression was found upregulated in patients with diabetes (73), and in aged rats it was found downregulated, but this could be reverted or upregulated by caloric restriction (35, 46). These data together with the presence of FOXO

binding elements in the promoter region of the clusterin gene clearly suggest a direct role for FOXO in transcriptional regulation of clusterin. Another gene implied in tumorigenesis is matrix metalloproteinase 10 (**MMP10**), a proteolytic enzyme involved in regulation of the extracellular matrix. MMP10 expression is downregulated by PKB and upregulated by FOXO in endothelial cells (22). Although both DL23 arrays did not identify MMP10 as a possible FOXO target the presence of one perfect and seven imperfect FOXO binding elements in its promoter region combined with the reciprocal regulation by PKB and FOXO strongly suggest MMP10 to be a direct FOXO target. The related **MMP7** was also found transcriptionally upregulated in FOXO arrays, namely in endothelial cells (22), and in renal carcinoma cells (60).

The gene product **TP73** is a member of the p53 family, and through differential splicing the various gene products can either have positive effects or negative effects on p53-mediated signaling (reviewed in (65)). A role for TP73 has been suggested in tumorigenesis but it is controversial as to whether it can truly act as tumor suppressor or as proto-oncogene (discussed in (49)). Although no other FOXO arrays resulted in TP73 as putative target it was found regulated in the DL23 arrays and its important function in regulating p53 signaling makes TP73 an interesting potential FOXO target.

Kruppel-like transcription factor 5 (**KLF5**) positively regulates proliferation (67), and mediates oncogenic activities of H-Ras (55). However, its expression is lost or severely inhibited in breast and prostate tumors (14, 15). In our DL23 and the Schulze arrays, KLF5 expression was found upregulated and in addition its expression was elevated by FOXO1 in endothelial cells (22).

Phospholipase D1 (**PLD1**) is an enzyme that catalyzes the hydrolysis of phosphatidylcholine to generate the lipid second messenger phosphatidate (PA) and choline, and can be activated by G-protein coupled receptor and receptor tyrosine kinase signaling (reviewed in (18)). By generating PA it can lead to PIP5 kinase activation thereby regulating membrane traffic and actin dynamics. PLD1 expression was found upregulated in the DL23 arrays and also arrays analyzing genes regulated during caloric restriction (46). In line with that, PLD1 expression was reduced in senescent fibroblasts (85).

The S100 calcium-binding protein P (**S100P**) is member of the S100 family and is involved in protein binding. Via Receptor for Activated Glycation End products (RAGE), S100P can stimulate cell proliferation and survival (6). Expression of S100P is increased upon immortalization and in early breast cancer (30). In addition, high expression levels of S100P are associated with a bad prognosis for patients with lung adenocarcinomas (8). S100P was found downregulated with cellular senescence (75) and in both the DL23 arrays and Schulze arrays its expression level is increased.

In conclusion, *in silico* analysis for the discovery of novel FOXO-mediated transcription provides promising putative putative target genes. The retrieval of various previously described target genes implies that it can act as a valid method to identify novel FOXO target genes. Since this analysis utilizes a variety of previously described microarrays with different FOXO transcription factors and experimental setups, it is likely that the recovered target genes will be general targets. The presence of FOXO binding elements within the promoter suggests that the putative target genes might act in a direct fashion whereas absence of binding elements would argue that FOXOs are not involved or, more likely, act through co-factors.

References

1. **Ahmad, S. R., E. A. Lidington, R. Ohta, N. Okada, M. G. Robson, K. A. Davies, M. Leitges, C. L. Harris, D. O. Haskard, and J. C. Mason.** 2003. Decay-accelerating factor induction by tumour necrosis factor-alpha, through a phosphatidylinositol-3 kinase and protein kinase C-dependent pathway, protects murine vascular endothelial cells against complement deposition. Immunology **110:**258-68.
2. **Al Moustafa, A. E., M. A. Alaoui-Jamali, G. Batist, M. Hernandez-Perez, C. Serruya, L. Alpert, M. J. Black, R. Sladek, and W. D. Foulkes.** 2002. Identification of genes associated with head and neck carcinogenesis by cDNA microarray comparison between matched primary normal epithelial and squamous carcinoma cells. Oncogene **21:**2634-40.
3. **Al-Taie, O. H., N. Uceyler, U. Eubner, F. Jakob, H. Mork, M. Scheurlen, R. Brigelius-Flohe, K. Schottker, J. Abel, A. Thalheimer, T. Katzenberger, B. Illert, R. Melcher, and J. Kohrle.** 2004. Expression Profiling and Genetic Alterations of the Selenoproteins GI-GPx and SePP in Colorectal Carcinogenesis. Nutr Cancer **48:**6-14.
4. **Alessi, D. R., F. B. Caudwell, M. Andjelkovic, B. A. Hemmings, and P. Cohen.** 1996. Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. FEBS Lett **399:**333-8.
5. **Andersen, B., M. D. Schonemann, R. V. Pearse, 2nd, K. Jenne, J. Sugarman, and M. G. Rosenfeld.** 1993. Brn-5 is a divergent POU domain factor highly expressed in layer IV of the neocortex. J Biol Chem **268:**23390-8.
6. **Arumugam, T., D. M. Simeone, A. M. Schmidt, and C. D. Logsdon.** 2004. S100P stimulates cell proliferation and survival via receptor for activated glycation end products (RAGE). J Biol Chem **279:**5059-65.
7. **Banham, A. H., N. Beasley, E. Campo, P. L. Fernandez, C. Fidler, K. Gatter, M. Jones, D. Y. Mason, J. E. Prime, P. Trougouboff, K. Wood, and J. L. Cordell.** 2001. The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p. Cancer Res **61:**8820-9.
8. **Beer, D. G., S. L. Kardia, C. C. Huang, T. J. Giordano, A. M. Levin, D. E. Misek, L. Lin, G. Chen, T. G. Gharib, D. G. Thomas, M. L. Lizyness, R. Kuick, S. Hayasaka, J. M. Taylor, M. D. Iannettoni, M. B. Orringer, and S. Hanash.** 2002. Gene-expression profiles predict survival of patients with lung adenocarcinoma. Nat Med **8:**816-24.
9. **Brown-Borg, H. M., K. E. Borg, C. J. Meliska, and A. Bartke.** 1996. Dwarf mice and the ageing process. Nature **384:**33.
10. **Burgering, B. M., and R. H. Medema.** 2003. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J Leukoc Biol **73:**689-701.
11. **Burk, R. F., K. E. Hill, J. A. Awad, J. D. Morrow, and P. R. Lyons.** 1995. Liver and kidney necrosis in selenium-deficient rats depleted of glutathione. Lab Invest **72:**723-30.
12. **Calvo, A., N. Xiao, J. Kang, C. J. Best, I. Leiva, M. R. Emmert-Buck, C. Jorcyk, and J. E. Green.** 2002. Alterations in gene expression profiles during prostate cancer progression: functional correlations to tumorigenicity and down-regulation of selenoprotein-P in mouse and human tumors. Cancer Res **62:**5325-35.
13. **Cardone, M. H., N. Roy, H. R. Stennicke, G. S. Salvesen, T. F. Franke, E. Stanbridge, S. Frisch, and J. C. Reed.** 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science **282:**1318-21.
14. **Chen, C., H. V. Bhalala, H. Qiao, and J. T. Dong.** 2002. A possible tumor suppressor role of the KLF5 transcription factor in human breast cancer. Oncogene **21:**6567-72.
15. **Chen, C., H. V. Bhalala, R. L. Vessella, and J. T. Dong.** 2003. KLF5 is frequently deleted and down-regulated but rarely mutated in prostate cancer. Prostate **55:**81-8.
16. **Chen, R. H., C. Abate, and J. Blenis.** 1993. Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. Proc Natl Acad Sci U S A **90:**10952-6.
17. **Christophe, D., C. Christophe-Hobertus, and B. Pichon.** 2000. Nuclear targeting of proteins: how many different signals? Cell Signal **12:**337-41.
18. **Cockcroft, S.** 2001. Signalling roles of mammalian phospholipase D1 and D2. Cell Mol Life Sci **58:**1674-87.
19. **Cohen, A. W., B. Razani, W. Schubert, T. M. Williams, X. B. Wang, P. Iyengar, D. L. Brasaemle, P. E. Scherer, and M. P. Lisanti.** 2004. Role of caveolin-1 in the modulation of lipolysis and lipid droplet formation. Diabetes **53:**1261-70.
20. **Cross, D. A., D. R. Alessi, P. Cohen, M. Andjelkovich, and B. A. Hemmings.** 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature **378:**785-9.
21. **Cui, H., and R. F. Bulleit.** 1998. Expression of the POU transcription factor Brn-5 is an early event in the terminal differentiation of CNS neurons. J Neurosci Res **52:**625-32.

22. **Daly, C., V. Wong, E. Burova, Y. Wei, S. Zabski, J. Griffiths, K. M. Lai, H. C. Lin, E. Ioffe, G. D. Yancopoulos, and J. S. Rudge.** 2004. Angiopoietin-1 modulates endothelial cell function and gene expression via the transcription factor FKHR (FOXO1). Genes Dev **18:**1060-71.
23. **Flurkey, K., J. Papaconstantinou, and D. E. Harrison.** 2002. The Snell dwarf mutation Pit1(dw) can increase life span in mice. Mech Ageing Dev **123:**121-30.
24. **Fox, S. B., P. Brown, C. Han, S. Ashe, R. D. Leek, A. L. Harris, and A. H. Banham.** 2004. Expression of the forkhead transcription factor FOXP1 is associated with estrogen receptor alpha and improved survival in primary human breast carcinomas. Clin Cancer Res **10:**3521-7.
25. **Frodin, M., and S. Gammeltoft.** 1999. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. Mol Cell Endocrinol **151:**65-77.
26. **Fujita, E., A. Jinbo, H. Matuzaki, H. Konishi, U. Kikkawa, and T. Momoi.** 1999. Akt phosphorylation site found in human caspase-9 is absent in mouse caspase-9. Biochem Biophys Res Commun **264:**550-5.
27. **Furukawa-Hibi, Y., K. Yoshida-Araki, T. Ohta, K. Ikeda, and N. Motoyama.** 2002. FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress. J Biol Chem **277:**26729-32.
28. **Furuyama, T., T. Nakazawa, I. Nakano, and N. Mori.** 2000. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J **349:**629-34.
29. **Furuyama, T., H. Yamashita, K. Kitayama, Y. Higami, I. Shimokawa, and N. Mori.** 2002. Effects of aging and caloric restriction on the gene expression of Foxo1, 3, and 4 (FKHR, FKHRL1, and AFX) in the rat skeletal muscles. Microsc Res Tech **59:**331-4.
30. **Guerreiro Da Silva, I. D., Y. F. Hu, I. H. Russo, X. Ao, A. M. Salicioni, X. Yang, and J. Russo.** 2000. S100P calcium-binding protein overexpression is associated with immortalization of human breast epithelial cells in vitro and early stages of breast cancer development in vivo. Int J Oncol **16:**231-40.
31. **Hosaka, T., W. H. Biggs, 3rd, D. Tieu, A. D. Boyer, N. M. Varki, W. K. Cavenee, and K. C. Arden.** 2004. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc Natl Acad Sci U S A **101:**2975-80.
32. **Ikeda, S., N. Shiva, A. Ikeda, R. S. Smith, S. Nusinowitz, G. Yan, T. R. Lin, S. Chu, J. R. Heckenlively, M. A. North, J. K. Naggert, P. M. Nishina, and M. P. Duyao.** 2000. Retinal degeneration but not obesity is observed in null mutants of the tubby-like protein 1 gene. Hum Mol Genet **9:**155-63.
33. **Imae, M., Z. Fu, A. Yoshida, T. Noguchi, and H. Kato.** 2003. Nutritional and hormonal factors control the gene expression of FoxOs, the mammalian homologues of DAF-16. J Mol Endocrinol **30:**253-62.
34. **Inoki, K., Y. Li, T. Zhu, J. Wu, and K. L. Guan.** 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol **4:**648-57.
35. **Jervis, K. M., and B. Robaire.** 2003. Effects of caloric restriction on gene expression along the epididymis of the Brown Norway rat during aging. Exp Gerontol **38:**549-60.
36. **Joel, P. B., J. Smith, T. W. Sturgill, T. L. Fisher, J. Blenis, and D. A. Lannigan.** 1998. pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. Mol Cell Biol **18:**1978-84.
37. **Junger, M. A., F. Rintelen, H. Stocker, J. D. Wasserman, M. Vegh, T. Radimerski, M. E. Greenberg, and E. Hafen.** 2003. The Drosophila Forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. J Biol **2:**20.
38. **Kaestner, K. H., W. Knochel, and D. E. Martinez.** 2000. Unified nomenclature for the winged helix/forkhead transcription factors. Genes Dev **14:**142-6.
39. **Kapeller, R., A. Moriarty, A. Strauss, H. Stubdal, K. Theriault, E. Siebert, T. Chickering, J. P. Morgenstern, L. A. Tartaglia, and J. Lillie.** 1999. Tyrosine phosphorylation of tub and its association with Src homology 2 domain-containing proteins implicate tub in intracellular signaling by insulin. J Biol Chem **274:**24980-6.
40. **Kauraniemi, P., M. Barlund, O. Monni, and A. Kallioniemi.** 2001. New amplified and highly expressed genes discovered in the ERBB2 amplicon in breast cancer by cDNA microarrays. Cancer Res **61:**8235-40.
41. **Kirschfink, M.** 1997. Controlling the complement system in inflammation. Immunopharmacology **38:**51-62.
42. **Kops, G. J., T. B. Dansen, P. E. Polderman, I. Saarloos, K. W. Wirtz, P. J. Coffer, T. T. Huang, J. L. Bos, R. H. Medema, and B. M. Burgering.** 2002. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature **419:**316-21.
43. **Kops, G. J., N. D. de Ruiter, A. M. De Vries-Smits, D. R. Powell, J. L. Bos, and B. M. Burgering.** 1999. Direct control of the Forkhead

transcription factor AFX by protein kinase B. Nature **398:**630-4.

44. **Kops, G. J., R. H. Medema, J. Glassford, M. A. Essers, P. F. Dijkers, P. J. Coffer, E. W. Lam, and B. M. Burgering.** 2002. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol Cell Biol **22:**2025-36.
45. **Lee, A. V., X. Cui, and S. Oesterreich.** 2001. Cross-talk among estrogen receptor, epidermal growth factor, and insulin-like growth factor signaling in breast cancer. Clin Cancer Res **7:**4429s-4435s; discussion 4411s-4412s.
46. **Lee, C. K., D. B. Allison, J. Brand, R. Weindruch, and T. A. Prolla.** 2002. Transcriptional profiles associated with aging and middle age-onset caloric restriction in mouse hearts. Proc Natl Acad Sci U S A **99:**14988-93.
47. **Lee, C. K., R. G. Klopp, R. Weindruch, and T. A. Prolla.** 1999. Gene expression profile of aging and its retardation by caloric restriction. Science **285:**1390-3.
48. **Mayo, L. D., and D. B. Donner.** 2001. A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc Natl Acad Sci U S A **98:**11598-603.
49. **Melino, G., V. De Laurenzi, and K. H. Vousden.** 2002. p73: Friend or foe in tumorigenesis. Nat Rev Cancer **2:**605-15.
50. **Modur, V., R. Nagarajan, B. M. Evers, and J. Milbrandt.** 2002. FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. J Biol Chem **277:**47928-37.
51. **Mora, S., and J. E. Pessin.** 2000. The MEF2A isoform is required for striated muscle-specific expression of the insulin-responsive GLUT4 glucose transporter. J Biol Chem **275:**16323-8.
52. **Moraes, R. C., A. Blondet, K. Birkenkamp-Demtroeder, J. Tirard, T. F. Orntoft, A. Gertler, P. Durand, D. Naville, and M. Begeot.** 2003. Study of the alteration of gene expression in adipose tissue of diet-induced obese mice by microarray and reverse transcription-polymerase chain reaction analyses. Endocrinology **144:**4773-82.
53. **Mostert, V., I. Dreher, J. Kohrle, S. Wolff, and J. Abel.** 2001. Modulation of selenoprotein P expression by TGF-beta(1) is mediated by Smad proteins. Biofactors **14:**135-42.
54. **Nakajima, T., A. Fukamizu, J. Takahashi, F. H. Gage, T. Fisher, J. Blenis, and M. R. Montminy.** 1996. The signal-dependent coactivator CBP is a nuclear target for pp90RSK. Cell **86:**465-74.
55. **Nandan, M. O., H. S. Yoon, W. Zhao, L. A. Ouko, S. Chanchevalap, and V. W. Yang.** 2004. Kruppel-like factor 5 mediates the transforming activity of oncogenic H-Ras. Oncogene **23:**3404-13.
56. **Nemoto, S., and T. Finkel.** 2002. Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. Science **295:**2450-2. Epub 2002 Mar 7.
57. **Pfaffle, R. W., C. Kim, O. Blankenstein, and H. Kentrup.** 1999. GH transcription factors. J Pediatr Endocrinol Metab **12:**311-7.
58. **Potter, C. J., L. G. Pedraza, and T. Xu.** 2002. Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol **4:**658-65.
59. **Puig, O., M. T. Marr, M. L. Ruhf, and R. Tjian.** 2003. Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes Dev **17:**2006-20.
60. **Ramaswamy, S., N. Nakamura, I. Sansal, L. Bergeron, and W. R. Sellers.** 2002. A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. Cancer Cell **2:**81-91.
61. **Richards, S. A., V. C. Dreisbach, L. O. Murphy, and J. Blenis.** 2001. Characterization of regulatory events associated with membrane targeting of p90 ribosomal S6 kinase 1. Mol Cell Biol **21:**7470-80.
62. **Santagata, S., T. J. Boggon, C. L. Baird, C. A. Gomez, J. Zhao, W. S. Shan, D. G. Myszka, and L. Shapiro.** 2001. G-protein signaling through tubby proteins. Science **292:**2041-50.
63. **Seoane, J., H. V. Le, L. Shen, S. A. Anderson, and J. Massague.** 2004. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell **117:**211-23.
64. **Sreekumar, R., J. Unnikrishnan, A. Fu, J. Nygren, K. R. Short, J. Schimke, R. Barazzoni, and K. S. Nair.** 2002. Effects of caloric restriction on mitochondrial function and gene transcripts in rat muscle. Am J Physiol Endocrinol Metab **283:**E38-43.
65. **Stiewe, T., and B. M. Putzer.** 2002. Role of p73 in malignancy: tumor suppressor or oncogene? Cell Death Differ **9:**237-45.
66. **Stubdal, H., C. A. Lynch, A. Moriarty, Q. Fang, T. Chickering, J. D. Deeds, V. Fairchild-Huntress, O. Charlat, J. H. Dunmore, P. Kleyn, D. Huszar, and R. Kapeller.** 2000. Targeted deletion of the tub mouse obesity gene reveals that tubby is a loss-of-function mutation. Mol Cell Biol **20:**878-82.
67. **Sun, R., X. Chen, and V. W. Yang.** 2001. Intestinal-enriched Kruppel-like factor (Kruppel-like factor 5) is a positive regulator of cellular proliferation. J Biol Chem **276:**6897-900.

