Cell-Cycle Control by Protein Kinase B

Celcyclus controle door proteïne kinase B

(met een samenvatting in het Nederlands)

Proefschrift

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Abbreviations

4OHT	4-hydroxy-tamoxifen
ALL	acute lymphoid leukemia
CAT	chloramphenicol acetyl transferase
Cdk	cell-cycle-dependent kinase
CEF	chicken embryo fibroblast
Cu/ZnSOD	cupper/zinc superoxide dismutase
DAF	dauer formation
DAPI	4',6-diamidino-2-phenylindole
DBE	DAF-16-binding element
EGF	epidermal growth factor
ER	estrogen receptor
FasL	Fas ligand
FKHR	Forkhead in rhabdomyosarcoma
FKHR-L1	FKHR-like 1
FYVE	Fab1p/YOTB/Vac1p/EEA1
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanosine triphosphate
GSK3	glycogen synthase kinase 3
IGF-I	insulin-like growth factor I
IGFBP-1	insulin-like growth factor binding protein 1
InsR	insulin receptor
IRE	insulin response element
LMB	leptomycin B
MEF	mouse embryo fibroblast
MLL	mixed-lineage leukemia
MnSOD	manganese superoxide dismutase
mSOS	mammalian Son of Sevenless
NES	nuclear export signal
NLS	nuclear localization signal
PDGF	platelet-derived growth factor
PDK1	$PI(3,4,5)P_3$-dependent kinase 1
PH	pleckstrin homology
PI(3)K	phosphatidylinositol-3OH-kinase
PI(3)P	phosphatidylinositol (3) phosphate
$PI(3,4)P_2$	phosphatidylinositol (3,4) diphosphate
$PI(3,4,5)P_3$	phosphatidylinositol (3,4,5) triphosphate
PKB	protein kinase B
PKC	protein kinase C
PLCγ	phospholipase C-γ
pRb	retinoblastoma tumor suppressor protein
PTEN	phosphatase and tensin homologue on chromosome 10
RalGDS	Ral guanine nucleotide dissociation stimulator
RBD	Ras-binding domain
Rlf	RalGDS-like factor
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SGK	serum- and glucocorticoid-inducible kinase
SH2	src homolgy 2

Welcome to this world of Fools
Of pink champagne and swimming pools
Well, all you have to lose is your virginity
Perhaps we'll have some fun tonight
So stick around and take a bite of life
We don't need feebleness in this proximity

-Les Claypool-

CHAPTER 1

General Introduction

The emergence of a cancer cell

Before a normal somatic cell has evolved into a malignant cancer cell it has acquired several traits that contribute to uncontrolled proliferation. For instance, the programmed cell death (apoptotic) machinery is inactivated. An important component of this machinery is the p53 tumor suppressor which can induce apoptosis in a cell containing severely damaged DNA, thereby preventing a cell that harbours potential oncogenic mutations from remaining in a tissue. Consequently, p53 has been found mutated to an inactive form in approximately fifty percent of all cancers (reviewed in (1)). In addition, some cancer cells contain constitutively activated signal transduction pathways that transduce anti-apoptotic signals. The phosphatidylinositol-3-OH kinase (PI(3)K) pathway, for instance, is normally controlled by growth factors and can antagonize apoptosis (2). Hence, mutations or deletions in the gene encoding a negative regulator of this pathway, phosphatase and tensin homologue deleted from chromosome 10 (PTEN, also known as MMAC1 or TEP1), are found in a variety of cancers (reviewed in (3)).

Many cancer cells have become insensitive to anti-growth signals. Certain extracellular ligands like transforming growth factor β (TGFβ) inhibit cellular proliferation by increasing protein expression of cell-cycle inhibitors such as $p15^{INK4B}$. One way in which cancer cells evade this inhibitory signal is by deleting the $p15^{INK4B}$ locus (reviewed in (4, 5)).

A cancer cell has acquired the potential for autonomous proliferation. During normal cell proliferation extracellular growth factors provide the signal required for the initiation of DNA synthesis via a cascades of protein modifications that, amongst others, ultimately inactivate the retinoblastoma protein (pRb). One of the few well described pRb-controlling pathways activated by growth factors is the one controlled by the Ras GTPase (6). In about fifteen percent of all cancers, Ras is mutated to an active form, giving rise to growth factor-independent proliferation (reviewed in (7)). Besides Ras-directed signalling, other cascades, including the previously mentioned PI(3)K cascade, control cellular proliferation and certain proteins in these cascades (e.g. PTEN) are sometimes found mutated. Last but not least, some tumor cells produce their own growth factors (8).

A cancer cell has acquired the ability to undergo more cell doublings than its non-transformed counterpart. In normal circumstances, human cells have limited replicative capacity due to the shortening of chromosome ends after each cell division. When these chromosome ends or telomeres reach a specific minimal length, the cell activates its apoptotic program, presumably via p53 (9, 10). One of the ways in which the length of the telomeres can be maintained is via the telomerase enzyme that adds hexanucleotide repeats to the telomeric DNA. In general, the amount of telomerase activity present in a cell correlates with its capacity to proliferate (11). Stem cells, for instance, have high levels of telomerase activity and in principle have the ability to divide indefinitely. Also, when lymphocytes are presented with antigens a rapid proliferative response is induced and the amount of telomerase protein is increased (12). Importantly, cancer cells have upregulated the expression of telomerase ensuring that the telomeres never reach their minimal length (reviewed in (13)).

In addition to all previously mentioned traits, a malignant cancer cell has acquired the capacity to metastasize and provide itself with the required oxygen and nutrients. However, the minimal amount of properties and the order in which a cell acquires them in order to become a cancer cell, malignant or benign, is unclear. Several of the previously mentioned processes have a large overlap. Survival induced by PI(3)K activity, for example, often goes hand in hand

with the induction of cell-cycle progression (3), and inactivating mutations in p53 accomplish the same as overexpression of telomerase: the inhibition of apoptosis upon telomere shortening. Nevertheless, one can create a human tumor cell by ectopic expression of oncogenic Ras, telomerase and SV40 large T, which inhibits p53 and pRb (14). This indicates that, despite some overlap, most of the characteristics discussed above contribute a unique feature to the rise of a cancer cell that the other traits cannot contribute, but what that unique contribution is and whether these four elements need to be dysregulated in all human tumor cells remains to be investigated. In conclusion, before a cell divides in an uncontrolled fashion, it has accumulated hits in multiple crucial genes.

Cancer and the cell-cycle

The division of one cell into two daughter cells requires several distinct processes that can be subdivided into four stages (figure 1). In G1 phase a cell is provided with an external growth signal that initiates cellular growth and permits the cell to prepare for DNA replication. During S phase the cell duplicates its entire genome, after which it prepares for cellular division during G2 phase. Finally, nuclear division (mitosis) and cytoplasmic division (cytokinesis) is achieved in M phase. When M phase is done and the cell is provided with the appropriate external cues, the whole process can repeat itself. However, when a cell in G1 does not receive a growth signal, it can enter the alternative state of cellular quiescence, also referred to as the G0 phase. Altogether, this sequence of events is termed the cell-cycle (figure 1) and is strictly regulated to ensure that cells do not divide without permission.

At various stages of the cell-cycle, checkpoints control its proper progression and arrest the ongoing cell division whenever a mishap is sensed. In G2 and M phases, for instance, the DNA damage checkpoint, which includes p53, ensures that the cell-cycle does not continue when DNA is damaged (reviewed in (15)). Furthermore, in mitosis the spindle assembly checkpoint halts cell-cycle progression in case certain chromosomes are misaligned or not properly attached to the spindle microtubules (reviewed in (16)). The G1-checkpoint, also referred to as the restriction point (R in figure 1), is activated in late M phase and cannot be inactivated unless the cell receives the right external growth signals. In this checkpoint, the E2F-1 transcription factor plays a crucial role. Its activity is required for progression of the cell-cycle into S phase but is inhibited by pRb (reviewed in (17)). This pRb protein is one of the targets of the growth factor signals delivered in early G1. When cells are provided with such signals, a cascade of events ensures the activation of cyclin/cdk complexes that hyperphosphorylate pRb resulting in its release from E2F-1 and subsequent S-phase entry (figure 2). The cyclin/cdk complexes in turn can be inhibited by cdk-inhibitors (CKI's) that directly bind and

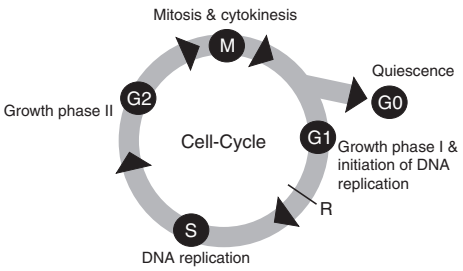

Figure 1. *Simplified scheme of the cell-cycle.* When cells receive a growth factor right after mitosis or during a state of quiescence (G0), G1 phase progresses and the initiation of DNA replication is licensed. Beyond the restriction point (R, or 'point of no return'), the cell will be committed to proceed with the cell-cycle and will start with DNA replication (S-phase) after which a growth phase (G2) prepares the cell for mitosis and cytokinesis (M-phase). If the cell is again provided with growth factor shortly after M-phase, it will proceed with another cell-division, but if not, it will enter quiescence.

inactivate the cyclin/cdk complex. Growth factor signalling can interfere with the activity of the checkpoint at multiple levels ensuring initiation of DNA synthesis and continuation of the cell-cycle. For example, growth factors can increase the activity or expression of the cyclin subunits of cyclin/cdk complexes, can relocalize cdk proteins from the cytoplasm to the nucleus and can decrease the activity or expression of CKI's (18, 19, 20, 21). This is one of the reasons why changes in growth factor-controlled signal transduction pathways that render them constitutively active are oncogenic: continuous activation of such pathways consistently inactivates the G1 checkpoint.

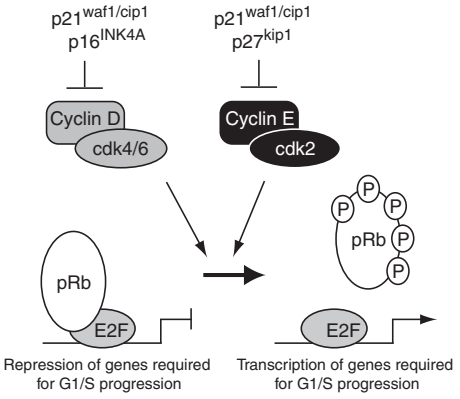

Figure 2. *Regulation of G1/S transition.* In early G1, pRb is hypophosphorylated and bound to E2F-1. This complex actively represses transcription of genes required for G1/S progression. Activation of cyclin D/cdk4/6 and cyclin E/cdk2 complexes by growth factors and E2F-1-mediated transcription causes pRb to be hyperphosphorylated. E2F-1 is then released from the inhibitory constraints imposed by pRb and actively transcribes genes required for G1/S transition. Conversely, G1/S progression can be inhibited by the $p27^{kip1}$, $p16^{INK4A}$ and $p21^{waf1/cip1}$ families of cdk inhibitors that directly bind and inhibit the cdk's and thus keep pRb in its hypophosphorylated form.

Growth factor signalling

Transduction of a growth factor signal across the plasma membrane of a cell normally requires a transmembrane receptor, one class of which is the receptor tyrosine kinase (RTK). Extracellular ligand-binding by the RTK triggers dimerization and a conformational change in the intracellular part of the RTK that results in the activation of its kinase domains. As a result, the receptor autophosphorylates, creating docking sites for intracellular signalling proteins that subsequently bind the receptor. Ultimately, this leads to activation, through either RTK-mediated phosphorylation or relocalization, of a variety of signalling pathways that control processes such as glucose metabolism, actin dynamics, DNA synthesis and apoptosis (reviewed in (22)). Cytoplasmic tyrosine kinases of the Src-family, for example, associate with the RTKs through a Src-homology 2 (SH2) domain which leads to their phosphorylation and thus activation (reviewed in (23)). Src-like kinases are then released into the cytoplasm where they tyrosine-phosphorylate substrates to regulate cytoskeletal organization, proliferation, and mitosis. Another important molecule activated by RTKs is phospholipase C-γ (PLCγ). As Src, PLCγ directly binds to and is activated by the RTK and subsequently converts phosphatidylinositol-(4,5)-diphosphate to diacylglycerol and inositol-triphosphate, which activates classical protein kinase C enzymes (PKCs) and mobilizes intracellular calcium, respectively (reviewed in (24)). Additional intracellular signalling cascades activated by RTKs include the JAK/STAT, Ras and PI(3)K pathways, the latter two of which are a subject of investigation in this thesis.

Growth factor signalling and the Ras cascade

Ras is a member of the superfamily of small GTPases that is active when bound to guanosine triphosphate (GTP) but inactive when bound to guanosine diphosphate (GDP). Such GTPases are activated by guanine nucleotide exchange factors (GEFs) which replace the bound GDP nucleotide for a GTP nucleotide. Ras

then undergoes a conformational change enabling it to interact in a GTP-specific manner with a variety of Ras binding domain (RBD)-containing effector molecules that transduce the signal further downstream. Inactivation of the GTPase occurs through hydrolysis of GTP to GDP by its intrinsic GTPase activity which is stimulated by GTPase-activating proteins (GAPs) (reviewed in (25)).

Upon activation of a RTK by a growth factor, the adaptor protein Grb2 is recruited to the plasma membrane, either directly or via another adaptor Shc, through interaction of its SH2 domain with a phospho-tyrosine docking site on the RTK (26). Grb2 is complexed with mSOS, a GEF for Ras. Binding of Grb2 to the RTK thus causes translocation of mSOS to the plasma membrane where Ras is located and hence increases the rate of nucleotide exchange on Ras leading to its activation. (reviewed in (27)). In this way, Ras can be activated by a wide variety of growth factors including platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and insulin.

As mentioned earlier, activated Ras can recruit several proteins to the plasma membrane, but only three effectors have been firmly established as *in vivo* targets for Ras-GTP (figure 3). By binding the Raf serine/threonine kinases, Ras activates the MEK/MAPK pathway that controls transcription, chromosome separation and proliferation (28, 29, 30). Through recruitment of GEFs for the Ras-like GTPase Ral to the plasma membrane (reviewed in (31)), Ras activity causes GTP-loading of Ral leading to activation of phospholipase D, activation of the c-jun transcription factor, and inhibition of AFX, a member of the superfamily of Forkhead transcription factors (32, 33, 34). Finally, in certain cases, active Ras can bind and activate PI(3)K, a lipid kinase that will be extensively discussed furtheron (35). However, to date involvement of Ras in PI(3)K activation is only seen in neuronal cells after stimulation with nerve growth factor (NGF) or upon overexpression of (oncogenic) Ras in several other cell types (35, 36, 37). Inhibition of endogenous Ras in mouse fibroblasts, for instance, does not significantly interfere with PDGF-, EGF- and insulin-induced PI(3)K activation (38), suggesting that Ras-mediated activation of PI(3)K might be restricted to specific cell types and/or growth factors.

Interestingly, artificial membrane targeting of all mentioned Ras effectors by adding a carboxy (C)-terminal CAAX localization motif from Ki-Ras constitutively activates them (39, 40, 41). This indicates that Ras-GTP may function in general to recruit its effectors to specific sites in the plasma membrane where substrates or additional activators are present (42). Alternatively, the interaction of Ras-GTP with its effectors may lead to activation via altering the conformation of the effector, as was recently shown for the interaction of PI(3)Kγ and Ras-GTP (43).