68. **Sun, X. F.** 2002. p73 overexpression is a prognostic factor in patients with colorectal adenocarcinoma. Clin Cancer Res **8:**165-70.
69. **Tamir, Y., and E. Bengal.** 2000. Phosphoinositide 3-kinase induces the transcriptional activity of MEF2 proteins during muscle differentiation. J Biol Chem **275:**34424-32.
70. **Tang, T. T., D. Dowbenko, A. Jackson, L. Toney, D. A. Lewin, A. L. Dent, and L. A. Lasky.** 2002. The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. J Biol Chem **277:**14255-65.
71. **Tran, H., A. Brunet, J. M. Grenier, S. R. Datta, A. J. Fornace, Jr., P. S. DiStefano, L. W. Chiang, and M. E. Greenberg.** 2002. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science **296:**530-4.
72. **Trougakos, I. P., and E. S. Gonos.** 2002. Clusterin/apolipoprotein J in human aging and cancer. Int J Biochem Cell Biol **34:**1430-48.
73. **Trougakos, I. P., M. Poulakou, M. Stathatos, A. Chalikia, A. Melidonis, and E. S. Gonos.** 2002. Serum levels of the senescence biomarker clusterin/apolipoprotein J increase significantly in diabetes type II and during development of coronary heart disease or at myocardial infarction. Exp Gerontol **37:**1175-87.
74. **Tulchinsky, E.** 2000. Fos family members: regulation, structure and role in oncogenic transformation. Histol Histopathol **15:**921-8.
75. **Untergasser, G., H. B. Koch, A. Menssen, and H. Hermeking.** 2002. Characterization of epithelial senescence by serial analysis of gene expression: identification of genes potentially involved in prostate cancer. Cancer Res **62:**6255-62.
76. **van de Peppel, J., P. Kemmeren, H. van Bakel, M. Radonjic, D. van Leenen, and F. C. Holstege.** 2003. Monitoring global messenger RNA changes in externally controlled microarray experiments. EMBO Rep **4:**387-93.
77. **van den Heuvel, A. P., A. M. de Vries-Smits, P. C. van Weeren, P. F. Dijkers, K. M. de Bruyn, J. A. Riedl, and B. M. Burgering.** 2002. Binding of protein kinase B to the plakin family member periplakin. J Cell Sci **115:**3957-66.
78. **Weindruch, R., T. Kayo, C. K. Lee, and T. A. Prolla.** 2002. Gene expression profiling of aging using DNA microarrays. Mech Ageing Dev **123:**177-93.
79. **Weindruch, R., T. Kayo, C. K. Lee, and T. A. Prolla.** 2001. Microarray profiling of gene expression in aging and its alteration by caloric restriction in mice. J Nutr **131:**918S-923S.
80. **Westley, J., H. Adler, L. Westley, and C. Nishida.** 1983. The sulfurtransferases. Fundam Appl Toxicol **3:**377-82.
81. **Wikman, H., J. K. Seppanen, V. K. Sarhadi, E. Kettunen, K. Salmenkivi, E. Kuosma, K. Vainio-Siukola, B. Nagy, A. Karjalainen, T. Sioris, J. Salo, J. Hollmen, S. Knuutila, and S. Anttila.** 2004. Caveolins as tumour markers in lung cancer detected by combined use of cDNA and tissue microarrays. J Pathol **203:**584-93.
82. **Wu, R., M. Jurek, S. Sundarababu, and D. E. Weinstein.** 2001. The POU gene Brn-5 is induced by neuregulin and is restricted to myelinating Schwann cells. Mol Cell Neurosci **17:**683-95.
83. **Yang, X., P. A. Jansson, I. Nagaev, M. M. Jack, E. Carvalho, K. S. Sunnerhagen, M. C. Cam, S. W. Cushman, and U. Smith.** 2004. Evidence of impaired adipogenesis in insulin resistance. Biochem Biophys Res Commun **317:**1045-51.
84. **Yao, R., Y. Wang, R. A. Lubet, and M. You.** 2002. Differentially expressed genes associated with mouse lung tumor progression. Oncogene **21:**5814-21.
85. **Yeo, E. J., I. S. Jang, H. K. Lim, K. S. Ha, and S. C. Park.** 2002. Agonist-specific differential changes of cellular signal transduction pathways in senescent human diploid fibroblasts. Exp Gerontol **37:**871-83.
86. **Zhou, B. P., Y. Liao, W. Xia, Y. Zou, B. Spohn, and M. C. Hung.** 2001. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol **3:**973-82.

Chapter

4

Protein kinase B phosphorylates pituitary-specific homeodomain transcription factors

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Protein kinase B (PKB or c-Akt) is a Ser/Thr kinase involved in a variety of cellular processes including cell cycle regulation, differentiation and cell survival. In addition, a role for PKB in the process of aging is suggested. PKB phosphorylates proteins within a consensus motif (R-X-R-X-X-S/T). Since the pituitary-specific transcription factors Prop-1 and Pit-1 contain such a motif and they have been described to play a role in aging by regulating hormone expression we investigated whether PKB has a direct role in regulating these transcription factors. PKB can phosphorylate both Prop-1 and Pit-1 *in vitro*, but no regulation of phosphorylation *in vivo* by PKB was observed. Furthermore, we show that both Prop-1 and Pit-1 are constitutively present in the nucleus and are not translocated upon regulation of the insulin-PI3K-PKB pathway.

Introduction

In the brain, the pituitary is responsible for the production and secretion of various hormones. It consists of two main lobes and a smaller intermediate lobe. In the anterior part of the pituitary, lactotropic cells secrete prolactin, somatotropic cells secrete growth hormone and thyrotropic cells secrete thyroid-stimulating hormone (TSH) (reviewed in (16)). Furthermore, the anterior lobe produces hormones including luteinizing hormone (LH) and follicle-stimulating hormone (FSH). A complex network of transcription factors is responsible for controlling embryonic pituitary development, differentiation of cell lineages, and hormone production and secretion.

Pituitary transcription factor-1 (Pit-1) is a key transcriptional regulator in the pituitary gland (reviewed in (6)). It is also known as growth hormone factor-1 (GHF-1) and is officially referred to as POU1F1. This protein is specifically expressed in the lactotropic, somatotropic and thyrotropic cells in the anterior pituitary gland, although modest expression can also be found in non-pituitary tissues or tumor cell lines (9). Pit-1 is a homeodomain-containing protein that contains an additional domain, also found in Oct-1, Oct-2 and UNC-86, and therefore bares the name POU-domain. Both domains are responsible for binding to DNA, while the POU domain also mediates interactions with other proteins. Pit-1 can homodimerize or bind to other heterologous DNA-binding proteins such as nuclear receptors (10, 20), or co-regulatory proteins such as CBP (5). Binding of Pit-1 to other proteins often leads to synergistic transcriptional activation. There are two mouse strains known to carry a mutation in the Pit-1 gene. The Snell mice are homozygous for a missense mutation in Pit-1 that prevents binding to DNA (13). They lack growth hormone, prolactin and TSH production and have hypoplastic anterior pituitary glands. Furthermore these mice are much smaller in size compared to their normal littermates. The Jackson mice carry a complex rearrangement of the gene encoding Pit-1 leading to the absence of a functional Pit-1 protein (8). These mice show a similar phenotype to that observed in the Snell mice. The Snell mice show an increased lifespan of 25 – 50%. In man several mutations have been identified within the Pit-1 gene leading to amino acid changes or loss of expression (17). All these mutations show the same pathological phenotype termed combined pituitary hormone deficiency (CPHD), which includes hypoplasia of the pituitary gland and reduction or absence of the pituitary hormones growth hormone, prolactin, TSH, LH and FSH.

Pit-1 can be phosphorylated, and this can modulate its activity. Protein kinase A (PKA) can phosphorylate Pit-1 on several residues and this leads to different effects on its DNA binding capacity, depending on the phosphorylated site and the interacting DNA

sequence. *In vitro* phosphorylation of Thr220 leads to reduced binding of Pit-1 to the growth hormone promoter (11). Mutating the PKA phosphorylation motifs however still leads to cAMP-dependent regulation of prolactin expression (14). Basal expression of growth hormone and prolactin as well as induced prolactin expression is also independent of Pit-1 phoshorylation by PKA (7). The effects observed on hormone expression via Pit-1 phosphorylation may therefore be independent of PKA. Phosphorylation of Thr220 regulates binding of Pit-1 to its cofactor Ets-1 (1). This might be accomplished by the activation of a cell cycle-dependent protein kinase since Pit-1 is phosphorylated in the M-phase of the cell cycle independent of PKA by a yet unidentified kinase leading to inhibition of DNA binding (4).

Expression of Pit-1 is preceded by and dependent on the expression of another pituitary-specific transcription factor called Prophet of pit-1 (Prop-1). Prop-1 was first characterized as the gene responsible for the phenotype observed in Ames dwarf mice. These mice carry a point mutation in Prop-1, and fail to activate the Pit-1 gene and lack a full development of the pituitary (18). This leads to the absence of growth hormone, prolactin and TSH causing reduced levels of insulin-like growth factor-1 (IGF-1), dwarfism and a 55% increase in lifespan (2).

Prop-1 is a transcription factor that contains a paired-like homeodomain responsible for dimerization of Prop-1 and interaction with DNA (18). It is expressed solely in the pituitary, both in pituitary tumors as well as normal tissues. Besides inducing Pit-1 expression in the post-natal pituitary it is also involved in early embryonic development where it is expressed from day 11 until day 15.5 in the dorsal part of the gland. In humans, mutations in Prop-1 can lead to CPHD and a dramatic reduction in growth hormone, prolactin and TSH. Furthermore, no expression of LH and FSH is observed (17, 19). These mutations are found both in- and outside the homeodomain and lead to decrease in DNA binding and transcriptional activity. Two of these mutations are heterozygous and affect the same amino acid, Arg71, that is changed to His or to Cys (15). Thus far, no post-translational modifications or regulation of Prop-1 have been found.

Protein kinase B (PKB) is an important mediator of growth factor signaling and involved in multiple cellular processes including cell cycle regulation, cell survival and longevity (reviewed in (3)). It can regulate the activity of downstream targets by phosphorylation of a serine or threonine residue within a specific PKB consensus motif (RxRxxS or RxRxxT). Both Prop-1 and Pit-1 contain a conserved PKB consensus motif. Therefore, we performed for both proteins phosphorylation assays and functional studies to determine whether they could be regulated insulin/PI3K/PKB signaling.

Materials and methods

Constructs

Prop1-WT was cloned into pMT2-HA vector from pcDNA3-myc-Prop1 (obtained from T. Usui (Kyoto, Japan)) using a SalI-site containing 5' primer (GTCGACGGAAGCAGAAAGGAGGCG) and a Not1 site containing 3' primer (GCGGCCGCTCAGTTCCAGGACTTGGATG). Prop1-T74A was made by site-directed mutagenesis using 5' primer CACCGCGCCACCTCC and its complementary 3' primer. Prop1-S68A and -A2 were cloned with 5' primer CCGCACGCCCGGCGCCGCCAC and its complementary 3' primer using Prop1-WT or Prop1-T74A as template respectively. All constructed plasmids were verified by sequence analysis. HA-FOXO4 has been described before (12). HA-Pit1-WT and HA-Pit1-T220A were generous gifts from A. Gutierrez-Hartmann (Denver CO).

Cell culture

HEK293T (human embryonic kidney cells stably transfected with large T antigen) and A14 cells (NIH3T3 mouse fibroblasts stably expressing insulin receptor) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Bio-Whittaker,

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                                        68      74
H. sapiens      GGGRSRFSPQGGQ**RGRPHS** **RRRHRT** TFSPVQLEQLESAFGR
C. familiaris   GVRRPRLSPQGGQ**RGRLHS** **RRRHRT** TFNPGQLEQLETAFGR
M. musculus     ELGRPKLCPQ---**RGRPHS** **RRRHRT** TFNPAQLEQLESAFGR
D. melanogaster N-EDGGFPGDGDDDSSAAK **RRRSRT** NFNSWQLEELERAFSA
C. elegans      EDDGIALEDDNDTGESAAK **RRRTRT** NFSGWQLEELESAFEA
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Figure 1: Amino acid sequence comparison of Prop-1 homologues. PKB motifs (RxRxxS or RxRxxT) are shown in bold.

Belgium), 1% penicillin/streptomycin (Bio-Whittaker) and 2 mM L-Gln (Bio-Whittaker). HEK293T and A14 cells were transfected using Fugene6 and $CaPO_4$ method respectively. Cells were treated with 10 μM H89, 10 μM SB203580, 50 μM PD98059, 1 μM staurosporin, 100 nM wortmannin, 10 μM LY294002, 1 μM insulin, 20 ng/ml EGF and/or 20 μM forskolin in medium without serum for the indicated durations.

Immunoprecipitation and western blot analysis

Cells grown in 5 cm dishes were lysed and scraped in 0.5 ml RIPA lysis buffer (20 mM Tris pH 8.0, 1% Triton X-100, 0.5% Na-DOC, 0.1% SDS, 10 mM EDTA, 150 mM NaCl, 1 mM NaF, 1 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and 2 μg/ml aprotinin). Lysates were then incubated with anti-HA 12CA5 antibody and protA-beads for 2 h, after which precipitations were washed 4 times in RIPA buffer and either Laemmli-buffer was added or they were used for *in vitro* kinase assays. Immunoprecipitations were analyzed by SDS-PAGE and immunoblotting for HA with monoclonal anti-HA 12CA5 (1:5000) and HRP-conjugated anti-mouse secondary antibody (1:10,000).

In vitro kinase assay

Immunoprecipitated protein samples were washed twice with washing buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$) and subsequently incubated with active baculo-PKB or PDK-1, or PKA (Pierce), and 5 μCi γ-^{32}P-ATP in reaction buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 50 μM ATP and 1 mM DTT). For the PKA assay the provided buffer

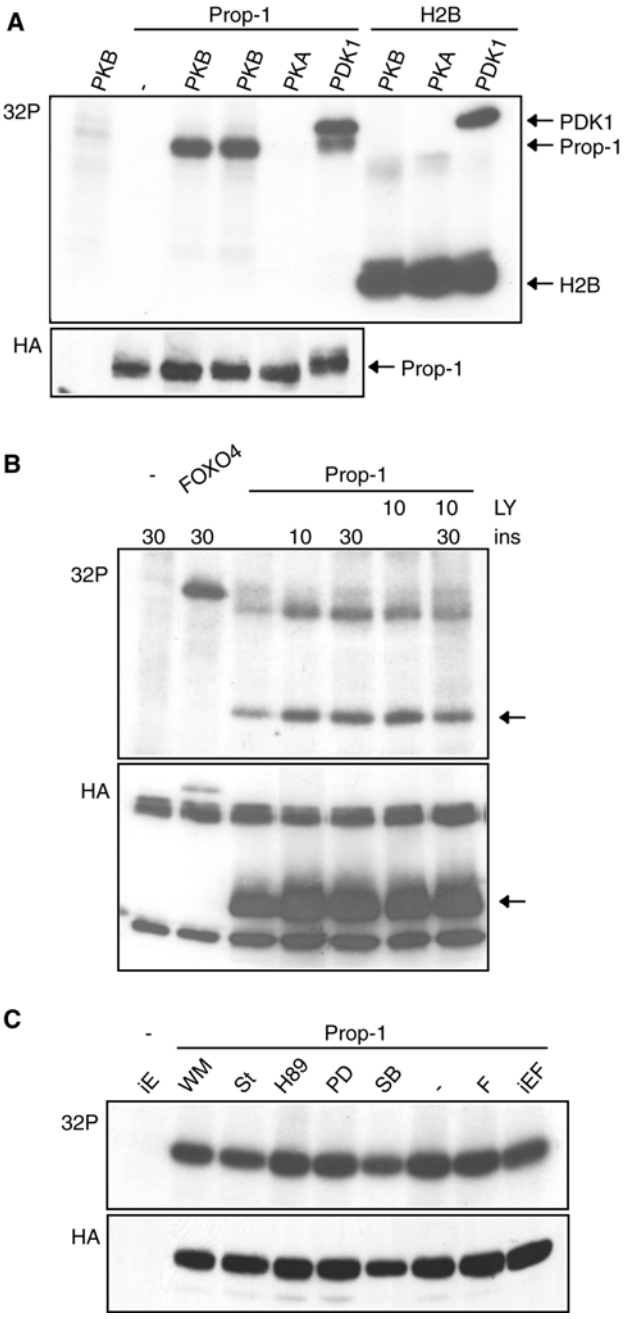

Figure 2: Prop-1 is phosphorylated by PKB *in vitro* but not *invivo* by the insulin pathway.
(A) HA-Prop1 was immunoprecipitated from transfected A14 cells using anti-HA 12CA5. An *in vitro* kinase assay was performed with no kinase (-) or active PKB, PKA or PDK1 using either Prop1 or H2B as substrates. HA-Prop1 levels were analyzed by western blotting using the 12CA5 antibody. (B) A14 cells were transiently transfected with HA-Prop1 treated with 1 μM insulin with or without pretreatment of 10 μM LY294002 for the indicated times after which immunoprecipitations with the anti-HA 12CA5 were performed. Samples were used in an *in vivo* kinase assay and analyzed by SDS-PAGE with autoradiography and immunoblotting using the 12CA5 antibody. (C) An *in vivo* kinase assay was performed with transiently HA-Prop1-transfected HEK293T cells. Cells were treated with various kinase inhibitors (wortmannin (WM), staurosporin (St), H89, PD98059 (PD), SB203580 (SB)) or activators (iE: insulin/EGF, F: forskolin) for 30 min. Subsequently immunoprecipitated HA-Prop1 was analyzed by SDS-PAGE using autoradiography and immunoblotting for HA.

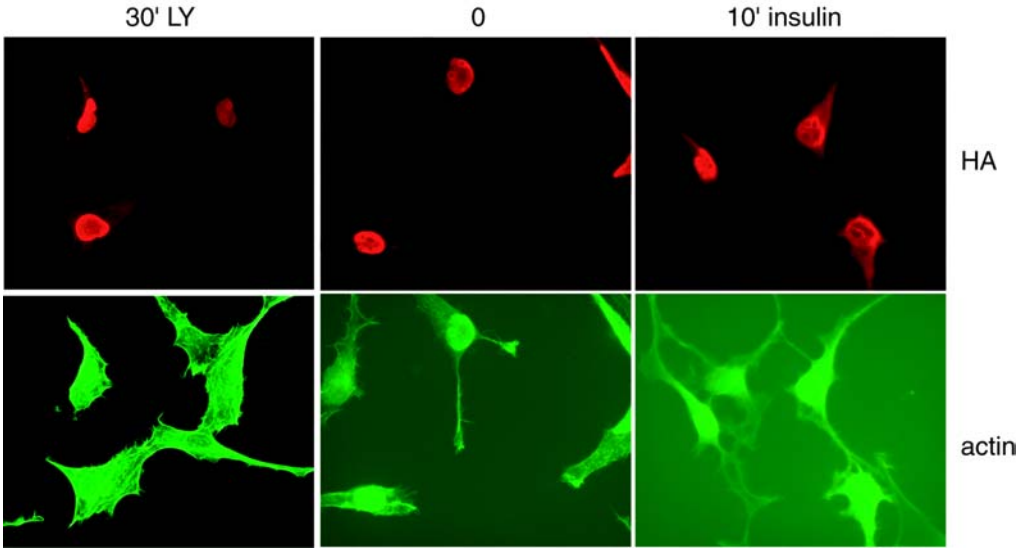

Figure 3: Prop-1 is localized in the nucleus.
A14 fibroblasts were transiently transfected with HA-tagged Prop-1. Cells were stimulated for 30 minutes with either LY or insulin and then fixed, permeabilized and stained for the HA epitope.

was used (Pierce). Unless indicated otherwise, after 30 min at 4°C 7 µl 5x Laemmli buffer was added to stop the reaction.

In vivo kinase assay
Cells were incubated in phosphate-free medium containing 1 mCi ^{32}P-orthophosphate for 4 hours. Then cells were treated for 30 min unless indicated otherwise and immunoprecipitations were performed.

Immunofluorescence
A14 fibroblasts seeded on coverslips were transfected with HA-tagged constructs by the $CaPO_4$ method. At 24 hours after transfection, cells were serum-starved overnight, and subsequently treated with 10 µM LY294002 or 1 µM insulin. After treatments cells were fixated in 4% paraformaldehyde for 30 min at 4°C and permeabilized with 0.1% Triton X-100 in the presence of 0.5% BSA for 30 min at 4ºC. Staining was performed with anti-HA for 2 h at 4ºC, washed in PBS with 0.1% Triton X-100 and 0.01% BSA, and then incubated for 1 h with anti-mouse FITC-conjugated secondary antibody. After further washing coverslips were mounted in Immu-mount (Shandon) and staining was visualized using a Zeiss Axioskop 2 microscope and Zeiss Vision software.

Results and discussion

PKB is a serine/threonine protein kinase that can phosphorylate proteins in a so-called PKB motif (RxRxxS/T). From a BLAST search for RxRxxS and RxRxxT motifs in Swiss-PROT a small selection of potential PKB substrates was made based on two criteria (see Chapter 3). First, whether the protein is involved in one or more of the known molecular or physiological processes that are also regulated by PKB signaling, and second, if there exists conservation of this PKB motif from lower to higher organisms. Both criteria are met by Prop-1 and Pit-1. PKB has been shown to exert its role in aging via cell-autonomous regulation of FOXO factors. Regulation of Prop-1 and or Pit-1 by PKB would provide a novel non-cell autonomous mechanism by which PKB could regulate aging, namely through regulating pituitary-specific hormone levels. Therefore both Prop-1 and Pit-1 were studied in greater detail.

PKB phosphorylates Prop-1.

Prop-1 orthologues from both higher and lower eukaryotic organisms were aligned with Clustal X. Alignment of the PKB motif is shown in figure 1. The alignment shows perfect conservation of the RxRxxT motif containing Thr74 between all species including *D. melanogaster* and *C. elegans*. Proximal to the conserved motif there was an additional PKB motif found in higher eukaryotes containing Ser68.

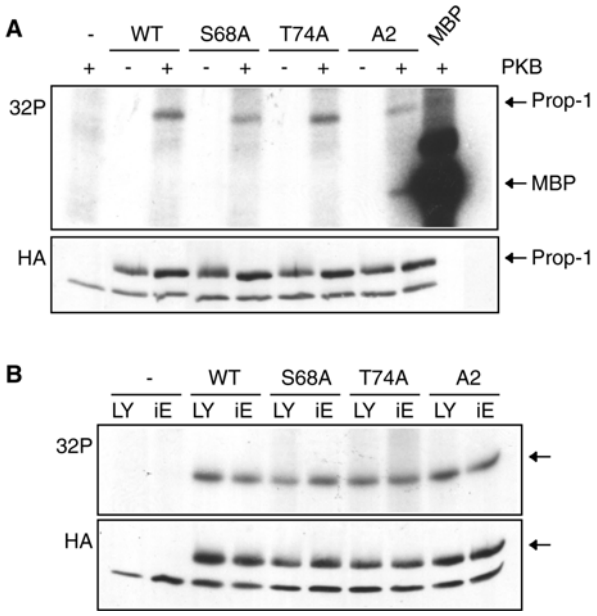

Figure 4: Phosphorylation of Prop-1 is not dependent on Ser68 and Thr74.
HEK293T cells were transiently transfected with either pMT2-empty vector or pMT2-HA-Prop1-WT, -S68A, -T74A or –A2. (A) An *in vitro* kinase assay was performed with immunoprecipitated HA-tagged proteins and active baculo-PKB. MBP was used as a positive control. Analysis was done by SDS-PAGE with autoradiography and western blotting using anti-HA. (B) An *in vivo* assay was performed treating the cells either with LY294002 (LY) or insulin/EGF (iE) for 30 min. Samples were analyzed as in (A).

To test whether Prop-1 could be phosphorylated by PKB *in vitro* we transiently transfected HA-tagged Prop-1 into A14 cells and immunoprecipitated Prop-1 using an anti-HA epitope antibody. These IPs were incubated with active forms of PKB, PKA and PDK1 (figure 2A). Both PKB and PDK1, but not PKA, are able to phosphorylate Prop-1 *in vitro*. To determine whether PKB-induced phosphorylation of Prop-1 also occurs *in vivo* we transfected HA-Prop-1 into A14 cells and treated cells with LY and/or insulin to regulate PKB activity (figure 2B). Prop-1 is phosphorylated but this does not seem to be regulated by insulin signaling. To determine which signaling pathways might be responsible for Prop-1 phosphorylation, we performed an *in vivo* kinase assay with various protein kinase inhibitors and activators (figure 2C). However, no inhibition or induction of Prop-1 phosphorylation could be observed.

The observed phosphorylation levels show the same pattern as the expression levels of Prop-1 indicating that basal Prop-1 phosphorylation is already high and that none of the kinases that are activated or inactivated by the various treatments have an effect on the phosphorylation of Prop-1.

It has been described for a great variety of proteins that they can translocate upon cellular stimulation, either or not dependent on phosphorylation. To investigate whether Prop-1 may relocate upon activation or inhibition of the insulin pathway we transiently transfected A14 fibroblasts with HA-tagged Prop-1 and performed immunofluorescence visualizing Prop-1 (figure 3). Prop-1 is present in the nucleus and does not relocate after inhibition or activation of PI3K. Furthermore, we also investigated whether PKB activation would result in altered Prop-1 transcriptional activity. To address this question, luciferase assays were performed using a vector with five Prop-1-binding elements cloned in front of the luciferase gene. No clear consistent results were obtained when modulating the insulin-PI3K pathway or when ectopically expressing active PKB (data not shown).

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                                    220
H. sapiens      ALYNEKVGANERK RKRRTT TISIAAKDALERHF
C. familiaris   ALYNEKVGANERK RKRRTT TISIAAKDALERHF
R. norvegicus   ALYNEKVGANERK RKRRTT TISIAAKDALERHF
S. porcus       ALYNEKVGANERK RKRRTT TISIAAKDALERHF
G. domesticus   ALYNEKVGVNERK RKRRTT TISISAKEALERHF
D. melanogaster PTSIDKIAAQGRK RKKRTS SIEVSVKGALEQHF
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Figure 5: Amino acid sequence comparison of Pit-1 homologues.
The PKB motif (RxRxxT) is shown in bold.

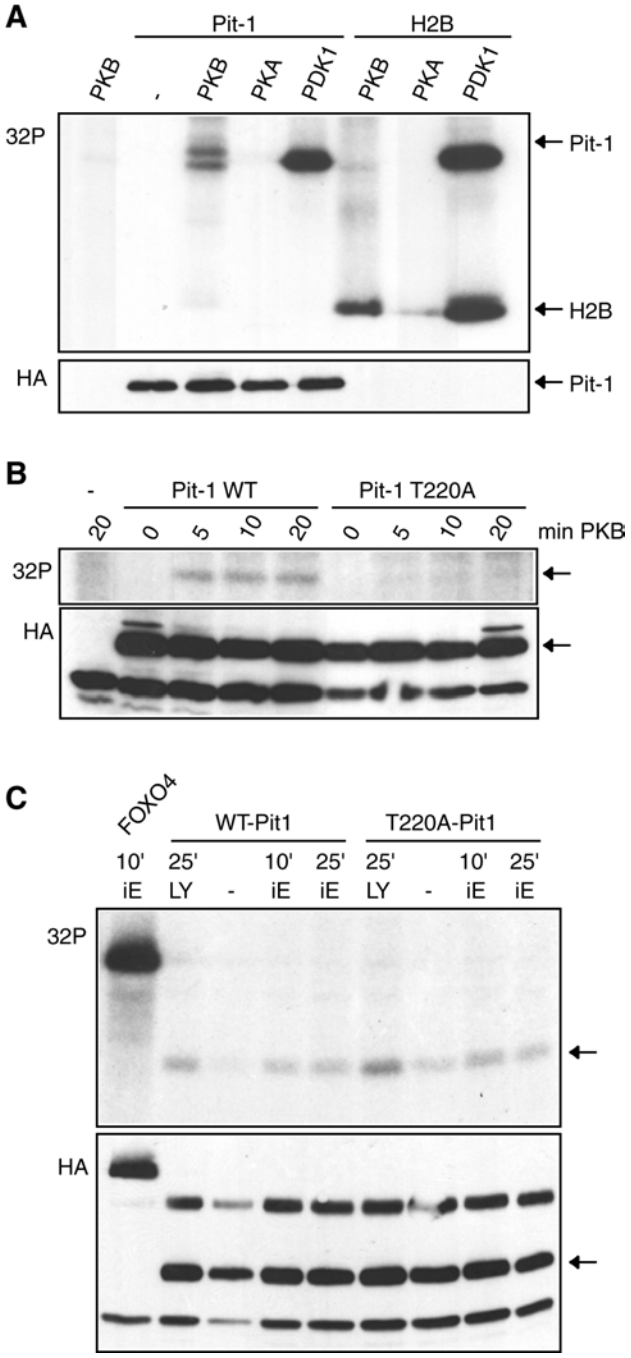

Figure 6: Pit-1 is phosphorylated *in vitro* by PKB but not *in vivo* by the insulin pathway.
(A) HA-Pit1 was immunoprecipitated from transiently transfected A14 fibroblasts using the 12CA5 antibody. An *in vitro* assay was performed using active baculo-PKB, PKA or PDK1. As a positive control H2B was used as a substrate. Immunoprecipitated proteins were analyzed after SDS-PAGE by autoradiography and immunoblotting with anti-HA. (B) HEK293T cells were transfected with either HA-Pit1-WT or HA-Pit1-T220A and immunoprecipitations were performed using anti-HA 12CA5. These were incubated subsequently with active baculo-PKB and radiolabeled ATP for different times. The samples were analyzed as described in (A). (C) HEK293T cells were transiently transfected with HA-FOXO4, HA-Pit1-WT or HA-Pit1-T220A and an *in vivo* kinase assay was performed, treating the cells with either LY294002 (LY) or insulin/EGF (iE) for the indicated times. Immunoprecipitated proteins were analyzed with SDS-PAGE by autoradiography and blotting for HA.

We next wanted to investigate whether phosphorylation of Prop-1 occurs via the putative PKB motifs. To do this we constructed Prop-1 mutants in which Ser68 and/or Thr74 were mutated to alanines by site-directed mutagenesis. As can be seen in figure 4A, the S68A and S68A/T74A (A2) mutants give a reduction in phosphorylation compared to wild-type and T74A Prop-1. However this inhibition of Prop-1 phosphorylation was not observed *in vivo*. None of the constructs show any change in phosphorylation upon treatment with LY or insulin/EGF, and also no differences could be seen between wild-type and the various mutants (figure 4B). Together, these data show that there is no role for PKB in Prop-1 phosphorylation.