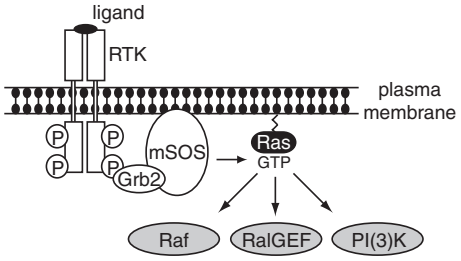

Figure 3. *The Ras signalling cascade.* Upon ligand binding to its receptor, the RTK autophosphorylates, creating binding sites for SH2 domain-containing proteins. The Grb2-mSOS complex is recruited to the plasma membrane which results in an exchange of GDP for GTP nucleotide on the Ras GTPase. GTP-bound Ras is capable of binding RBD-containing effector proteins which results in their activation through relocalization and/or conformational alteration. In this way, Ras can activate the serine/threonine kinase Raf, the family of Ral exchange factors (RalGEF) and PI(3)K.

Growth factor signalling and the PI(3)K cascade

The classical form of the heterodimeric lipid kinase PI(3)K consists of a p85 regulatory subunit and a p110 catalytic subunit. Several mammalian isoforms of both subunits have been identified and it is thought that the specific isoforms function in designated developmental stages and in certain distinct processes including vesicular transport and heterotrimeric G-protein signalling ((44, 45, 46) and reviewed in (47)). PI(3)K can be activated by cytokines, G-protein-linked receptors, several kinds of cellular stress including heat shock and hypoxia, and by growth factors (reviewed in (48)). Upon RTK autophosphorylation, two phosphotyrosine docking sites in a YMXM motif that allow binding and activation of PI(3)K are created on the receptor itself, as is the case for the PDGF receptor, or on a general adaptor protein, such as IRS-1 in the case for the insulin receptor (InsR) (49, 50). The p85 protein directly binds the phosphotyrosines on the receptor through its two SH2 domains thereby bringing the p110 subunit in the vicinity of the plasma membrane (reviewed in (51)). However, the events that follow this relocalization and that eventually lead to full activation of PI(3)K are poorly understood. It has been suggested that RTK-mediated phosphorylation of the p85 subunit is required (52), but additional regulatory mechanisms may include autophosphorylation, dimerization, p110 conformational stabilization and binding to and possibly phosphorylation by Src-family kinases (53, 54, 55, 56). Once activated, PI(3)K phosphorylates the membrane phospholipid phosphoinositol (PI) on the 3' position of the inositol ring producing PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 (reviewed in (57)). These lipids function as second messengers to recruit Fab1p/YOTB/Vac1p/EEA1 (FYVE)- and pleckstrin homology (PH)-domain containing proteins (reviewed in (57, 58)). In analogy to Ras signalling, binding of proteins that harbour a PH-domain to the 3' phosphorylated phosphoinositides leads to their activation at least in part via relocalization to the plasma membrane where additional activating molecules are present (reviewed in (59)). Again, as with the Ras effectors, artificial membrane targeting of the lipid-binding proteins leads to their constitutive activation (38, 60). Conversely, direct binding of effector proteins to the p85 or p110 subunits has been observed. The microtububle motor protein dynamin, for instance, can bind the Src-homolgy 3 (SH3) domain of p85, suggesting an involvement of PI(3)K in vesicle pinching (61). Proteins that bind to p110 include Ras and R-Ras (35, 62). Although most studies concerning the interaction between p110 and Ras have focussed on PI(3)K as being downstream of Ras, the *vice versa* situation has been reported as well: PI(3)K as an activator of Ras has been suggested by several groups although some studies find that this does not require growth factor-stimulated PI(3)K but rather basal PI(3)K activity (compare (63) with (64)). Also, a proline-rich region in the p85 subunit of PI(3)K can bind Grb2, which might suggest that p110 binds Ras and p85 recruits mSOS to activate Ras (65). Nevertheless, it remains to be investigated in what circumstances one activates the other.

Signalling through 3' phosphorylated phosphoinositides

In general, FYVE-domain containing proteins bind PI(3)P lipids and proteins that harbour a PH domain bind with high affinity to PI(3,4)P_2 and PI(3,4,5)P_3 lipids (reviewed in (57, 58)). The use of specific inhibitors of PI(3)K activity, the fungal metabolite wortmannin and the structurally unrelated compound LY294002, greatly helped the identification of proteins that *in vivo* are downstream of PI(3)K activity (figure 4) (66, 67). Using these compounds it was shown that PI(3)K contributes to Rab5-mediated

endosome fusion via early endosome antigen 1 (EEA1) that binds PI(3)P via its FYVE-domain (68). In addition, some other FYVE-domain-containing proteins that bind PI(3)P, such as the tyrosine kinase substrate Hrs and the yeast protein Vps27p, have been identified, but to date no role for these proteins in PI(3)K signal transduction has been demonstrated (figure 4) (69, 70). Through the $PI(3,4)P_2$ and $PI(3,4,5)P_3$ lipids, PI(3)K has been implicated in the activation of a variety of proteins. First, via PH domain-containing GEFs, PI(3)K can activate the GTPases Rac1 (GEF: Tiam-1), involved in cytoskeletal rearrangements; Arf (GEFs: ARNO, GRP1), involved in vesicular trafficking; and possibly even Ras (GEF: mSOS) (71, 72, 73). Second, PI(3)K activity has been shown to be required for growth factor induced activation of PLCγ, either directly or via bruton's tyrosine kinase (Btk) (74, 75). Third, some cytoskeleton binding proteins including profilin and spectrin have been shown to bind the $PI(3,4)P_2$ and $PI(3,4,5)P_3$ lipids (71, 76), and finally, several serine/threonine kinases are activated by PI(3)K lipid products (figure 4). The PKC family members PKCδ, PKCε, PKCη and PKCζ, for instance, are activated by direct binding to the lipids, and PKCδ and PKCζ can also be activated indirectly via PI(3)K-mediated activation of an upstream kinase (77, 78, 79). Besides PKCs, other kinases that are indirectly activated by PI(3)K include focal adhesion kinase (FAK), involved in cellular adhesion (80), and $p70^{S6k}$, involved in protein translation (81, 82).

Importantly, two lipid phosphatases have been identified that can terminate PI(3)K signalling by dephosphorylating the PI(3)K lipid products. The heamatopoietic-specific 5' phosphatases SHIP and SHIP-2 can inactivate some PI(3)K-dependent signalling pathways by converting $PI(3,4,5)P_3$ to $PI(3,4)P_2$ (83, 84). Especially interesting is the gene product of the *PTEN* gene. This gene was originally identified as being mutated in cells from patients with Cowden's disease, an inherited disorder characterized by hamartomas in a variety of tissues, and was subsequently found mutated or deleted in quite a number of cancers ((85) and reviewed in (3, 86, 87)). Recently, PTEN was found to have phosphatase activity towards the 3' phosphate of the inositol ring of the PI(3)K lipid products (88, 89, 90). The fact that PTEN is a tumor suppressor that presumably works via dephosphorylating phosphatidylinositol lipids at the very site that PI(3)K phosphorylates them implies that PI(3)K signalling pathways might contribute to tumor formation. Indeed, the p3k protein expressed by the ASV-16 virus encodes