PKB phosphorylates Pit-1.

Pit-1 was also selected as a putative PKB substrate (see Chapter 3). The alignment between human Pit-1 and orthologues from higher and lower eukaryotic organisms was performed with Clustal X and is shown in figure 5. Although there is conservation between higher eukaryotes the RxRxxT motif is not perfectly conserved in the lower eukaryote *D. melanogaster*. However, a lysine residue present instead of the second arginine residue is a close homologue.

To determine whether Pit-1 could be phosphorylated by PKB *in vitro* we transiently transfected HA-tagged Pit-1 into A14 cells. We performed an *in vitro* kinase assay using active forms of PKB, PKA and PDK1 (figure 6A). PKB was able to phosphorylate Pit-1 *in vitro*.

Although it has been reported that PKA can phosphorylate Pit-1 (11), this could not be observed in our experiment, probably due to the low activity as can be seen in lack of H2B phosphorylation. To test whether phosphorylation of Pit-1 by PKB occurs on Thr220 within the PKB motif we used wild-type and T220A Pit-1, and an *in vitro* kinase assay was performed (figure 6B). Whereas wild-type Pit-1 is phosphorylated, this is inhibited in mutant Pit-1. This indicates that Thr220 is important for Pit-1 phosphorylation by PKB *in vitro*. To investigate whether phosphorylation of Pit-1 by PKB also occurs *in vivo*, a kinase assay was performed utilizing HEK293T cells transiently transfected with wild-type or mutant Pit-1 (figure 6C). The results show clearly that phosphorylation of Pit-1 cannot be inhibited by LY or induced by insulin/EGF. Furthermore, the Pit-1 T220A mutant still shows similar levels of phosphorylation *in vivo* compared to wild-type Pit-1. These results demonstrate that Pit-1 is not an *in vivo* PKB substrate, even though Pit-1 is phosphorylated by PKB *in vitro*.

Since PKB-dependent phosphorylation of Pit-1 could not be shown we investigated other functional aspects of Pit-1 regulation. To see whether modulating insulin-induced signaling would lead to a change in localization of Pit-1 immunofluorescence experiments were performed using A14 fibroblasts and HA-tagged Pit-1. As can be seen in figure 7 no obvious Pit-1 relocation from or to the nucleus could be observed when cells were treated with either LY or insulin. Furthermore, upon activating or inactivating the insulin pathway neither changes in DNA binding, investigated with electro-mobility shift assays, nor clear effects on transcriptional activity, investigated Prop-1 and Pit-1 responsive luciferase assays, were found (data not shown). The only effect observed with luciferase assays was that co-transfection of active PKB (myr-PKB) with active Ras (RasV12) in pituitary cells led to a decrease in Ras-induced transcriptional activity on the prolactin promoter (D. Duval and A. Gutierrez-Hartmann, personal communication). It could be hypothesized that this effect is via Ets-1, which is a cofactor for Pit-1, since Ets-1 is known to be regulated by the Ras pathway and that PKB somehow regulates Ets-1 activity.

Taken together, it can be concluded that both Prop-1 and Pit-1 are not physiological substrates of PKB. Although phosphorylation *in*

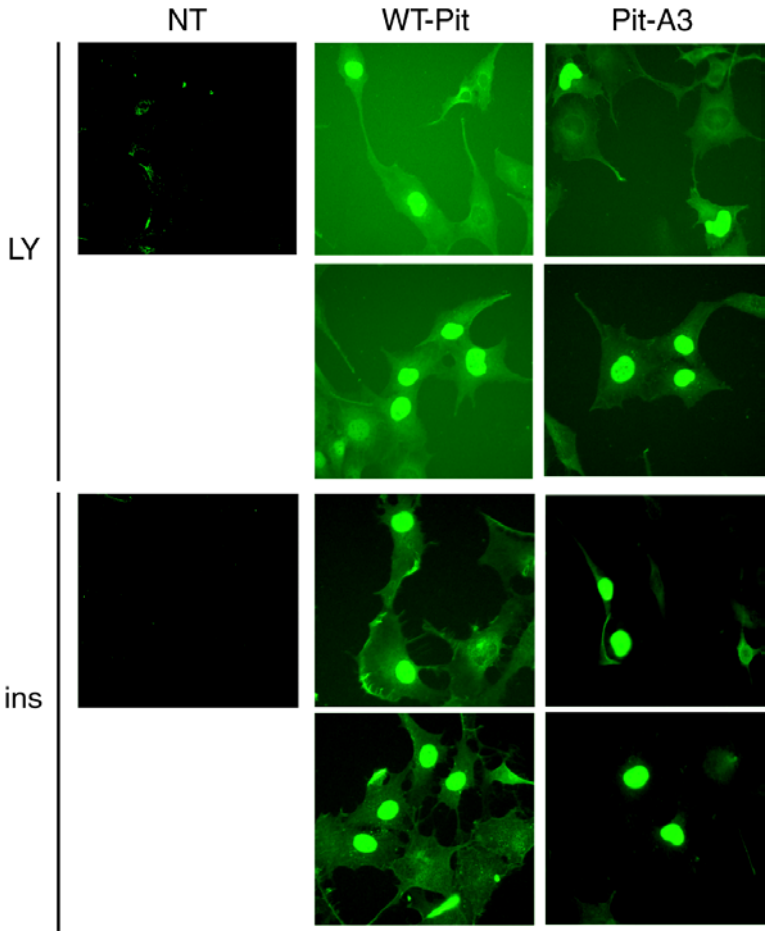

Figure 7: Pit-1 is localized in the nucleus.
A14 cells were transiently transfected with HA-Pit-1. Cells were treated with LY or insulin for 30 minutes and subsequently fixed, permeabilized and stained with anti-HA.

vitro of both proteins was found, no changes of phosphorylation status could be detected *in vivo*. Furthermore, no obvious effects were visible in various functional assays.

References

1. **Augustijn, K. D., D. L. Duval, R. Wechselberger, R. Kaptein, A. Gutierrez-Hartmann, and P. C. van der Vliet.** 2002. Structural characterization of the PIT-1/ETS-1 interaction: PIT-1 phosphorylation regulates PIT-1/ETS-1 binding. Proc Natl Acad Sci U S A **99:**12657-62.
2. **Brown-Borg, H. M., K. E. Borg, C. J. Meliska, and A. Bartke.** 1996. Dwarf mice and the ageing process. Nature **384:**33.
3. **Burgering, B. M., and R. H. Medema.** 2003. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J Leukoc Biol **73:**689-701.
4. **Caelles, C., H. Hennemann, and M. Karin.** 1995. M-phase-specific phosphorylation of the POU transcription factor GHF-1 by a cell cycle-regulated protein kinase inhibits DNA binding. Mol Cell Biol **15:**6694-701.
5. **Cohen, L. E., Y. Hashimoto, K. Zanger, F. Wondisford, and S. Radovick.** 1999. CREB-independent regulation by CBP is a novel mechanism of human growth hormone gene expression. J Clin Invest **104:**1123-30.
6. **Dattani, M. T., and I. C. Robinson.** 2000. The molecular basis for developmental disorders of the pituitary gland in man. Clin Genet **57:**337-46.
7. **Fischberg, D. J., X. H. Chen, and C. Bancroft.** 1994. A Pit-1 phosphorylation mutant can mediate both basal and induced prolactin and growth hormone promoter activity. Mol Endocrinol **8:**1566-73.
8. **Flurkey, K., J. Papaconstantinou, and D. E. Harrison.** 2002. The Snell dwarf mutation Pit1(dw) can increase life span in mice. Mech Ageing Dev **123:**121-30.
9. **Gil-Puig, C., M. Blanco, T. Garcia-Caballero, C. Segura, and R. Perez-Fernandez.** 2002. Pit-1/GHF-1 and GH expression in the MCF-7 human breast adenocarcinoma cell line. J Endocrinol **173:**161-7.
10. **Gonzalez, M. M., and C. Carlberg.** 2002. Cross-repression, a functional consequence of the physical interaction of non-liganded nuclear receptors and POU domain transcription factors. J Biol Chem **277:**18501-9. Epub 2002 Mar 12.
11. **Kapiloff, M. S., Y. Farkash, M. Wegner, and M. G. Rosenfeld.** 1991. Variable effects of phosphorylation of Pit-1 dictated by the DNA response elements. Science **253:**786-9.
12. **Kops, G. J., N. D. de Ruiter, A. M. De Vries-Smits, D. R. Powell, J. L. Bos, and B. M. Burgering.** 1999. Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature **398:**630-4.
13. **Li, S., E. B. Crenshaw, 3rd, E. J. Rawson, D. M. Simmons, L. W. Swanson, and M. G. Rosenfeld.** 1990. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene pit-1. Nature **347:**528-33.
14. **Okimura, Y., P. W. Howard, and R. A. Maurer.** 1994. Pit-1 binding sites mediate transcriptional responses to cyclic adenosine 3',5'-monophosphate through a mechanism that does not require inducible phosphorylation of Pit-1. Mol Endocrinol **8:**1559-65.
15. **Paracchini, R., M. Giordano, A. Corrias, S. Mellone, P. Matarazzo, J. Bellone, P. Momigliano-Richiardi, and G. Bona.** 2003. Two new PROP1 gene mutations responsible for compound pituitary hormone deficiency. Clin Genet **64:**142-7.
16. **Parks, J. S., M. E. Adess, and M. R. Brown.** 1997. Genes regulating hypothalamic and pituitary development. Acta Paediatr Suppl **423:**28-32.
17. **Rodriguez, R., and B. Andersen.** 2003. Cellular determination in the anterior pituitary gland: PIT-1 and PROP-1 mutations as causes of human combined pituitary hormone deficiency. Minerva Endocrinol **28:**123-33.
18. **Sornson, M. W., W. Wu, J. S. Dasen, S. E. Flynn, D. J. Norman, S. M. O'Connell, I. Gukovsky, C. Carriere, A. K. Ryan, A. P. Miller, L. Zuo, A. S. Gleiberman, B. Andersen, W. G. Beamer, and M. G. Rosenfeld.** 1996. Pituitary lineage determination by the Prophet of Pit-1 homeodomain factor defective in Ames dwarfism. Nature **384:**327-33.
19. **Wu, W., J. D. Cogan, R. W. Pfaffle, J. S. Dasen, H. Frisch, S. M. O'Connell, S. E. Flynn, M. R. Brown, P. E. Mullis, J. S. Parks, J. A. Phillips, 3rd, and M. G. Rosenfeld.** 1998. Mutations in PROP1 cause familial combined pituitary hormone deficiency. Nat Genet **18:**147-9.
20. **Ying, C., and D. H. Lin.** 2000. Estrogen-modulated estrogen receptor x Pit-1 protein complex formation and prolactin gene activation require novel protein synthesis. J Biol Chem **275:**15407-12.

Chapter

5

Direct control of caveolin-1 expression by FOXO transcription factors

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Protein kinase B can phoshorylate and thereby inactivate the FOXO family of transcription factors. When active, FOXO factors can bind to DNA within promoter sequences and subsequently regulate gene expression. We have used DNA micro-array analysis to identify potential gene targets of FOXO. Here we demonstrate that caveolin-1 expression is directly controlled by FOXO. First, caveolin-1 expression was increased upon induction or overexpression of FOXO factors at both mRNA and protein levels. Secondly we show that regulation of endogenous FOXO activity regulates caveolin-1 levels and that this can be inhibited by dominant-negative FOXO. Thirdly, FOXO activates transcription from the caveolin-1 promoter and with chromatin immunoprecipitations we demonstrate that this activation occurs via direct interaction of FOXO with the promoter. Finally we demonstrate FOXO-mediated attenuation of EGF-induced signaling, a phenomenon also observed with increased caveolin-1 expression. These findings suggest a novel mechanism by which FOXO factors can exert their cellular effects via transcriptional activation of caveolin-1.

Introduction

Forkhead box O (FOXO) transcription factors are the human homologues of the nematode *Caenorhabditis elegans* transcription factor DAF-16. Both FOXO and DAF-16 are winged-helix domain-containing proteins that are under the direct control of the insulin/phosphoinositide-3-kinase (PI3K)/ protein kinase B (PKB) signaling cascade (reviewed in (8)). FOXOs and DAF-16 are phosphorylated by PKB within conserved consensus phosphorylation motifs leading to nuclear exclusion and subsequent transcriptional inactivation. Besides by PKB-mediated phosphorylation it is now clear that transcriptional activity can also be regulated by phosphorylation by other kinases and by other post-translational modifications such as acetylation ((6, 18, 48) and reviewed in (8)). An increasing number of publications implicates this pathway and in particular the FOXO proteins in a variety of cellular processes including apoptosis, differentiation and cell cycle arrest and via these processes in phenomena such as aging (reviewed in (8)). For instance in *C. elegans* mutations in DAF-16 cause animals to suppress DAF-2-induced dauer arrest, metabolic shift to fat storage and longevity phenotype. In mice FOXO3a-deficiency causes follicular activation leading to oocyte death, premature follicle depletion and thereby secondary fertility (11). FOXO proteins can bind promoters containing consensus binding elements (5'-TTGTTTAC-3') and thereby regulate gene expression (19). Several target genes have been identified thus far. By inducing gene transcription of pro-apoptotic genes such as Bim and FasL, but also others like Bcl6, FOXOs can induce apoptosis in haematopoietic cells (5, 14, 46). In other cell types FOXO activation leads to a G1 cell cycle arrest via regulation of $p27^{kip1}$ and cyclin D, or a G2 arrest via GADD45 regulation (33, 43, 47). By regulating expression levels of proteins in the insulin pathway, such as the insulin receptor, it can also create a negative feedback loop (37).

Caveolin-1 is the main constituent of microdomains localized within the cellular membrane called caveolae. Within caveolae, caveolin-1 interacts with growth factor receptors like EGF and insulin receptors, and signaling molecules such as protein kinase A, Src kinases and H-Ras (29, 41). Through this interaction and subsequent sequestering these proteins are mostly negatively regulated with respect to their activity. The human caveolin-1 gene is situated on gene locus 7q31.1 and through alternative translation initiation sites,

transcription results in an α and β isoform of 24 and 22 kDa respectively. This gene locus has been implied in tumorigenesis, but it is still controversial to what extend caveolin-1 plays a role in this. Studies demonstrate a correlation between increased caveolin-1 expression, tumorigenesis and metastatic capacity in various types of primary tumors such as lung adenomas, esophageal squamous cell carcinomas, colon and pancreatic adenocarcinomas (17, 21, 23, 45). In prostate tumors high expression of caveolin-1 results in cell survival and enhanced metastasis (28). However others have shown in several tumor cell lines and some whole tumors that caveolin-1 expression is downregulated and that re-expression of caveolin-1 leads to inhibition of cell growth (1, 2, 27, 38, 50, 51). Caveolin-$1^{-/-}$ mice are viable but do show hyperproliferative abnormalities and the deficiency leads to the acceleration of mammary lesion formation in tumor-prone transgenic mice (40, 52). This can be due in part to the elevated cyclin D levels and inhibition of apoptosis (30, 52). Furthermore transformation of NIH3T3 cells by a variety of oncogenes such as HPV, v-abl and H-ras results in a decrease in, or even loss of caveolin-1 expression (15, 39). This decrease leads to an exit from cell cycle arrest whereas overexpression of caveolin-1 induced by hydrogen peroxide leads to G0/G1 cell cycle arrest and premature cellular senescence (20, 49). Furthermore in senescent cells caveolin-1 expression is elevated and leads to attenuation of EGF signaling with respect to mitogen-activated protein kinase (MAPK) phosphorylation (36). This senescent phenotype can however be reversed by reducing the expression levels of caveolin-1 (12), suggesting that either the senescent state is a reversible phenotype or that these cells are actually quiescent rather than senescent.

The molecular mechanisms regulating caveolin-1 expression are largely unknown. Various signaling pathways have been implicated but a coherent picture is still lacking. It is known that forskolin via cAMP can downregulate caveolin-1 mRNA levels in a dose-dependent manner (54). C-myc can directly regulate expression via the INR sequence present in the caveolin-1 promoter (35). Furthermore, PKC epsilon signaling can enhance caveolin-1 expression as well (53). Certain physiological processes are also able to regulate caveolin-1 expression like lactation, which occurs through prolactin-Ras-dependent signaling, and adipogenesis/differentiation (34, 42). The latter process may be regulated PPARγ since it can upregulate caveolin-1 expression and plays a critical role in adipogenesis (10). Furthermore caveolin-1 is downregulated by E2F and p53 in the S-phase of dividing cells and upregulated by free cholesterol via two sterol regulatory element-like sequences (3, 4, 16).

To identify novel target genes of FOXO transcription factors, DNA micro-array analysis was performed using a human colon carcinoma cell line stably expressing an inducible active FOXO3a. One of the genes that was found to be upregulated upon FOXO activation was caveolin-1. Here we show that caveolin-1 expression is directly regulated by FOXO and transcriptional activation of the caveolin-1 promoter by FOXO leads to attenuation of EGF-induced signaling.

Materials and methods

Constructs

Retroviral pBabe-puro expressing dominant-negative FOXO4 (DB) was constructed by subcloning from pMT2-HA-DB (33). pBabe-puro without insert was used as negative control in retroviral infections. pGL2-cav1 was constructed by cloning the ChIP cav1 promoter PCR fragment with flanking Sac1/XhoI sites in pGEM-T. Subsequently the SacI/XhoI fragment was subcloned into SacI/XhoI-digested pGL2-basic (Promega). The following constructs have been described: pBabe-HA-FOXO3a-A3 (25), pMT2-HA-FOXO4 (33). pBabe-p16 was a gift from R. Medema (32). pRL-Tk (Tk renilla luciferase) was purchased from Promega.

Cell culture and transient transfection
DLD1 and DL23 cells were grown in RPMI. HEK293T, C2C12, A14 and the MEF cells (WT, TKO) were grown in Dulbecco's modified Eagle's medium and Mcf7 cells in DF12 medium. Media were supplemented with 10% fetal calf serum (Bio-Whittaker, Belgium), 1% penicillin/streptomycin (Bio-Whittaker) and 2 mM L-Gln (Bio-Whittaker). DLD1 and DL23 cells were treated with 0.5 μM 4OHT where indicated. EGF was added to a final concentration of 20 ng/ml. Cells were treated overnight with 1 μM insulin or 10 μM LY294002 in medium without serum. TKO MEF cells were a gift from H. te Riele (13). A14 cells were transiently transfected using the $CaPO_4$ method and HEK293T cells using PEI (Polysciences).

Retroviral infection
Retroviruses were obtained by transfecting Phoenix cells with the relevant retroviral construct. 36 hours after transfection conditioned medium from these cells was collected, filtered and diluted 1:1 with fresh medium and 6 μg/ml polybreen (Sigma) was added. The medium was added in two consecutive rounds to infect mouse embryonic fibroblasts and infected cells were selected by puromycin treatment and collected after 48 hours .

Northern blotting
Total RNA was extracted from cells using RNAzol (Tel-Test, Texas). 20 μg was electroforesed, blotted onto nitrocellulose and probed for caveolin-1 and GAPDH using α-^{32}P-dCTP-labeled cDNA probes.

Immunoblot analysis
Protein samples in Laemmli buffer were separated by SDS-PAGE on 12.5% gels and transferred to PVDF membrane (NEN). Western blots were blocked overnight at 4°C in TBS/0.1% Tween-20 (TBST) containing either 2% nonfat dried milk (Protifar, Nutricia) and 0.5% bovine serum albumin (Sigma) or for the anti-phosphorylation-specific antibodies 2% bovine serum albumin. The western blots were then incubated for 2 hours with the indicated primary antibodies in TBST using the dilutions recommended by the manufacturers or 1:8000 for the self-generated antibodies. After washing four times for 5 minutes with TBST blots were incubated with secondary antibodies anti-mouse HRP or anti-rabbit HRP (1:10,000) for 1 hour at 4°C. Blots were washing again four times for 5 minutes with PBS/0.1% Tween-20 and analyzed with chemiluminescence (ECL (NEN)). Primary antibodies used were anti-caveolin-1 from Santa Cruz (N-20) and BD Transduction Laboratories (clone 2297), anti-p27kip1 (BD Transduction Laboratories), anti-phospho p44/42 MAPK (Thr202/Tyr204) and anti-phospho PKB (Ser473) (Cell Signaling), anti-tubulin (Calbiochem), anti-HA (12CA5) and anti-PKB (5178) (7).

Chromatin immunoprecipitation
DL23 cells were incubated with or without 4OHT for 20 h before crosslinking with 1% formaldehyde for 10 min at RT. Then glycine was added to a final concentration of 125 mM after which cells were washed 3 times with PBS. Cells were subsequently treated with trypsin and collected by scraping in PBS/20%FCS. After centrifugation cells were collected in cell lysis buffer (5 mM PIPES pH8.0, 85 mM KCl, 0.5% NP40 plus proteinase inhibitors (Complete, Roche) and dounced 20 times. Subsequently the lysates were collected in nuclear lysis buffer (50 mM Tris pH8.0, 1% SDS, 10 mM EDTA, 0.5 mM PMSF plus proteinase inhibitors after centrifuging and sonicated 4 times 30 seconds on ice at 40% using an UP200S sonicator (Dr.Hielscher, GmbH). After centrifuging for 10 min at 14000rpm at 4°C 3% of the supernatant was taken as input sample after which the remainder was divided in two equal amounts that were diluted 5-fold in dilution buffer (20 mM Tris pH8.0, 1% SDS, 2 mM EDTA, 0.3 mM NaCl, herring sperm) and precleared 2 times using ProtA beads. For the immunoprecipitation the anti-HA 12CA5 antibody was used and anti-PKB 5178 antibody as negative control. After the 16 h incubation period with the primary antibody ProtA beads were added and incubated for another 45 min followed by various washing steps (i.e. 2 times in buffer 1 (20 mM Tris pH8.0, 0.1% TX100, 2 mM EDTA, 150 mM NaCl), 2 times in buffer 2 (20 mM Tris pH8.0, 1% TX100, 2 mM EDTA, 150 mM NaCl), 4 times in buffer 3 (20 mM Tris pH8.0, 1% TX100, 2 mM EDTA, 500 mM NaCl) and 2 times with TE. The immunocomplexes were subsequently eluted from the beads by vortexing in elution buffer (1% SDS, 50 mM $NaHCO_3$). RNAse and NaCl were added (to a final concentration of 0.1 μg/μl and 300 mM respectively) and heated for 16 hours at 65°C to dissociate the cross-linked portion. DNA was precipitated and treated with proteinase K at 42°C for 2 h after which phenol-chloroform extraction and precipitation were performed. The DNA was analyzed by PCR (50 ng of each primer set, 2 U Taq polymerase, At 60°C, 29 cycles). Primer sets used were 5'-GCTGCAGTGACCTATGAATG-3' and 5'-GAACTCATGGAAACAAATAGGG-3' for the cav1 PCR resulting in a 511 nt fragment encompassing the FOXO binding element, and 5'-GAATATCCGATCTAGCCTGG-3' and 5'-TGGCACTGTGCTTCCTGTAC-3' for the control PCR resulting in a 370 nt fragment.

Luciferase assay
Cells were transfected with 1 μg pGL2-cav1 along with 20 ng TK-renilla and various concentrations of HA-FOXO4 (0.5 and 2.0 μg for A14 cells and 0.2, 0.5 and 1.0 μg for HEK293T cells). Lysis and subsequent determination of luciferase counts were performed 40 hours after transfection in triplicate using the Dual-

Luciferase Reporter Assay (Promega). The assays were performed four times.

Results

Caveolin-1 expression is increased by FOXO activity.

To identify novel transcriptional targets of FOXO transcription factors a micro-array was performed using the DL23 cell line. This subclone from the DLD1 human colon carcinoma cell line expresses a conditionally active FOXO3a construct that is mutated in its PKB-phosphorylation motifs (26). This FOXO construct is fused to a modified form of the estrogen receptor hormone-binding domain so that FOXO3a-A3 is inactive unless cells are treated with 4-hydroxy tamoxifen (4-OHT). A putative target gene that was found to be upregulated upon 4-OHT treatment in the array was caveolin-1. Using the GenomeView program (NCBI Entrez Genome) we examined the region upstream of the Transcription Initiation Site (TIS) of the human caveolin-1 gene. This revealed one consensus FOXO binding sequence (5'-TTGTTTAC-3') at position –1814 upstream of the TIS and three nearly perfect sequences on positions –1584, –1069 and –679.

To verify the results from the micro-array we analyzed caveolin-1 expression following FOXO activation. Induction of FOXO activity by 4-OHT treatment in DL23 cells resulted in a time-dependent increase of caveolin-1 RNA and protein levels (figure 1A and 1B respectively). Using a different anti-caveolin-1 antibody recognizing both α and β isoforms of caveolin-1 we could show that the induction of caveolin-1 transcripts leads to an increase of both isoforms (figure 1B). Having verified our DNA micro-array result we next wanted to determine if FOXO could also induce caveolin-1 expression in an unrelated experimental system. To this end mouse embryonic fibroblasts (MEFs) were virally infected with the active FOXO construct. Protein analysis showed increase in caveolin-1 expression (figure 1C).

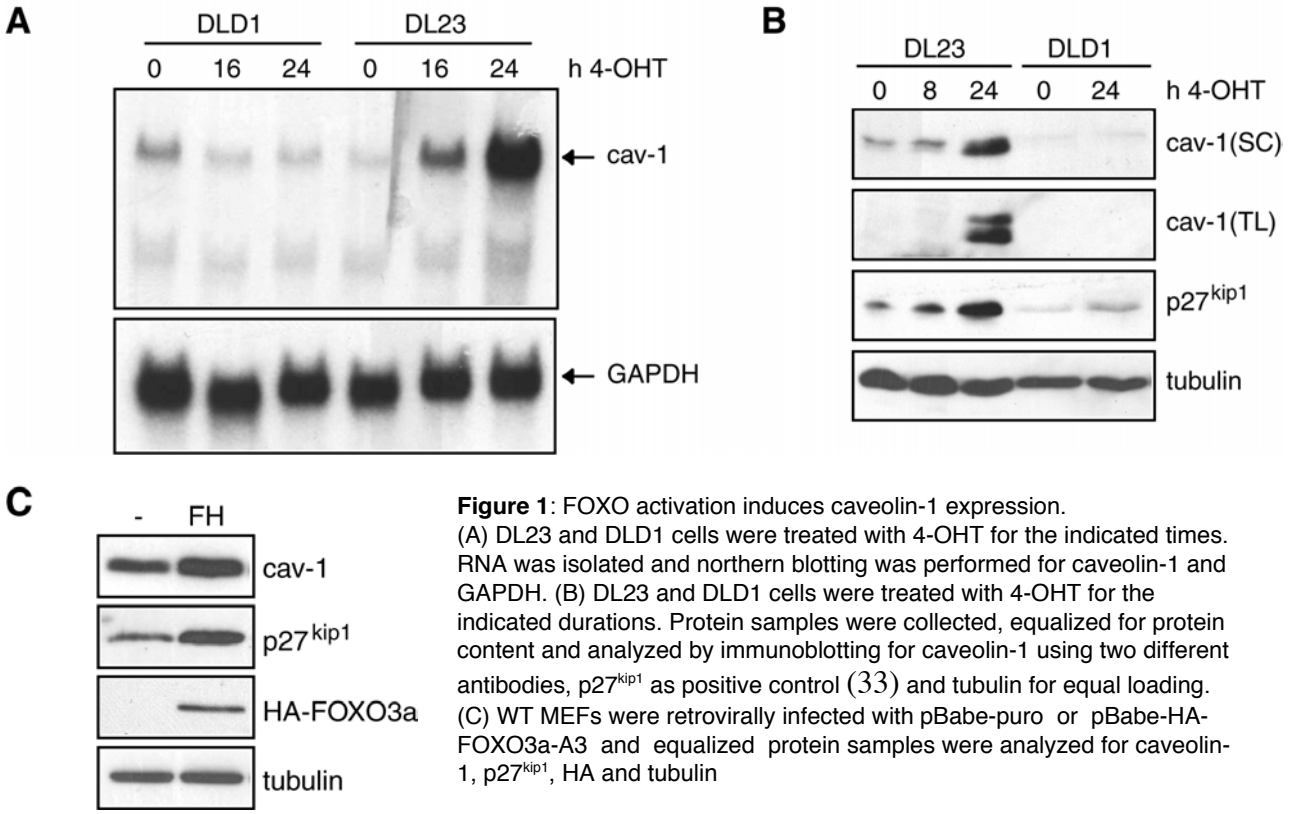


Figure 1: FOXO activation induces caveolin-1 expression.
(A) DL23 and DLD1 cells were treated with 4-OHT for the indicated times. RNA was isolated and northern blotting was performed for caveolin-1 and GAPDH. (B) DL23 and DLD1 cells were treated with 4-OHT for the indicated durations. Protein samples were collected, equalized for protein content and analyzed by immunoblotting for caveolin-1 using two different antibodies, $p27^{kip1}$ as positive control (33) and tubulin for equal loading. (C) WT MEFs were retrovirally infected with pBabe-puro or pBabe-HA-FOXO3a-A3 and equalized protein samples were analyzed for caveolin-1, $p27^{kip1}$, HA and tubulin

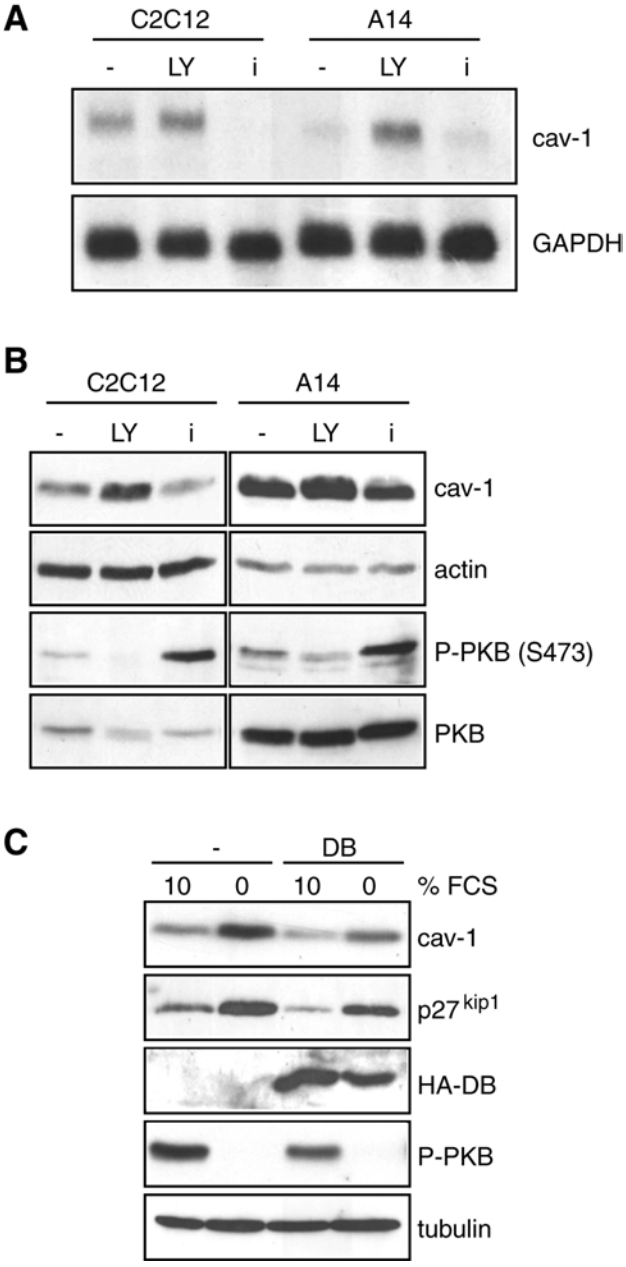


Figure 2: Endogenous FOXO activity regulates caveolin-1 expression.
(A) A14 and C2C12 cells were treated overnight with LY or insulin in serum-free medium. RNA samples were collected and analyzed using caveolin-1 and GAPDH-specific probes. (B) A14 and C2C12 cells were treated overnight with LY or insulin in serum-free medium. Equalized protein samples were immunoblotted for the presence of caveolin-1 and actin (upper two panels). As control for the regulation of the insulin pathway immunoblotting was performed for phosphorylated PKB (Ser473) and total PKB (lower two panels). (C) WT MEFs were retrovirally infected with pBabe-puro or pBabe-DB. Cells were subsequently left untreated or serum-starved for 20 hours. Protein samples were collected, equalized for protein content and analyzed using antibodies against caveolin-1 and the indicated proteins.

Thus, we conclude that ligand-independent FOXO activation results in an increase in caveolin-1 RNA and protein expression.

Regulation of caveolin-1 expression by cellular stimulation.