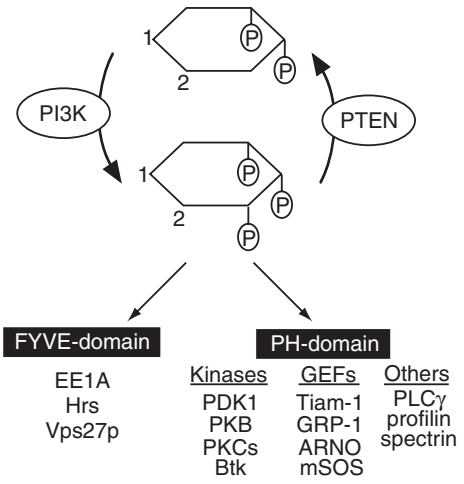

Figure 4. *Effectors of PI(3)K-generated lipidproducts.* Active PI(3)K converts PI, PI(4)P and $PI(4,5)P_2$ to PI(3)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$, respectively. These lipids act as second messengers to recruit a variety of effector proteins to the plasma membrane where these are activated. FYVE domain-containing proteins bind to PI(3)P but not to $PI(3,4)P_2$ and $PI(3,4,5)P_3$, and include EE1A, Hrs and the yeast Vps27p protein. Proteins that bind all three lipids but display preferential binding to $PI(3,4)P_2$ and $PI(3,4,5)P_3$ include several kinases, GEFs, actin-binding proteins and PLCγ. The PI(3)K-generated lipidproducts can be dephosphorylated by the 3' phosphatase and tumor suppressor PTEN that thereby terminates all PI(3)K-dependent signalling events.

a constitutively active p110 subunit of PI(3)K and is responsible for the transformation of chicken fibroblasts that are infected with this virus (91, 92). The importance of PI(3)K signalling in tumor formation will bemore extensively discussed in chapter 2.

Protein kinase B in PI(3)K signal transduction

Several years ago, two research groups simultaneously cloned a gene of which the product displayed high sequence homology to both the PKC and protein kinase A (PKA) family of serine/threonine kinases. Based on this characteristic, it was named protein kinase B (PKB) and RAC-PK (related to A and C protein kinases), respectively (93, 94). A third group isolated the same gene in a screen for cDNAs that show similarity to the *v-Akt* gene of the transforming AKT8 virus. Closer sequence comparisons revealed that v-Akt cDNA was identical to this new gene fused to the viral gag sequence and it was named c-Akt (95, 96). Since then, PKB-like genes have been found in many types of species, including *Drosophila melanogaster*, *Caenorhabditis elegans* and *homo sapiens* (93, 97, 98). Three PKB isoforms exist in mammalian cells: PKBα (AKT) PKBβ (AKT2) and PKBγ (AKT3) (figure 5) ((93, 95, 99, 100, 101, 102), references are for human sequences) and it has been suggested that the three PKB species play roles in distinct cellular processes. PKBγ is most prominent in neuronal and renal tissues whereas PKBα and PKBβ are more widely expressed. However, PKBβ-expression is relatively high in insulin-responsive tissues such as liver and muscle, suggesting that of the three isoforms PKBβ is particularly important for PI(3)K-mediated insulin responses in those tissues. This is in agreement with the recent observations that PKBβ but not PKBα functions in metabolic responses of 3T3-L1 adipocytes to insulin treatment despite the fact that both PKBα and PKBβ are activated by insulin in these cells (103, 104). Interestingly, all three genes encoding the PKB isozymes have been found amplified or upregulated in a number of human ovarian, mammary, gastric and pancreatic carcinomas with especially frequent amplifications in undifferentiated tumors (95, 105, 106, 107, 108, 109). This suggests that PKB has transforming capabilities and may contribute to tumor aggressiveness.

The real boost in PKB research interest came after the discovery in 1995 that PKB is a mediator of PI(3)K signal transduction, thereby putatively positioning PKB in all PI(3)K-mediated cellular responses (38, 110). In subsequent years the mechanism by which PI(3)K activates PKB has been largely resolved and appears to be similar for all three PKB isoforms (100). Upon formation of $PI(3,4)P_2$ and

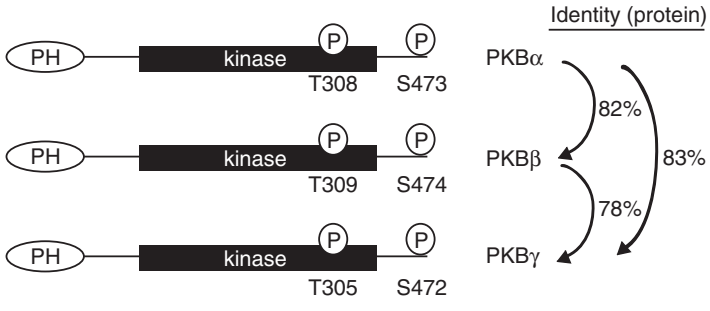

Figure 5. *Schematic representation of the three human PKB isozymes.* Depicted is the PH domain, the kinase domain and the two conserved regulatory phosphorylation sites. Percentage of identical sequences between the three enzymes at the protein level is shown on the right.

$PI(3,4,5)P_3$, PKB is recruited to the plasma membrane through its amino (N)-terminal PH domain (figure 6) ((111) and reviewed in (57)). Of these two lipid-products, $PI(3,4)P_2$ seems to be the *in vivo* activator of PKB, since PKB can bind this lipid with higher affinity than it binds to $PI(3,4,5)P_3$ and as a result is activated more efficiently by $PI(3,4)P_2$ (111, 112, 113). Once properly localized, PKB is phosphorylated on T308 and S473 (figures 5 and 6) (T309/S474 in PKBβ and T305/S472 in PKBγ) after which the kinase is fully active (38, 110, 114, 115). T308 is phosphorylated by the $PI(3,4,5)P_3$-dependent kinase 1 (PDK1) that itself binds the lipidproducts of PI(3)K activity (116, 117). Besides activating PKB, this PDK1 kinase appears to be a more general mediator of PI(3)K signalling, since it was shown that it is involved in PI(3)K-mediated activation of several PKCs, PKA, $p90^{rsk}$ and $p70^{S6k}$ (79, 118, 119, 120).

The kinase that phosphorylates S473 of PKB, conveniently termed PDK2, has yet to be identified, but a recent study suggests that by interaction with the PKC-related kinase-2 (PRK2), PDK1 can aquire PDK2-like activity (121). It has also been proposed that the integrin-linked kinase 4 (ILK4) is PDK2 (122). In addition to the regulation by PDKs, PH-domain-dependent multimerization of PKB is likely to be involved in PKB activation and recently a seemingly non-catalytic co-activator of PKB, the proto-oncogene TCL, was found in a T-cell leukemia cell-line (123, 124).

In the following chapter, the cellular functions of the PI(3)K/PKB pathway will be discussed. Its role in the regulation of metabolism, protein translation, survival, and cell-cycle progression will be examined, and particular focus will be put on its possible contributions to cellular transformation and tumor formation.

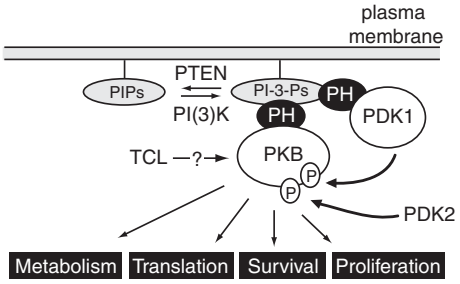

Figure 6. *Simplified scheme of PKB activation.* PI(3)K generates 3' phosphorylated phosphoinositides (PI-3-Ps) that recruit PKB and PDK1 to the plasma membrane by virtue of their PH domains. PKB is then phosphorylated by PDK1 and the still unidentified PDK2, resulting in its full activation. In addition to this catalytic activation of PKB, the non-catalytic TCL protein is likely to be involved in activating PKB but details of its function are largely unknown. Active PKB is released into the cytosol where it affects diverse cellular processes such as metabolism, protein translation, survival and cell-cycle progression.