As our results show that overexpression of FOXO can regulate caveolin-1 expression we next examined whether caveolin-1 expression could also be regulated by insulin/PI3-kinase/PKB/FOXO signaling. To this end we made use of C2C12 mouse myoblasts and A14 cells, NIH3T3 cells that are stably expressing the human insulin receptor (9). In both cell lines insulin treatment induces PKB activation as can be observed by western blotting using an antibody against phosphorylated PKB (figure 2B). In both cell lines the RNA and protein levels of caveolin-1 were upregulated by overnight serum deprivation (data not shown) or treatment with LY (figure 2A and 2B). On the other hand, insulin treatment resulted in a decrease in caveolin-1 expression (figure 2A and 2B). This indicates that caveolin-1 expression is regulated by insulin signaling in a PI3-kinase-dependent manner. To further demonstrate the involvement of FOXO in cellular regulation of caveolin-1 expression we virally infected MEFs to express the isolated DNA binding domain of FOXO4, which acts as a dominant-negative FOXO (33). Cells were then cultured in the presence of fetal calf serum or serum-deprived for 24 hours. As can be seen in figure 2C dominant-negative FOXO could inhibit both the serum-containing and serum-deprived conditions. However, quantification of three independent experiments using ImageQuant showed that in the presence of the dominant-negative FOXO the induction of caveolin-1 levels by serum-deprivation was reduced from 2.8+/-0.4 to 1.5+/-0.2-fold. This

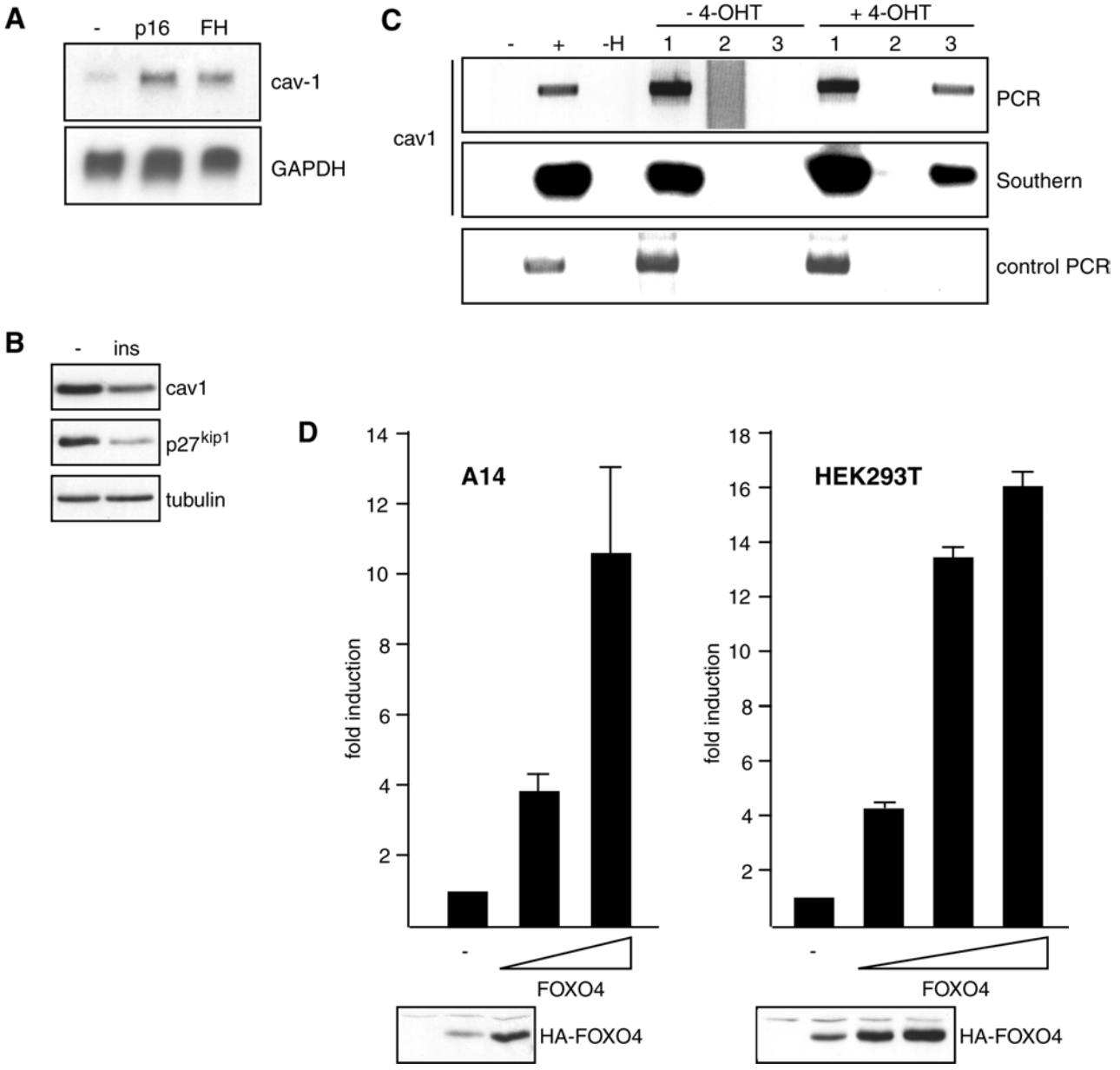
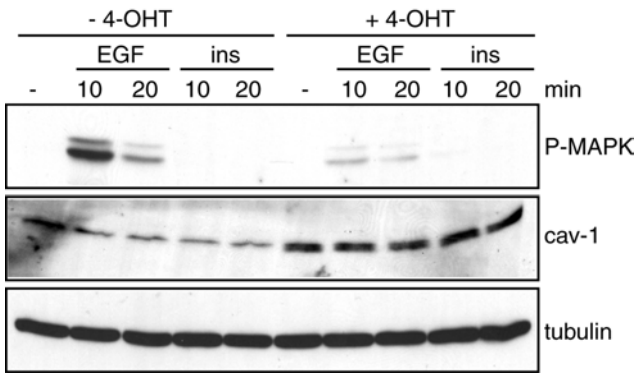


Figure 3: FOXO directly regulates caveolin-1 expression.
(A) WT MEFs were retrovirally infected with pBabe-puro, pBabe-p16 or pBabe-HA-FOXO3a-A3. RNA samples were collected and analyzed by northern blotting for caveolin-1 and GAPDH. (B) TKO mefs were serum-deprived for 24 hours in the absence or presence of insulin. Whole cell lysates were collected, equalized for protein content and analyzed for the presence of caveolin-1, $p27^{kip1}$ and tubulin. (C) Chromatin immunoprecipitations were performed using DL23 cells treated with or without 4-OHT for 20 hours. Negative and positive controls were milliQ and genomic DNA from DL23 cells respectively. –H: anti-HA IP performed on nuclear lysis buffer/dilution buffer; 1: input; 2: anti-PKB IP; 3: anti-HA IP. (D) A14 and HEK293T cells were transiently transfected with cav1-luciferase construct, TK renilla and increasing amounts of HA-FOXO4. Cells were lysed and luciferase activity was measured. Results were analyzed using One-way ANOVA and were highly significant (p-value < 0.001). Expression of HA-FOXO4 was analyzed using SDS-PAGE and anti-HA antibody.

reduction occurs even despite the small downregulation of PKB activity in DB-infected cells. From this we conclude that caveolin-1 expression is, at least in part, regulated by cellular signaling regulating FOXO.

Regulation of caveolin-1 expression is independent of cell cycle regulation.

It has been described that FOXO activation leads to cell cycle arrest and that this occurs via regulation of $p27^{kip1}$ and cyclin D gene transcription (33, 43). Furthermore it has been described that senescent cells have elevated levels of caveolin-1. Senescent cells are cells that are irreversibly arrested in the G0/G1 phase of the cell cycle. Viral infection of MEFs with FOXO3a-A3 but also with $p16^{ink4a}$, which is known to induce cell cycle arrest and senescence (22, 31), induce an increase in caveolin-1 mRNA levels (figure 3A). We therefore wanted to know whether caveolin-1 regulation by FOXO was via a direct mechanism or merely the result of a FOXO-induced cell cycle arrest. To study this we made use of TKO MEFs that are deficient for all three members of the Rb pocket protein family (Rb, p107 and p130). It has been described that these cells are insensitive to serum deprivation with respect to cell cycle regulation (13). We confirmed this and also found no regulation of the cell cycle by insulin in these cells (data not shown). Thus serum-deprived TKO MEFs were either left untreated or overnight stimulated with insulin. Caveolin-1 levels as well as the levels of the positive control $p27^{kip1}$ were decreased upon insulin treatment (figure 3B). From this we conclude that the insulin/PI3K pathway regulates caveolin-1 expression in a cell cycle-independent manner.



FOXO factors directly regulate expression of caveolin-1.

As the results described above clearly suggest direct regulation of caveolin-1 expression by FOXO, we next performed chromatin immunoprecipitations (ChIPs) to demonstrate that FOXO indeed can bind directly to the caveolin-1 promoter. Chromatin-bound DNA was isolated from DL23 cells that were left untreated or stimulated with 4-OHT for 20 hours. ChIPs were carried out using an anti-HA antibody to pull down HA-tagged FOXO3a-A3, or anti-PKB antibody as a negative control. Figure 3C shows that only in the cells stimulated with 4-OHT FOXO3a was able to bind the caveolin-1 promoter. This was visualized by PCR utilizing a caveolin-1 promoter fragment containing the consensus FOXO binding site and subsequent Southern blotting using a labeled caveolin-1 promoter fragment obtained by PCR. A control PCR encompassing an a-specific genomic region nearby the caveolin-1 gene displayed only positive amplification in the input lanes, indicating that the caveolin-1 promoter immunoprecipitation was specific.

Having determined that FOXO binds to the caveolin-1 promoter we wanted to know whether this binding would indeed lead to transcriptional activation of caveolin-1 gene expression. To this end the PCR product obtained, encompassing the FOXO binding element at –1814, was cloned into a luciferase vector (pGL2-cav1). A14 and HEK293T cells were transiently transfected with pGL2-cav1 together with increasing amounts of HA-tagged FOXO4. In both cell lines expression of

Figure 4: FOXO activation attenuates EGF-induced MAPK phosphorylation.
DL23 cells were treated with or without 4-OHT for 20 hours and subsequently stimulated with or without EGF for 10 or 20 minutes. Whole cell lysates were collected, equalized for protein content were then analyzed for phosphorylated MAPK, caveolin-1 and tubulin.

FOXO4 induces transcriptional activity in a dose-dependent manner (figure 3D). This shows that FOXO binding to this region can confer transcriptional regulation of caveolin-1 gene expression by FOXO. However, the luciferase reporter used contains a fragment of the caveolin-1 promoter encompassing the one perfect consensus binding element for FOXO, and it is well possible that the other three imperfect FOXO elements that are also present within the promoter may play a role in the transcriptional activation by FOXO as well.

FOXO activation attenuates EGF-induced MAPK phosphorylation.

It is known that caveolin-1 negatively affects EGF signaling by attenuating downstream signaling i.e. MAPK phosphorylation and subsequent effects. This desensitization of growth factor signaling is a hallmark of senescence but may equally apply to quiescence (36). A FOXO-mediated increase in caveolin-1 expression may therefore result in attenuation of EGF signaling. To study whether FOXO activation could indeed attenuate growth factor-induced signaling we used DL23 cells treated with or without 4-OHT. As shown before (figure 1) cells treated with 4-OHT showed elevated caveolin-1 expression, and MAPK phosphorylation upon EGF stimulation was inhibited by FOXO activation (figure 4). In keeping with previous results (36), this suggests that activation of FOXO attenuates EGF signaling through upregulating caveolin-1 expression.

Discussion

In this study we show that FOXO overexpression induces caveolin-1 expression and that regulation of caveolin-1 by growth factor signaling is FOXO-dependent. Furthermore we demonstrate that regulation of caveolin-1 gene expression by FOXO is through direct binding of FOXO to the caveolin-1 promoter region. This upregulation of caveolin-1 protein by FOXO results in the apparent downregulation of EGF-induced MAPK activity.

The insulin/PI3K/PKB pathway regulates multiple cellular functions. It has been shown that FOXO signaling and downregulation of this signaling by the insulin pathway has great effects on cellular behavior. We have previously demonstrated that FOXOs can induce cell cycle arrest via multiple transcriptional mechanisms (33, 43). Furthermore we have provided evidence that FOXO can subsequently trigger cells to become quiescent, a reversible cellular state of cell cycle arrest, by increasing p130 levels (26). Here we have shown that caveolin-1 levels are increased by FOXO factors. Groups have shown that increased caveolin-1 expression causes cell cycle arrest and senescence (20, 49). Caveolin-1 levels are high in senescent cells but the fact that the senescent phenotype can be reverted by lowering the caveolin-1 levels suggests that the cells are not in a complete senescent state since they have the capacity to enter cell cycle again (12). So we suggest that it is more likely that the FOXO-induced caveolin-1 expression is contributing to establishing quiescence rather than senescence.

It has been shown that cAMP signaling can regulate caveolin-1 levels, although it is not clear whether this involves transcriptional regulation (54). In some cell types cAMP is known to induce a cell cycle arrest whereas in other cells cAMP can induce cell cycle progression, depending on whether cAMP inhibits or activates the ERK pathway respectively (reviewed in (44)). It is not known what the effect of cAMP is on cell cycle regulation in the cells that do show regulation of caveolin- expression, but in cells that show a cAMP-induced cell cycle arrest, cAMP can inhibit phosphorylation of PKB by sequestering PDK-1 (24). This will lead to subsequent activation of FOXO factors. This appears

important for the cAMP-induced cell cycle arrest in cell types such as DL23 and MEF cell lines (Kuiperij et al, submitted). Our findings suggest a model in which the forskolin/cAMP-regulated caveolin-1 expression is through PDK1/PKB and is mediated by the activation of FOXO.

It is highly tempting to postulate a role for FOXO-induced caveolin-1 expression with respect to tumorigenesis. However, some groups demonstrate elevated caveolin-1 expression, whereas others show loss of caveolin-1 expression and suggest caveolin-1 to have tumor suppressive qualities. It is interesting to notice however that the majority of papers that show an increase in caveolin-1 expression do so by analyzing whole primary tumors, whereas the link between downregulation of caveolin-1 and tumorigenesis has been mainly shown in tumor cell lines. It could be that downregulation of caveolin-1 in these cell lines is in fact a way to prevent replicative senescence, which is thus far only a clearly established process in cultured cells.

In conclusion, we show here direct transcriptional control of caveolin-1 expression by FOXO. FOXO activation results in attenuated EGF signaling, suggested to be mediated by upregulation of caveolin-1 expression, and this may contribute to FOXO-mediated induction of cellular quiescence.

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References

1. **Bagnoli, M., A. Tomassetti, M. Figini, S. Flati, V. Dolo, S. Canevari, and S. Miotti.** 2000. Downmodulation of caveolin-1 expression in human ovarian carcinoma is directly related to alpha-folate receptor overexpression. Oncogene **19:**4754-63.
2. **Bender, F. C., M. A. Reymond, C. Bron, and A. F. Quest.** 2000. Caveolin-1 levels are down-regulated in human colon tumors, and ectopic expression of caveolin-1 in colon carcinoma cell lines reduces cell tumorigenicity. Cancer Res **60:**5870-8.
3. **Bist, A., C. J. Fielding, and P. E. Fielding.** 2000. p53 regulates caveolin gene transcription, cell cholesterol, and growth by a novel mechanism. Biochemistry **39:**1966-72.
4. **Bist, A., P. E. Fielding, and C. J. Fielding.** 1997. Two sterol regulatory element-like sequences mediate up-regulation of caveolin gene transcription in response to low density lipoprotein free cholesterol. Proc Natl Acad Sci U S A **94:**10693-8.
5. **Brunet, A., A. Bonni, M. J. Zigmond, M. Z. Lin, P. Juo, L. S. Hu, M. J. Anderson, K. C. Arden, J. Blenis, and M. E. Greenberg.** 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell **96:**857-68.
6. **Brunet, A., L. B. Sweeney, J. F. Sturgill, K. F. Chua, P. L. Greer, Y. Lin, H. Tran, S. E. Ross, R. Mostoslavsky, H. Y. Cohen, L. S. Hu, H. L. Cheng, M. P. Jedrychowski, S. P. Gygi, D. A. Sinclair, F. W. Alt, and M. E. Greenberg.** 2004. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science **303:**2011-5.
7. **Burgering, B. M., and P. J. Coffer.** 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature **376:**599-602.
8. **Burgering, B. M., and R. H. Medema.** 2003. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J Leukoc Biol **73:**689-701.
9. **Burgering, B. M., R. H. Medema, J. A. Maassen, M. L. van de Wetering, A. J. van der Eb, F. McCormick, and J. L. Bos.** 1991. Insulin stimulation of gene expression mediated by p21ras activation. Embo J **10:**1103-9.
10. **Burgermeister, E., L. Tencer, and M. Liscovitch.** 2003. Peroxisome proliferator-activated receptor-gamma upregulates caveolin-1 and caveolin-2 expression in human carcinoma cells. Oncogene **22:**3888-900.
11. **Castrillon, D. H., L. Miao, R. Kollipara, J. W. Horner, and R. A. DePinho.** 2003. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science **301:**215-8.
12. **Cho, K. A., S. J. Ryu, J. S. Park, I. S. Jang, J. S. Ahn, K. T. Kim, and S. C. Park.** 2003. Senescent phenotype can be reversed by reduction of caveolin status. J Biol Chem **278:**27789-95.
13. **Dannenberg, J. H., A. van Rossum, L. Schuijff, and H. te Riele.** 2000. Ablation of the retinoblastoma gene family deregulates G(1) control

causing immortalization and increased cell turnover under growth-restricting conditions. Genes Dev **14:**3051-64.