References

1. A. J. Levine, p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-31 (1997).
2. R. Yao, G. M. Cooper, Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* **267**, 2003-6 (1995).
3. A. Di Cristofano, P. P. Pandolfi, The multiple roles of PTEN in tumor suppression. *Cell* **100**, 387-90 (2000).
4. T. M. Fynan, M. Reiss, Resistance to inhibition of cell growth by transforming growth factor-beta and its role in oncogenesis. *Crit Rev Oncog* **4**, 493-540 (1993).
5. L. Chin, J. Pomerantz, R. A. DePinho, The INK4a/ARF tumor suppressor: one gene—two products—two pathways. *Trends Biochem Sci* **23**, 291-6 (1998).
6. D. S. Peeper, et al., Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. *Nature* **386**, 177-81 (1997).
7. J. L. Bos, ras oncogenes in human cancer: a review. *Cancer Res* **49**, 4682-9 (1989).
8. P. Fedi, S. R. Tronick, S. A. Aaronson, *Growth Factors*. J. F. Holland, et al., Eds., Cancer Medicine (1997).
9. L. Chin, et al., p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell* **97**, 527-38 (1999).
10. J. Karlseder, D. Broccoli, Y. Dai, S. Hardy, T. de Lange, p53- and ATM-dependent apoptosis induced by telomeres lacking TRF2. *Science* **283**, 1321-5 (1999).

11. N. W. Kim, et al., Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011-5 (1994).
12. K. Hiyama, et al., Activation of telomerase in human lymphocytes and hematopoietic rogenitor cells. *J Immunol* **155**, 3711-5 (1995).
13. T. M. Bryan, T. R. Cech, Telomerase and the maintenance of chromosome ends. *Curr Opin Cell Biol* **11**, 318-24 (1999).
14. W. C. Hahn, et al., Creation of human tumour cells with defined genetic elements. *Nature* **400**, 464-8 (1999).
15. M. J. O'Connell, N. C. Walworth, A. M. Carr, The G2-phase DNA-damage checkpoint. *Trends Cell Biol* **10**, 296-303 (2000).
16. A. D. Rudner, A. W. Murray, The spindle assembly checkpoint. *Curr Opin Cell Biol* **8**, 773-80 (1996).
17. R. A. Weinberg, The retinoblastoma protein and cell cycle control. *Cell* **81**, 323-30 (1995).
18. P. Brennan, et al., Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity* **7**, 679-89 (1997).
19. J. A. Diehl, M. Cheng, M. F. Roussel, C. J. Sherr, Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* **12**, 3499-511 (1998).
20. H. Gille, J. Downward, Multiple ras effector pathways contribute to G(1) cell cycle progression. *J Biol Chem* **274**, 22033-40 (1999).
21. R. H. Medema, G. J. Kops, J. L. Bos, B. M. Burgering, AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782-7 (2000).
22. T. Hunter, Signaling—2000 and beyond. *Cell* **100**, 113-27 (2000).
23. T. Erpel, S. A. Courtneidge, Src family protein tyrosine kinases and cellular signal transduction pathways. *Curr Opin Cell Biol* **7**, 176-82 (1995).
24. G. Carpenter, Q. Ji, Phospholipase C-gamma as a signal-transducing element. *Exp Cell Res* **253**, 15-24 (1999).
25. J. L. Bos, Ras-like GTPases. *Biochim Biophys Acta* **1333**, M19-31 (1997).
26. M. Rozakis-Adcock, et al., Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* **360**, 689-92 (1992).
27. J. Downward, Control of ras activation. *Cancer Surv* **27**, 87-100 (1996).
28. R. Marais, J. Wynne, R. Treisman, The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**, 381-93 (1993).
29. M. Zecevic, et al., Active MAP kinase in mitosis: localization at kinetochores and association with the motor protein CENP-E. *J Cell Biol* **142**, 1547-58 (1998).
30. C. J. Marshall, Small GTPases and cell cycle regulation. *Biochem Soc Trans* **27**, 363-70 (1999).
31. R. M. Wolthuis, J. L. Bos, Ras caught in another affair: the exchange factors for Ral. *Curr Opin Genet Dev* **9**, 112-7 (1999).
32. H. Jiang, et al., Involvement of Ral GTPase in v-Src-induced phospholipase D activation. *Nature* **378**, 409-12 (1995).
33. N. D. de Ruiter, R. M. Wolthuis, H. van Dam, B. M. Burgering, J. L. Bos, Ras-dependent regulation of c-Jun phosphorylation is mediated by the ral guanine nucleotide exchange factor-Ral pathway . *Mol Cell Biol* **20**, 8480-8 (2000).
34. G. J. Kops, et al., Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**, 630-4 (1999).
35. P. Rodriguez-Viciana, et al., Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* **370**, 527-32 (1994).
36. P. Rodriguez-Viciana, et al., Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. *Cell* **89**, 457-67 (1997).
37. B. Hallberg, M. Ashcroft, D. M. Loeb, D. R. Kaplan, J. Downward, Nerve growth factor induced stimulation of Ras requires Trk interaction with Shc but does not involve phosphoinositide 3-OH kinase. *Oncogene* **17**, 691-7 (1998).
38. B. M. Burgering, P. J. Coffer, Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602 (1995).
39. D. Stokoe, S. G. Macdonald, K. Cadwallader, M. Symons, J. F. Hancock, Activation of Raf as a result of recruitment to the plasma membrane. *Science* **264**, 1463-7 (1994).
40. S. A. Didichenko, B. Tilton, B. A. Hemmings, K. Ballmer-Hofer, M. Thelen, Constitutive activation of protein kinase B and phosphorylation of p47phox by a membrane-targeted phosphoinositide 3-kinase. *Curr Biol* **6**, 1271-8 (1996).
41. R. M. Wolthuis, N. D. de Ruiter, R. H. Cool, J. L. Bos, Stimulation of gene induction and cell growth by the Ras effector Rlf. *Embo J* **16**, 6748-61 (1997).
42. R. Marais, Y. Light, H. F. Paterson, C. J. Marshall, Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *Embo J* **14**, 3136-45 (1995).
43. M. E. Pacold, et al., Crystal structure and functional analysis of ras binding to its effector phosphoinositide 3-kinase gamma. *Cell* **103**, 931-43. (2000).
44. S. Pons, et al., The structure and function of p55PIK reveal a new regulatory subunit for phosphatidylinositol 3-kinase. *Mol Cell Biol* **15**, 4453-65 (1995).
45. B. Stoyanov, et al., Cloning and characterization of a G protein-activated human phosphoinositide-3 kinase. *Science* **269**, 690-3 (1995).
46. S. Volinia, et al., A human phosphatidylinositol 3-