14. **Dijkers, P. F., R. H. Medema, J. W. Lammers, L. Koenderman, and P. J. Coffer.** 2000. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr Biol **10:**1201-4.
15. **Engelman, J. A., C. C. Wykoff, S. Yasuhara, K. S. Song, T. Okamoto, and M. P. Lisanti.** 1997. Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. J Biol Chem **272:**16374-81.
16. **Fielding, C. J., A. Bist, and P. E. Fielding.** 1999. Intracellular cholesterol transport in synchronized human skin fibroblasts. Biochemistry **38:**2506-13.
17. **Fine, S. W., M. P. Lisanti, F. Galbiati, and M. Li.** 2001. Elevated expression of caveolin-1 in adenocarcinoma of the colon. Am J Clin Pathol **115:**719-24.
18. **Fukuoka, M., H. Daitoku, M. Hatta, H. Matsuzaki, S. Umemura, and A. Fukamizu.** 2003. Negative regulation of forkhead transcription factor AFX (Foxo4) by CBP-induced acetylation. Int J Mol Med **12:**503-8.
19. **Furuyama, T., T. Nakazawa, I. Nakano, and N. Mori.** 2000. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem J **349:**629-34.
20. **Galbiati, F., D. Volonte, J. Liu, F. Capozza, P. G. Frank, L. Zhu, R. G. Pestell, and M. P. Lisanti.** 2001. Caveolin-1 expression negatively regulates cell cycle progression by inducing G(0)/G(1) arrest via a p53/p21(WAF1/Cip1)-dependent mechanism. Mol Biol Cell **12:**2229-44.
21. **Ho, C. C., P. H. Huang, H. Y. Huang, Y. H. Chen, P. C. Yang, and S. M. Hsu.** 2002. Up-regulated caveolin-1 accentuates the metastasis capability of lung adenocarcinoma by inducing filopodia formation. Am J Pathol **161:**1647-56.
22. **Kato, D., K. Miyazawa, M. Ruas, M. Starborg, I. Wada, T. Oka, T. Sakai, G. Peters, and E. Hara.** 1998. Features of replicative senescence induced by direct addition of antennapedia-p16INK4A fusion protein to human diploid fibroblasts. FEBS Lett **427:**203-8.
23. **Kato, K., Y. Hida, M. Miyamoto, H. Hashida, T. Shinohara, T. Itoh, S. Okushiba, S. Kondo, and H. Katoh.** 2002. Overexpression of caveolin-1 in esophageal squamous cell carcinoma correlates with lymph node metastasis and pathologic stage. Cancer **94:**929-33.
24. **Kim, S., K. Jee, D. Kim, H. Koh, and J. Chung.** 2001. Cyclic AMP inhibits Akt activity by blocking the membrane localization of PDK1. J Biol Chem **276:**12864-70.
25. **Kops, G. J., T. B. Dansen, P. E. Polderman, I. Saarloos, K. W. Wirtz, P. J. Coffer, T. T. Huang, J. L. Bos, R. H. Medema, and B. M. Burgering.** 2002. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature **419:**316-21.
26. **Kops, G. J., R. H. Medema, J. Glassford, M. A. Essers, P. F. Dijkers, P. J. Coffer, E. W. Lam, and B. M. Burgering.** 2002. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol Cell Biol **22:**2025-36.
27. **Lee, S. W., C. L. Reimer, P. Oh, D. B. Campbell, and J. E. Schnitzer.** 1998. Tumor cell growth inhibition by caveolin re-expression in human breast cancer cells. Oncogene **16:**1391-7.
28. **Li, L., G. Yang, S. Ebara, T. Satoh, Y. Nasu, T. L. Timme, C. Ren, J. Wang, S. A. Tahir, and T. C. Thompson.** 2001. Caveolin-1 mediates testosterone-stimulated survival/clonal growth and promotes metastatic activities in prostate cancer cells. Cancer Res **61:**4386-92.
29. **Li, S., J. Couet, and M. P. Lisanti.** 1996. Src tyrosine kinases, Galpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. J Biol Chem **271:**29182-90.
30. **Liu, J., P. Lee, F. Galbiati, R. N. Kitsis, and M. P. Lisanti.** 2001. Caveolin-1 expression sensitizes fibroblastic and epithelial cells to apoptotic stimulation. Am J Physiol Cell Physiol **280:**C823-35.
31. **McConnell, B. B., M. Starborg, S. Brookes, and G. Peters.** 1998. Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. Curr Biol **8:**351-4.
32. **Medema, R. H., R. E. Herrera, F. Lam, and R. A. Weinberg.** 1995. Growth suppression by p16ink4 requires functional retinoblastoma protein. Proc Natl Acad Sci U S A **92:**6289-93.
33. **Medema, R. H., G. J. Kops, J. L. Bos, and B. M. Burgering.** 2000. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature **404:**782-7.
34. **Park, D. S., H. Lee, C. Riedel, J. Hulit, P. E. Scherer, R. G. Pestell, and M. P. Lisanti.** 2001. Prolactin negatively regulates caveolin-1 gene expression in the mammary gland during lactation, via a Ras-dependent mechanism. J Biol Chem **276:**48389-97.
35. **Park, D. S., B. Razani, A. Lasorella, N. Schreiber-Agus, R. G. Pestell, A. Iavarone, and**

M. P. Lisanti. 2001. Evidence that Myc isoforms transcriptionally repress caveolin-1 gene expression via an INR-dependent mechanism. Biochemistry **40:**3354-62.

36. **Park, W. Y., J. S. Park, K. A. Cho, D. I. Kim, Y. G. Ko, J. S. Seo, and S. C. Park.** 2000. Up-regulation of caveolin attenuates epidermal growth factor signaling in senescent cells. J Biol Chem **275:**20847-52.
37. **Puig, O., M. T. Marr, M. L. Ruhf, and R. Tjian.** 2003. Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes Dev **17:**2006-20.
38. **Racine, C., M. Belanger, H. Hirabayashi, M. Boucher, J. Chakir, and J. Couet.** 1999. Reduction of caveolin 1 gene expression in lung carcinoma cell lines. Biochem Biophys Res Commun **255:**580-6.
39. **Razani, B., Y. Altschuler, L. Zhu, R. G. Pestell, K. E. Mostov, and M. P. Lisanti.** 2000. Caveolin-1 expression is down-regulated in cells transformed by the human papilloma virus in a p53-dependent manner. Replacement of caveolin-1 expression suppresses HPV-mediated cell transformation. Biochemistry **39:**13916-24.
40. **Razani, B., J. A. Engelman, X. B. Wang, W. Schubert, X. L. Zhang, C. B. Marks, F. Macaluso, R. G. Russell, M. Li, R. G. Pestell, D. Di Vizio, H. Hou, Jr., B. Kneitz, G. Lagaud, G. J. Christ, W. Edelmann, and M. P. Lisanti.** 2001. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. J Biol Chem **276:**38121-38.
41. **Razani, B., and M. P. Lisanti.** 2001. Two distinct caveolin-1 domains mediate the functional interaction of caveolin-1 with protein kinase A. Am J Physiol Cell Physiol **281:**C1241-50.
42. **Scherer, P. E., M. P. Lisanti, G. Baldini, M. Sargiacomo, C. C. Mastick, and H. F. Lodish.** 1994. Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. J Cell Biol **127:**1233-43.
43. **Schmidt, M., S. Fernandez de Mattos, A. van der Horst, R. Klompmaker, G. J. Kops, E. W. Lam, B. M. Burgering, and R. H. Medema.** 2002. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol Cell Biol **22:**7842-52.
44. **Stork, P. J., and J. M. Schmitt.** 2002. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol **12:**258-66.
45. **Suzuoki, M., M. Miyamoto, K. Kato, K. Hiraoka, T. Oshikiri, Y. Nakakubo, A. Fukunaga, T. Shichinohe, T. Shinohara, T. Itoh, S. Kondo, and H. Katoh.** 2002. Impact of caveolin-1 expression on prognosis of pancreatic ductal adenocarcinoma. Br J Cancer **87:**1140-4.
46. **Tang, T. T., D. Dowbenko, A. Jackson, L. Toney, D. A. Lewin, A. L. Dent, and L. A. Lasky.** 2002. The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. J Biol Chem **277:**14255-65.
47. **Tran, H., A. Brunet, J. M. Grenier, S. R. Datta, A. J. Fornace, Jr., P. S. DiStefano, L. W. Chiang, and M. E. Greenberg.** 2002. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science **296:**530-4.
48. **Van Der Horst, A., L. G. Tertoolen, L. M. De Vries-Smits, R. A. Frye, R. H. Medema, and B. M. Burgering.** 2004. FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2SIRT1. J Biol Chem **4:**4.
49. **Volonte, D., K. Zhang, M. P. Lisanti, and F. Galbiati.** 2002. Expression of caveolin-1 induces premature cellular senescence in primary cultures of murine fibroblasts. Mol Biol Cell **13:**2502-17.
50. **Wiechen, K., L. Diatchenko, A. Agoulnik, K. M. Scharff, H. Schober, K. Arlt, B. Zhumabayeva, P. D. Siebert, M. Dietel, R. Schafer, and C. Sers.** 2001. Caveolin-1 is down-regulated in human ovarian carcinoma and acts as a candidate tumor suppressor gene. Am J Pathol **159:**1635-43.
51. **Wiechen, K., C. Sers, A. Agoulnik, K. Arlt, M. Dietel, P. M. Schlag, and U. Schneider.** 2001. Down-regulation of caveolin-1, a candidate tumor suppressor gene, in sarcomas. Am J Pathol **158:**833-9.
52. **Williams, T. M., M. W. Cheung, D. S. Park, B. Razani, A. W. Cohen, W. J. Muller, D. Di Vizio, N. G. Chopra, R. G. Pestell, and M. P. Lisanti.** 2003. Loss of caveolin-1 gene expression accelerates the development of dysplastic mammary lesions in tumor-prone transgenic mice. Mol Biol Cell **14:**1027-42.
53. **Wu, D., and D. M. Terrian.** 2002. Regulation of caveolin-1 expression and secretion by a protein kinase cepsilon signaling pathway in human prostate cancer cells. J Biol Chem **277:**40449-55.
54. **Yamamoto, M., S. Okumura, N. Oka, C. Schwencke, and Y. Ishikawa.** 1999. Downregulation of caveolin expression by cAMP signal. Life Sci **64:**1349-57.

Chapter

6

Concluding remarks

The response of cells to extracellular stimuli can lead to a variety of cellular responses including proliferation and cell survival. One of the major pathways translating such an extracellular signal to a cellular effect is the PI3K/PKB pathway that leads to a subsequent inhibition of FOXO transcription factors. In this thesis, different aspects of this signaling pathway have been described.

The Ser/Thr kinase PKB is a critical mediator of PI3K signaling, and its activity is regulated on different levels including phosphorylation, localization, and binding to other proteins (see Chapter 1). To obtain a better understanding how PKB exerts its function, an Y2H screen was performed utilizing the PH domain of PKB. Periplakin was found to interact with PKB through its carboxy-terminus ((12) and Chapter 2). PKB is ubiquitously expressed, whereas periplakin is thought to be mainly expressed in epidermis or epithelial cell lines. Thus, the regulation that periplakin can exert on PKB activity can only occur in epithelial cells. Recently however, a tissue-wide survey for periplakin expression revealed that periplakin has a wider tissue distribution (7), resembling more the expression pattern of more widely expressed plectin than that of envoplakin. The authors also found that periplakin binds to keratin-8 and vimentin, which confirms our findings and those of others that periplakin interacts with vimentin. In addition, the regions of periplakin important for vimentin binding have been confirmed by others (1). Besides binding to the intermediate filament network via periplakin, PKB can also interact with the actin cytoskeleton (4). This suggests that PKB localization to cellular compartments is necessary for correct PKB function.

Besides cytoskeletal localization, periplakin can also be localized within the nuclear compartment ((12) and Chapter 2). It contains a putative bipartite nuclear localization signal (NLS), which is located within the carboxy-terminal part of the central rod domain. All plakin family members, including periplakin, plectin and Bpag1/BP180, contain such a central rod domain. Plectin contains a putative NLS in a similar region as for the periplakin and it can be localized to nuclear lamina (13). In addition, Bpag1 can localize to the nucleus (14). The authors describe that this might occur via a putative NLS in the plakin domain. With respect to plectin and periplakin, the presence of putative NLS sequences in the carboxy-terminal part makes it more likely that this putative NLS would indeed be a valid one. Since it is not known how PKB translocates to the nucleus upon activation, it is tempting to speculate that there is role for periplakin as PKB binding protein in this process. Further research might shed more light on this potential mechanism.

Periplakin has also been described to interact with other proteins. Most interactions occur through the carboxy-terminal part of the protein. An example of such an interaction is that with the μ-opioid receptor (5). Binding of periplakin to this receptor disrupts $G_i\alpha$ protein activation. More recently, a role for periplakin in haematopoietic cells has been discovered. The carboxy-terminus of periplakin can also interact with FcγRI and this negatively modulates ligand binding to the receptor (personal communications and (2, 3)). It should be noted however that the carboxy-terminal domain of periplakin is used in most cases as an epitope for antibodies that can recognize endogenous periplakin (1). Interactions of periplakin with other proteins might therefore block proper antibody recognition. Also, antibody recognition might be in part be blocked by hiding of the carboxy-terminal tail by crosslinking (1). The use of different antibodies might therefore be responsible for the differences found in localization and presence of periplakin in different tissues and cells.

PKB can regulate FOXO activity and through this it has been implicated in the regulation of aging. Besides these cell autonomous processes aging can also be controlled by paracrine signaling. Growth hormone production and secretion from the pituitary has been shown to affect the longevity of mammals. Transcription factors responsible for growth hormone production are Pit-1, which transcriptionally activates the growth hormone gene, and Prop-1, which is responsible for transcription from the Pit-1 gene. Both factors contain consensus PKB motifs that are conserved between human and mouse but also between higher and lower eukaryotes. The conserved PKB motifs and the function of Pit-1 and Prop-1 led to the investigation of these proteins as putative PKB substrates. PKB could phosphorylate Pit-1 and Prop-1 *in vitro* but no *in vivo* effects were observed. Therefore, we concluded that these proteins are not valid PKB substrates. From the data obtained, it can be concluded that *in vitro* phosphorylation does not always translate into physiologically occurring events, since *in vitro* phosphorylation might occur in a less specific fashion than *in vivo*. Another explanation could be that phosphorylation of certain substrates by PKB only can occur in the presence of essential co-factors that in some cases can be cell type-specific. Nevertheless it implies that some of the described PKB substrates may be questioned to a certain extent since only *in vitro* phosphorylation by PKB is shown.

FOXO is the main mediator from upstream PI3K/PKB signaling towards transcriptional regulation and subsequent cellular processes. In some cell types FOXO regulates several target genes that are involved in regulating apoptosis. In other cell types FOXO regulates the cell cycle with respect to the decision to proliferate or arrest in cell cycle. Cells that enter cell cycle arrest can subsequently be driven into a G0 phase from where the decisions can be made to differentiate, become senescent, or re-enter the cell cycle. This state is also referred to as quiescence, and a marker for this state is p130, a target gene of FOXO signaling. We found a novel gene to be regulated by FOXO, caveolin-1, and its upregulation leads to senescence. However, this senescent phenotype has been described to be reversible, suggesting that cells are rather quiescent than senescent. By regulating caveolin-1 and p130 expression, FOXO factors are able to keep cells in a quiescent state. The fact that caveolin-1 was selected by *in silico* analysis, and was validated as being a FOXO target gene, is a proof of principle that *in silico* analysis can be a useful tool in the discovery of novel players in signaling pathways.