kinase complex related to the yeast Vps34p-Vps15p protein sorting system. *Embo J* **14**, 3339-48 (1995).
47. B. Vanhaesebroeck, S. J. Leevers, G. Panayotou, M. D. Waterfield, Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem Sci* **22**, 267-72 (1997).
48. S. R. Datta, A. Brunet, M. E. Greenberg, Cellular survival: a play in three Akts. *Genes Dev* **13**, 2905-27 (1999).
49. A. Kashishian, A. Kazlauskas, J. A. Cooper, Phosphorylation sites in the PDGF receptor with different specificities for binding GAP and PI3 kinase in vivo. *Embo J* **11**, 1373-82 (1992).
50. M. G. Myers, Jr., et al., YMXM motifs and signaling by an insulin receptor substrate 1 molecule without tyrosine phosphorylation sites. *Mol Cell Biol* **16**, 4147-55 (1996).
51. C. L. Carpenter, L. C. Cantley, Phosphoinositide kinases. *Curr Opin Cell Biol* **8**, 153-8 (1996).
52. W. M. Kavanaugh, A. Klippel, J. A. Escobedo, L. T. Williams, Modification of the 85-kilodalton subunit of phosphatidylinositol-3 kinase in platelet-derived growth factor-stimulated cells. *Mol Cell Biol* **12**, 3415-24 (1992).
53. R. Dhand, et al., PI 3-kinase is a dual specificity enzyme: autoregulation by an intrinsic protein-serine kinase activity. *Embo J* **13**, 522-33 (1994).
54. C. M. Pleiman, W. M. Hertz, J. C. Cambier, Activation of phosphatidylinositol-3' kinase by Src-family kinase SH3 binding to the p85 subunit. *Science* **263**, 1609-12 (1994).
55. M. J. Layton, A. G. Harpur, G. Panayotou, P. I. Bastiaens, M. D. Waterfield, Binding of a diphosphotyrosine-containing peptide that mimics activated platelet-derived growth factor receptor beta induces oligomerization of phosphatidylinositol 3-kinase. *J Biol Chem* **273**, 33379-85 (1998).
56. J. Yu, et al., Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. *Mol Cell Biol* **18**, 1379-87 (1998).
57. A. Toker, L. C. Cantley, Signalling through the lipid products of phosphoinositide-3-OH kinase. *Nature* **387**, 673-6 (1997).
58. S. J. Leevers, B. Vanhaesebroeck, M. D. Waterfield, Signalling through phosphoinositide 3-kinases: the lipids take centre stage. *Curr Opin Cell Biol* **11**, 219-25 (1999).
59. T. O. Chan, S. E. Rittenhouse, P. N. Tsichlis, AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* **68**, 965-1014 (1999).
60. M. Andjelkovic, et al., Role of translocation in the activation and function of protein kinase B. *J Biol Chem* **272**, 31515-24 (1997).
61. I. Gout, et al., The GTPase dynamin binds to and is activated by a subset of SH3 domains. *Cell* **75**, 25-36 (1993).
62. B. M. Marte, P. Rodriguez-Viciana, S. Wennstrom, P. H. Warne, J. Downward, R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways. *Curr Biol* **7**, 63-70 (1997).
63. S. Wennstrom, J. Downward, Role of phosphoinositide 3-kinase in activation of ras and mitogen-activated protein kinase by epidermal growth factor. *Mol Cell Biol* **19**, 4279-88 (1999).
64. Q. Hu, A. Klippel, A. J. Muslin, W. J. Fantl, L. T. Williams, Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science* **268**, 100-2 (1995).
65. J. Wang, K. R. Auger, L. Jarvis, Y. Shi, T. M. Roberts, Direct association of Grb2 with the p85 subunit of phosphatidylinositol 3-kinase. *J Biol Chem* **270**, 12774-80 (1995).
66. A. Arcaro, M. P. Wymann, Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. *Biochem J* **296**, 297-301 (1993).
67. C. J. Vlahos, W. F. Matter, K. Y. Hui, R. F. Brown, A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* **269**, 5241-8 (1994).
68. A. Simonsen, et al., EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* **394**, 494-8 (1998).
69. C. G. Burd, S. D. Emr, Phosphatidylinositol(3)-phosphate signaling mediated by specific binding to RING FYVE domains. *Mol Cell* **2**, 157-62 (1998).
70. J. M. Gaullier, et al., FYVE fingers bind PtdIns(3)P. *Nature* **394**, 432-3 (1998).
71. L. E. Rameh, et al., A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *J Biol Chem* **272**, 22059-66 (1997).
72. J. K. Klarlund, et al., Regulation of GRP1-catalyzed ADP ribosylation factor guanine nucleotide exchange by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273**, 1859-62 that distinct PH domains select for different phosphoinositides. (1998).
73. K. Venkateswarlu, P. B. Oatey, J. M. Tavare, P. J. Cullen, Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. *Curr Biol* **8**, 463-6 (1998).
74. Y. S. Bae, et al., Activation of phospholipase C-gamma by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273**, 4465-9 (1998).
75. M. Falasca, et al., Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. *Embo J* **17**, 414-22 (1998).
76. A. Lambrechts, et al., The mammalian profilin

isoforms display complementary affinities for PIP2 and proline-rich sequences. *Embo J* **16**, 484-94 (1997).

77. A. Toker, et al., Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. *J Biol Chem* **269**, 32358-67 and regulate subsequent plasma membrane cycling events. (1994).
78. M. L. Standaert, et al., Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes. Potential role in glucose transport. *J Biol Chem* **272**, 30075-82 (1997).
79. J. A. Le Good, et al., Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* **281**, 2042-5 (1998).
80. H. C. Chen, J. L. Guan, Stimulation of phosphatidylinositol 3'-kinase association with foca adhesion kinase by platelet-derived growth factor. *J Biol Chem* **269**, 31229-33 (1994).
81. Q. P. Weng, et al., Phosphatidylinositol 3-kinase signals activation of p70 S6 kinase in situ through site-specific p70 phosphorylation. *Proc Natl Acad Sci U S A* **92**, 5744-8 (1995).
82. R. Mendez, M. G. Myers, Jr., M. F. White, R. E. Rhoads, Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase. *Mol Cell Biol* **16**, 2857-64 (1996).
83. M. J. Aman, T. D. Lamkin, H. Okada, T. Kurosaki, K. S. Ravichandran, The inositol phosphatase SHIP inhibits Akt/PKB activation in B cells. *J Biol Chem* **273**, 33922-8 (1998).
84. V. Taylor, et al., 5' phospholipid phosphatase SHIP-2 causes protein kinase B inactivation and cell cycle arrest in glioblastoma cells. *Mol Cell Biol* **20**, 6860-71 (2000).
85. A. Di Cristofano, B. Pesce, C. Cordon-Cardo, P. P. Pandolfi, Pten is essential for embryonic development and tumour suppression. *Nat Genet* **19**, 348-55 Spain. (1998).
86. L. C. Cantley, B. G. Neel, New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A* **96**, 4240-5 (1999).
87. D. Bonneau, M. Longy, Mutations of the human PTEN gene. *Hum Mutat* **16**, 109-22 (2000).
88. T. Maehama, J. E. Dixon, The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273**, 13375-8 (1998).
89. M. P. Myers, et al., The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc Natl Acad Sci U S A* **95**, 13513-8 (1998).
90. V. Stambolic, et al., Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39 (1998).
91. H. W. Chang, et al., Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. *Science* **276**, 1848-50 (1997).
92. M. Aoki, et al., The catalytic subunit of phosphoinositide 3-kinase: requirements for oncogenicity. *J Biol Chem* **275**, 6267-75 (2000).
93. P. J. Coffer, J. R. Woodgett, 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 (1991).
94. P. F. Jones, T. Jakubowicz, F. J. Pitossi, F. Maurer, B. A. Hemmings, 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 (1991).
95. S. P. Staal, 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 (1987).
96. A. Bellacosa, J. R. Testa, S. P. Staal, P. N. Tsichlis, A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* **254**, 274-7 (1991).
97. T. F. Franke, K. D. Tartof, P. N. Tsichlis, The SH2-like Akt homology (AH) domain of c-akt is present in multiple copies in the genome of vertebrate and invertebrate eucaryotes. Cloning and characterization of the Drosophila melanogaster c-akt homolog Dakt1. *Oncogene* **9**, 141-8 (1994).
98. S. Paradis, G. Ruvkun, 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 (1998).
99. D. Brodbeck, P. Cron, B. A. Hemmings, A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain. *J Biol Chem* **274**, 9133-6 (1999).
100. S. Masure, et al., Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3. *Eur J Biochem* **265**, 353-60 (1999).
101. K. Nakatani, H. Sakaue, D. A. Thompson, R. J. Weigel, R. A. Roth, Identification of a human Akt3 (protein kinase B gamma) which contains the regulatory serine phosphorylation site. *Biochem Biophys Res Commun* **257**, 906-10 (1999).
102. J. Okano, I. Gaslightwala, M. J. Birnbaum, A. K. Rustgi, H. Nakagawa, Akt/Protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. *J Biol Chem* **275**, 30934-42 (2000).
103. K. S. Walker, et al., Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with protein kinase B alpha. *Biochem J* **331**, 299-308 (1998).
104. M. M. Hill, et al., A role for protein kinase Bbeta/Akt2 in insulin-stimulated GLUT4 translocation in

adipocytes. *Mol Cell Biol* **19**, 7771-81 (1999).
105. J. Q. Cheng, et al., AKT2, a putative oncogene encoding a member of a subfamily of protein- serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci U S A* **89**, 9267-71 (1992).
106. A. Bellacosa, et al., Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int J Cancer* **64**, 280-5 (1995).
107. J. Q. Cheng, et al., Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. *Proc Natl Acad Sci U S A* **93**, 3636-41 (1996).
108. W. Miwa, et al., Isolation of DNA sequences amplified at chromosome 19q13.1-q13.2 including the AKT2 locus in human pancreatic cancer. *Biochem Biophys Res Commun* **225**, 968-74 (1996).
109. K. Nakatani, et al., Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. *J Biol Chem* **274**, 21528-32 (1999).
110. T. F. Franke, et al., The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**, 727-36 (1995).
111. T. F. Franke, D. R. Kaplan, L. C. Cantley, A. Toker, Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* **275**, 665-8 (1997).
112. M. Frech, et al., High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. *J Biol Chem* **272**, 8474-81 (1997).
113. A. Klippel, W. M. Kavanaugh, D. Pot, L. T. Williams, A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol Cell Biol* **17**, 338-44 (1997).
114. D. R. Alessi, et al., Mechanism of activation of protein kinase B by insulin and IGF-1. *Embo J* **15**, 6541-51 (1996).
115. D. Stokoe, et al., Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* **277**, 567-70 (1997).
116. D. R. Alessi, et al., Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* **7**, 261-9 (1997).
117. L. Stephens, et al., Protein kinase B kinases that mediate phosphatidylinositol 3,4,5- trisphosphate-dependent activation of protein kinase B. *Science* **279**, 710-4 (1998).
118. X. Cheng, Y. Ma, M. Moore, B. A. Hemmings, S. S. Taylor, Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase. *Proc Natl Acad Sci U S A* **95**, 9849-54 (1998).
119. N. Pullen, et al., Phosphorylation and activation of p70s6k by PDK1. *Science* **279**, 707-10 (1998).
120. M. R. Williams, et al., The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr Biol* **10**, 439-48 (2000).
121. A. Balendran, et al., PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr Biol* **9**, 393-404 (1999).
122. M. Delcommenne, et al., 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 (1998).
123. K. Datta, et al., AH/PH domain-mediated interaction between Akt molecules and its potential role in Akt regulation. *Mol Cell Biol* **15**, 2304-10 (1995).
124. J. Laine, G. Kunstle, T. Obata, M. Sha, M. Noguchi, The protooncogene TCL1 is an Akt kinase coactivator. *Mol Cell* **6**, 395-407 (2000).

CHAPTER 2

Cellular Transformation by PKB: A Review

Introduction

The notion that PI(3)K-dependent signalling might be involved in cellular transformation originates from several independent findings. First, a p65-form of the p85 regulatory subunit of PI(3)K was recently identified in a thymic lymphoma and shown to contribute to tumor formation in transgenic mice expressing p65 in T-lymphocytes (1, 2). Second, a viral oncogene (p3k) in the ASV-16 virus represents a constitutively active version of the catalytic subunit of PI(3)K and is a potent transforming gene in cultured chicken embryo fibroblasts (CEFs) (3). Third, the gene for the p110α subunit of PI(3)K (*PIK3CA*) is found amplified in cervical cancer cell lines and the transformed phenotype of these cells is significantly affected by treatment with the two specific PI(3)K inhibitors wortmannin and LY294002 (4). Fourth, these same specific inhibitors of PI(3)K activity are able to revert the transformed phenotype of a variety of cancer cell lines (see for example (5)), and fifth, the tumor suppressor PTEN is a phosphatase for 3' phosphorylated phosphoinositides and it was recently shown that re-expression of PTEN in PTEN-negative tumor cells decreases PI(3)K signalling and reverts the transformed phenotype of the tumor cells (6, 7).

The scale of PI(3)K involvement in human tumor formation is demonstrated by studies that have examined mutations and deletions of the *PTEN* locus in a variety of tumors and tumor cell-lines (see (8) and references therein). Mutations and deletions occurred in 30% of all primary glioblastomas, >50% of all melanoma cell-lines, 30-50% of all advanced prostate cancers and of all endometrial carcinomas. Despite the observations that PTEN mutations are found predominantly in advanced glial and prostate tumors, mutations also occur in a significant percentage (10%) of breast cancer cell lines. Finally, occasional mutations in the *PTEN* gene are reported in head and neck and thyroid cancers. Overall, PTEN appears a quite common target for mutation in human cancer, with a mutation frequency which approaches that of p53.

One of the critical mediators of PI(3)K signalling with respect to cellular transformation is PKB, as suggested by a number of observations. Besides amplifications of the gene encoding PKB found in some cancers and the fact that PKB was originally identified as the transforming gene of the AKT8 oncovirus (see chapter 1), kinase-deficient PKB inhibits BCR/ABL-dependent transformation of murine bone marrow cells *in vitro* and suppresses leukemia development in severe combined immuno-deficiency (SCID) mice, presumably by somehow negatively affecting endogenous PKB function (5). In another study it was found that several constitutively active versions of PKB can induce transformation of CEFs and moreover that a dominant-negative mutant of PKB inhibits transformation of CEFs induced by p3k (9). Finally, a conditionally active version of PKB is able to transform Rat1 fibroblasts (10), and overexpression of active forms of PKB can override the reversal of transformation accomplished by PTEN re-expression in PTEN-negative cell-lines or mouse embryo fibroblasts (MEFs) from *mPTEN* -/- mice (7, 11). Altogether, these studies point to PKB as a major mediator of PI(3)K-induced cellular transformation and suggest that identifying its substrates and cellular functions might aid in understanding the tumorigenic effect of the PI(3)K/PKB pathway.

Control of metabolism and protein translation by PKB

Insulin stimulates the cellular uptake of glucose by recruiting glucose transporters of the GLUT family to the plasma membrane, and insulin can stimulate the conversion of glucose to pyruvate during the process of glycolysis. PKB

Table 1. *Putative substrates of PKB*

Substrate	Sequence	*in vitro*/*in vivo*#	Reference
Consensus	*RXRXX**S**/**T***		(127)
GSK3β	RARTS**S**^{9}	both	(14, 15)
BAD	RGRSR**S**^{112}	*in vitro***	(42)
Caspase-9	RRRFS**S**^{196}	*in vitro**	(46)
eNOS	RIRTQ**S**^{1177}	*in vitro**	(55)
IKKα	RERLGT^{23}	*in vitro***	(52)
AFX	RRRAA**S**^{193}	both	(106)
	RPRSS**S**^{258}	both	(106)
FKHR	RPRSC**T**^{24}	*in vitro**	(105)
	RRRAA**S**^{256}	*in vitro**	(105)
	RPRTS**S**^{319}	*in vitro**	(105)
FKHR-L1	RPRSC**T**^{32}	*in vitro**	(103)
	RRRAV**S**^{253}	*in vitro**	(103)
	RSRTN**S**^{315}	*in vitro*	(103)
hTERT[a]	RIRGK**S**^{824}	*in vitro*	(128)
Rac	RIRPL**S**^{71}	*in vitro*	(129)
BRCA1[b]	RKRRP**T**^{509}	*in vitro***	(130)
PFK2	RMRRN**S**^{466}	*in vitro*	(16)
	RPRNY**S**^{483}	*in vitro*	(16)
PDE-3B	RPRRR**S**^{273}	both	(18)
Raf	RQRST**S**^{259}	*in vitro***	(83)
IRS-1	RPRSK**S**^{265}	*in vitro***^	(131)
	RSRTE**S**^{302}		(131)
	RVRAS**S**^{325}		(131)
	RHRGS**S**^{358}		(131)
ASK1	RGRGS**S**^{83}	*in vitro***	(62)
mTOR	RTRTD**S**^{2448}	neither	(33)