Besides inhibiting MAPK phoshorylation, induced by epidermal growth factor (EGF) but also by arsenite, Neu differentiation factor (NDF) or binding to laminin (6, 10, 11), high caveolin-1 expression levels have also been reported to inhibit protein phosphatase 2A (PP2A) signaling, through direct binding of caveolin-1 to PP2A (8). When active, PP2A is able to dephosphorylate PKB and thus inhibit PKB activity. This inhibition of PP2A by caveolin-1 might also be the cause of the caveolin-induced high activity status of PKB (9, 11). This would provide a mechanism by which FOXO factors induce a negative feedback loop regulating their activity. Besides transcriptionally increasing insulin receptor expression, FOXOs would be able to inhibit PP2A, the negative regulator of PKB activity. However, it has been shown that caveolin-1 can bind to receptor tyrosine kinases upon conditions of pro-apoptotic stress such as ceramide (15). Binding of caveolin-1 to for instance the platelet-derived growth factor (PDGF) receptor or insulin receptor causes an inhibition of the downstream signal towards PI3K (15). Inhibition of PI3K activity results in a sensitization of cells to ceramide-induced cell death. The apparent discrepancy of caveolin-1

induced increase in PKB activity and lower PI3K signaling suggests that it would occur through PI3K-independent PKB activation. Through inhibition of PP2A, caveolin-1 can compensate for the inhibition of PI3K with respect to downstream signaling to PKB. It is shown that caveolin-induced increase in PKB activity can lead to a sensitization towards pro-apoptotic stimuli such as tumor necrosis factor α (TNFα) or arsenite resulting in cell death (9, 11). This is in contrast with the generally accepted anti-apoptotic properties of PKB. A clear explanation is at present not available, although it is plausible that a combination of high PKB activity with other altered signaling pathways might cause this phenomenon.

In conclusion, the findings described in this thesis provide a better insight in PKB – FOXO signaling and interesting directions for the future. Identification of novel PKB substrates might lead to place PKB in other signaling pathways and cellular processes, and thereby possibly provide additional mechanisms by which PKB plays a role in tumorigenesis and aging. Another interesting direction would be pursuing the roles of co-factors for FOXO transcription factors, since the picture emerges that these are very important in FOXO-mediated phenomena. For instance, co-factors could explain the specificity for each of the FOXOs by binding to one FOXO but not the other. Furthermore differences in the final outcome of cellular processes, apoptosis versus cell cycle arrest, might be caused by co-factors.

References

1. **Aho, S.** 2004. Many faces of periplakin: domain-specific antibodies detect the protein throughout the epidermis, explaining the multiple protein-protein interactions. Cell Tissue Res **316:**87-97.
2. **Beekman, J. M., J. E. Bakema, J. G. Van De Winkel, and J. H. Leusen.** 2004. Direct interaction between Fc{gamma}RI (CD64) and periplakin controls receptor endocytosis and ligand binding capacity. Proc Natl Acad Sci U S A **30:**30.
3. **Beekman, J. M., J. E. Bakema, J. Van Der Linden, B. Tops, M. Hinten, M. J. Van Vugt, J. G. Van De Winkel, and J. H. Leusen.** 2004. Modulation of Fcgamma RI (CD64) ligand binding by blocking peptides of periplakin. J Biol Chem **25:**25.
4. **Cenni, V., A. Sirri, M. Riccio, G. Lattanzi, S. Santi, A. de Pol, N. M. Maraldi, and S. Marmiroli.** 2003. Targeting of the Akt/PKB kinase to the actin skeleton. Cell Mol Life Sci **60:**2710-20.
5. **Feng, G. J., E. Kellett, C. A. Scorer, J. Wilde, J. H. White, and G. Milligan.** 2003. Selective interactions between helix VIII of the human mu-opioid receptors and the C terminus of periplakin disrupt G protein activation. J Biol Chem **278:**33400-7.
6. **Fiucci, G., D. Ravid, R. Reich, and M. Liscovitch.** 2002. Caveolin-1 inhibits anchorage-independent growth, anoikis and invasiveness in MCF-7 human breast cancer cells. Oncogene **21:**2365-75.
7. **Kazerounian, S., J. Uitto, and S. Aho.** 2002. Unique role for the periplakin tail in intermediate filament association: specific binding to keratin 8 and vimentin. Exp Dermatol **11:**428-38.
8. **Li, L., C. H. Ren, S. A. Tahir, C. Ren, and T. C. Thompson.** 2003. Caveolin-1 maintains activated Akt in prostate cancer cells through scaffolding domain binding site interactions with and inhibition of serine/threonine protein phosphatases PP1 and PP2A. Mol Cell Biol **23:**9389-404.
9. **Ono, K., Y. Iwanaga, M. Hirayama, T. Kawamura, N. Sowa, and K. Hasegawa.** 2004. Contribution of caveolin-1 alpha and Akt to TNF-alpha-induced cell death. Am J Physiol Lung Cell Mol Physiol **287:**L201-9.
10. **Park, W. Y., K. A. Cho, J. S. Park, D. I. Kim, and S. C. Park.** 2001. Attenuation of EGF signaling in senescent cells by caveolin. Ann N Y Acad Sci **928:**79-84.
11. **Shack, S., X. T. Wang, G. C. Kokkonen, M. Gorospe, D. L. Longo, and N. J. Holbrook.** 2003. Caveolin-induced activation of the phosphatidylinositol 3-kinase/Akt pathway increases arsenite cytotoxicity. Mol Cell Biol **23:**2407-14.
12. **van den Heuvel, A. P., A. M. de Vries-Smits, P. C. van Weeren, P. F. Dijkers, K. M. de Bruyn, J. A. Riedl, and B. M. Burgering.** 2002. Binding of protein kinase B to the plakin family member periplakin. J Cell Sci **115:**3957-66.
13. **Wiche, G.** 1998. Role of plectin in cytoskeleton organization and dynamics. J Cell Sci **111:**2477-86.
14. **Young, K. G., M. Pool, and R. Kothary.** 2003. Bpag1 localization to actin filaments and to the nucleus is regulated by its N-terminus. J Cell Sci **116:**4543-55.

15. **Zundel, W., L. M. Swiersz, and A. Giaccia.** 2000. Caveolin 1-mediated regulation of receptor tyrosine kinase-associated phosphatidylinositol 3-kinase activity by ceramide. Mol Cell Biol **20:**1507-14.

Summary

Cellular processes such as proliferation, apoptosis and differentiation are the basis for normal cellular behavior and are regulated by a number of extracellular stimuli such as hormones, cytokines and growth factors. Via binding to (transmembrane) receptors these factors lead to regulation of intracellular signaling pathways ultimately resulting in gene expression. When cells malfunction in one or more processes or when the balance between different processes is disturbed this can lead to abnormal cell behavior and pathological phenotypes such as cancer, obesity, diabetes and premature aging. This malfunctioning can arise when the activity of proteins involved in the pathways is changed due to altered expression, or mutations. The various extracellular factors can activate different intracellular pathways that in turn can converge or diverge between one another. One of the major kinases involved in insulin and other growth factor signaling is phosphoinositide-3-kinase (PI3K). This lipid kinase can recruit protein kinase B (PKB) to the plasma membrane where it is phosphorylated by other kinases. Upon activation PKB in turn can phosphorylate a variety of proteins in a so-called PKB motif (R-X-R-X-X-S/T), thereby regulating their activity. These PKB substrates act on cellular process such as proliferation, cell survival, protein translation but also on for instance transcription. Transcription factors that are described to be phosphorylated by PKB are members of the Forkhead box O (FOXO). Upon phosphorylation these factors are excluded from the nucleus and subsequently inactivated.

In **Chapter 2** the identification of a novel interaction partner for PKB is described. In a binding screen the plakin family member periplakin was found to interact with PKB. The interacting regions have been narrowed down to nine amino acids within the PH domain of PKB, and the carboxyterminus of periplakin. Periplakin is present in distinct cellular compartments such as nucleus, plasma membrane, mitochondria and the intermediate filament network through direct binding to vimentin. Binding of PKB to periplakin does not affect PKB activation but can inhibit PKB-mediated phosphorylation and subsequent inactivation of FOXO transcription factors.

Chapter 3 describes an *in silico* approach to identify novel substrates for PKB and target genes for FOXO factors. To identify downstream targets of PKB, proteins containing the consensus PKB motif (RXRXXS/T) were selected. This selection was narrowed down on the basis of their known function, and evolutionary conservation of the motifs was assessed. Two potential PKB substrates selected for further analysis are Pit-1 and Prop-1. To identify novel FOXO target genes, microarray analysis was performed using a cell line containing an inducible active FOXO3a. Results from this array were compared with other relevant microarrays and putative target genes were checked for the presence of FOXO binding elements in the promoter region.

In **Chapter 4**, the analysis of Pit-1 and Prop-1 as putative PKB substrates is described. Pit-1 and Prop-1 are pituirary-specific homeodomain transcription factors involved in the production of growth hormone and other pituitary-specific hormones. Both proteins contain PKB consensus motifs and are indeed phosphorylated *in vitro* by PKB. *In vivo* phosphorylation however is not observed neither are any changes in localization or transcriptional activity.

Chapter 5 deals with the validation of a novel FOXO target gene encoding for caveolin-1. Caveolin-1 expression is induced upon FOXO activation and downregulated by insulin-dependent PI3K/PKB signaling on both mRNA and protein levels. Regulation of caveolin-1 transcription occurs by direct binding of FOXO to binding elements in the caveolin-1 promoter region. The regulation of caveolin-1 expression results in functional attenuation of EGF signaling.

Samenvatting

Het menselijk lichaam bestaat uit miljarden cellen. Cellulaire processen zoals celdeling, celdood en differentiatie zijn de basis voor de normale gang van zaken in cellen en daarmee het menselijk lichaam. Deze processen worden geregeld door stimuli van buitenaf zoals hormonen en groeifactoren. Door binding aan receptoren kunnen deze factoren signaleringsroutes binnen de cel reguleren. Als er een fout in zo'n signaleringsroute zit, kan deze route zich abnormaal gaan gedragen wat kan leiden tot ziektes als kanker, obesitas en diabetes. Een van de belangrijkste eiwitten in de signaleringsroute van insuline is het zogenaamde Protein Kinase B (PKB). PKB is een eiwit dat, wanneer actief, andere eiwitten kan reguleren m.b.t. hun activiteit. PKB doet dat door die eiwitten (substraten) te fosforyleren: er een fosfaatgroep aan te zetten. Dit gebeurt binnen een zogenaamd PKB motief. Deze PKB substraten hebben belangrijke rollen in de cellulaire processen die gereguleerd worden door insuline en PKB, zoals celgroei, celdood en differentiatie. Een aantal PKB substraten behoren tot de genfamilie van Forkhead transcriptiefactoren (FOXO) wat inhoudt dat ze leiden tot het aflezen van DNA wat leidt tot aanmaak van uiteindelijk eiwitten.

In **hoofdstuk 2** is de identificatie van een nieuwe bindingspartner van PKB beschreven, namelijk periplakin. De delen van zowel PKB als periplakin die betrokken zijn bij deze binding zijn bepaald. Periplakin is aanwezig in specifieke compartimenten van de cel waar het onder andere betrokken is bij het verbinden van celnetwerken. Binding van PKB aan periplakin heeft geen effect op de activering van PKB maar remt wel PKB-gemedieerde processen zoals fosforylatie van FOXO factoren door PKB, wat leidt een relatief hoge activiteit van de FOXO factoren.

In **hoofdstuk 3** een manier wordt beschreven om nieuwe PKB substraten te identificeren alsmede nieuwe genen die gereguleerd worden door FOXO transcriptiefactoren. Voor de identificatie van nieuwe PKB substraten een lijst werd gegenereerd van eiwitten die het zogenaamde PKB motief bevatten. Van deze grote lijst een eiwitten werden geselecteerd die een belangrijke functie hadden (gentranscriptie) en vervolgens werd bepaald of de aanwezige PKB motieven geconserveerd waren in evolutie (dus ook in lagere diersoorten).

In **hoofdstuk 4** werden twee mogelijke PKB substraten onderzocht die voortkwamen uit de in hoofdstuk 3 gegenereerde lijst. Het gaat om twee eiwitten die alleen voorkomen in de hypofyse, en die daar essentieel zijn voor de aanmaak van groeihormoon. Beide eiwitten worden door PKB gefosforyleerd wanneer de proef gedaan wordt in een reageerbuis, maar in cellen kon geen fosforylatie van de eiwitten door PKB worden aangetoond. PKB activering in cellen leidde verder ook niet tot een verandering van lokalisatie of activiteit van beide eiwitten.

Hoofdstuk 5 gaat over de validatie van caveoline-1 als zijnde een FOXO target gen. Dit houdt in dat het gen coderend voor caveoline-1 transcriptioneel wordt gereguleerd door FOXO en daarmede ook door insuline en PKB. Insuline en PKB activiteit leiden tot een verlaging van caveoline-1 in de cel door remming van FOXO. Remming van insuline leidt tot FOXO activering en daarmee tot een verhoging van caveoline-1. Caveoline-1 is het hoofdbestanddeel van zgn. caveolae, microdomeinen in het celmembraan. Door een verhoging van caveoline-1 wordt signalering van groeifactoren zoals EGF, PDGF maar ook insuline gemoduleerd.

Curriculum vitae

Pieter van den Heuvel werd geboren op 5 februari 1975 te Nijmegen. In 1993 behaalde hij zijn VWO diploma aan het Gymnasium Bernrode te Bernheze. In datzelfde jaar begon hij aan de studie Biologie aan de Katholieke Universiteit Nijmegen, waarbij het propedeutisch examen werd afgelegd in 1995. Vervolgens werd onderzoekservaring opgedaan bij de afdeling Celbiologie aan de Katholieke Universiteit Nijmegen onder begeleiding van Dr. K. van Roozendaal en Prof. Dr. E.J.J. van Zoelen, en bij de Developmental Biology Section van de Craniofacial and Developmental Biology and Regeneration Branch aan het National Institute of Dental and Craniofacial Research, National Institutes of Health in Bethesda, USA onder begeleiding van Dr. E. Danen en Prof. Dr. K.M. Yamada. Het doctoraal-diploma werd behaald in oktober 1999. Het promotie-onderzoek, zoals beschreven in dit proefschrift, is gedaan in de periode van oktober 1999 tot juli 2004 onder begeleiding van Prof. Dr. Ir. B.M.T. Burgering bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum Utrecht.

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uhuh, vijf minuutjes ja. Succes met JOUW lab. Hola Margarita, veel success/plezier met de aankomende gezinsuitbreiding. Sanne, komt dat verslag van Boston nog? Succes met Naaatch, o nee, Jip. Miranda, m'n een-dag-in-de-week kamergenote. Blij dat je weer terug bent. Ingrid, succes in Amsterdam, Leiden of toch Bilthoven? Wendy, gewoon de wormen niets meer geven en kijken wat er dan gebeurt. Fried, de snelst pratende onderzoeker die ik ken. Dank je voor je wijze woorden en succes met de wormen! Jun, please keep that happy smile behind the microscope and in the hallway! And thank you for your laptop. Dames van het secretariaat: Saskia, Felicia, Marianne, Marit. Heerlijk bijpraten over van alles en nog wat. Bedankt voor de nodige ondersteuning. Piet, voor ons allebei geen onderwijstaken meer. Heerlijk. Fons, dank voor altijd genoeg orthofosfaat voor de in vivo's. Annelies, succes met de Cancer Genomics. Wim, ik verbaas me er over dat ik op dit punt ben zonder jou in de laatste fase. Hartstikke bedankt voor alle hulp eerder bij vele computerfrustraties.

Natuurlijk mogen ook sommige oud-collega's niet vergeten worden. Geert: Mr. FOXO, kom jij terug, ga ik die kant op. Biertje in december dan maar? Johan, bedankt voor het niet waarschuwen van Fiona. Toch nu maar mooi journal of CELL science. Nancy, mNNNN, Nen, waar komt die beach tent nu te staan? En zullen we ook sushi en kersenpitten serveren dan? Na jullie was de lat iets hoger komen te liggen. Bedankt voor het geven van het goede voorbeeld.

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Paul C, the English patient man. Een beter commissielid heb ik me niet kunnen wensen, althans met het nakijken. "A variety of" comments hebben geleid tot een ongetwijfeld beter begrijpbaar boekje.

Dear Arthur, thanks for your hospitality during my stay in Denver. Too bad that our results were not that what we hoped for but who knows, maybe in the future some day.

Vrienden uit het Nijmeegse, jasssseker! Bedankt voor jullie vriendschap! Ik hoop dat er nog vele avondjes uit, goede gesprekken, kerstdineetjes, vakanties en weekendjes mogen volgen!?! Serge en Miranda in het bijzonder, heel fijn dat jullie mijn paranimfen willen zijn.

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Carolina, mi schatje y pechocha. This last part I can write in Dutch, English or Spanish now (more or less) and you will understand it all. Thank you for coming into my life, and for being here with me, in the Netherlands. Your love and your great ability to make me smile were a great support for me. And thanks for sticking up with the every now and then stressy times. A future with you, wherever that may be, is such a great thing to look forward to.