#) *in vivo* is specified as analyzing PKB-dependent substrate phosphorylation in a tissue culture system using dominant-negative PKB (PKBcaax, PKB-AA or PKB-AAA)
*) *in vitro* and in cell-culture using catalytically active PKB
**) *in vitro* and in cell-culture using catalytically active PKB and kinase-dead PKB
^) Not measured for separate residues but for all four serines simultaneously
a) human telomerase reverse transcriptase subunit
b) breast cancer susceptibility gene 1

is capable of regulating the uptake of glucose by recruiting (muscle/fat-tissue specific) GLUT4 glucose transporters to the plasma membrane, and insulin-dependent increase in mRNA and protein expression of the (erythrocyte-specific) GLUT1 glucose transporter is dependent on PKB activity (12, 13). In addition, PKB can directly phosphorylate and inactivate, both *in vitro* and *in vivo*, glycogen synthase kinase 3 (GSK3), an enzyme involved in regulating glycogen synthesis (table 1) (14, 15). Subsequent observations further emphasized a role for PKB in controlling aspect of cellular metabolism, and showed that PKB *in vitro* directly controls the activity of an enzyme that takes part in glycolysis, namely phosphofructo-kinase 2 (PFK2) (table 1) (16), although a role for PKB in PFK2 function *in vivo* was recently questioned (17). Furthermore, insulin-induced activation of the cAMP-regulator phosphodiesterase 3B (PDE3B) in adipocytes was also shown to be mediated through direct phopshorylation by PKB (table 1) (18).

In addition to these direct forms of metabolic control, PKB was found to regulate the transcription of several insulin-responsive genes. Via an insulin-response element (IRE), the InsR/PI(3)K/PKB signalling route can inhibit phosphoenolpyruvaat-carboxykinase (PEPCK) and insulin-like growth factor-binding protein 1 (IGFBP-1) gene expression, and activate glucose-6-phosphatase and fatty acid synthase gene expression (19, 20, 21, 22).

The contribution of PKB-controlled metabolic processes to cellular transformation is likely to be of a permissive nature. None of the above mentioned genes have been implicated in the onset of tumor formation, but are nonetheless thought to facilitate growth by increasing the tumor's energy supply. With this respect it is interesting to note that GLUT1 expression, although normally restricted, is high in most tumor tissues (23). In addition, besides being inhibited by insulin, GSK3 negatively regulates the Wnt/β-catenin/T-cell factor (TCF)/cyclinD1 pathway implicated in the formation of >90% of all colon carcinomas (reviewed in (24)). GSK3 directly phosphorylates β-catenin which is then targetted for ubiquitin-mediated degradation via the Skp1/CulA/F-box (SCF) complex (25). Inactivation of GSK3 by the Wnt ligand via the frizzled receptor and the cytoplasmic dishevelled protein causes stabilization of β-catenin and activation of the TCF/LEF family of transcription factors (reviewed in (26)). In this way, GSK3 can regulate the expression of the genes for c-myc and cyclin D1, involved in proliferation (27, 28). This raises the interesting possibility that PKB might affect β-catenin stability and thus cell-cycle progression via the inhibition of GSK3. Despite the attractiveness of this, solid evidence is lacking and a recent report suggests that PKB-regulated GSK3 constitutes a different pool than the one regulated by Wnt signalling (29). Finally, GSK3 has been implicated in the regulation of c-jun transcriptional activity, involved in tumor formation (30) but no role for PKB in this process has yet been reported.

A role for PKB in protein translation is also suggested. PKB is able to phosphorylate and thereby inactivate the translational repressor 4E-binding protein 1 (4E-BP1) which in turn inactivates the translation initiation factor eIF-4E that facilitates RNA entry into ribosomes (31). Moreover, hyperactive PKB can activate $p70^{S6k}$, the kinase that phosphorylates the S6 ribosomal subunit to stimulate protein synthesis (32). PKB may accomplish the latter via direct phosphorylation of the $p70^{S6k}$ activator mammalian target of rapamycin (mTOR) (table 1) (33). Although it was recently shown that endogenous PKB does not play an essential role in insulin-stimulated, PI(3)K-dependent $p70^{S6k}$ activation (34), PKB might still activate $p70^{S6k}$ in the context of a transformed cell that contains hyperactive PKB. In agreement with this, the $p70^{S6k}$ inhibitor

rapamycin inhibits p3k/PKB-mediated transformation of CEFs, and a catalytically active PKB that is unable to inactivate 4E-BP1 or activate $p70^{S6k}$ does not mediate CEF transformation by p3k (35). Additionally, overexpression of eIF-4E in rodent fibroblasts causes cellular transformation, and subsequent overexpression of 4E-BP1 can revert this (36, 37). Altogether, this suggests that translational control may play an important role in cellular transformation induced by the PI(3)K/PKB pathway.

Survival signalling by PKB

NGF and insulin-like growth factor I (IGF-I) transduce survival signals to the cell by activating the PI(3)K/PKB cascade and these growth factors thereby oppose apoptotic signals that are elicited by serum starvation or the addition of toxic stimuli (38, 39, 40). Several mechanisms as to how PKB transduces the PI(3)K-mediated survival signal have been proposed. BAD, a pro-apoptotic member of the Bcl2 family can induce apoptosis via the release of cytochrome *c* from the mitochondrial membrane, thereby activating certain proteases termed caspases that are the executioners of cell death (figure 1) (41). PKB directly phosphorylates BAD on serine 136, creating binding sites for cytoplasmic 14-3-3 proteins (table 1 and figure 1) (42). Upon PKB-mediated phosphorylation, BAD complexes with 14-3-3 proteins and is thereby kept inactive (42, 43). However interestingly, BAD inactivation by PKB does not appear to be the major mechanism for PKB-mediated survival, since BAD is not ubiquitously expressed and certain cell-types that do not express BAD are still protected by PKB activity ((44) and see (45) for a critical review). PKB has also been reported to directly phosphorylate and inactivate caspase-9 in cells transformed by oncogenic Ras (table 1 and figure 1), although formal proof for involvement of caspase-9 phosphorylation in growth factor-mediated survival was not provided (46). In fact, the PKB phosphorylation site in caspase-9 is only present in the human protein but not in caspase-9 proteins of lower organisms including mouse and dog, suggesting that caspase-9 regulation by PKB is not a general phenomenon conserved throughout evolution, and that it is unlikely to be involved in survival signalling during development (47).

Other putative targets for PKB in growth factor-induced survival signalling include the cAMP-response element-binding protein (CREB), IκB kinase (IKK), endothelial nitric oxide synthase (eNOS) and the apoptosis signal-regulated kinase 1 (ASK1) (figure 1). Immunoprecipitated PKB can activate the CREB transcription factor through phosphorylation of serine 133, leading to enhanced transcription of the *bcl2* gene that protects cells from apoptosis

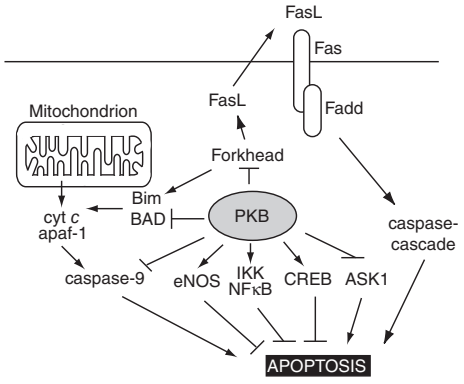

Figure 1. *Survival signalling by PKB.* Apoptotic signals can be antagonized by PKB in a variety of ways. Fas-mediated apoptosis can be inhibited by PKB via the inactivation of the Forkhead FKHR-L1 that transcribes the gene encoding FasL. Mitochondria-mediated apoptosis is inhibited by PKB via the inactivation of BAD and via the inhibition of Forkhead-mediated Bim expression. Additionally, PKB inhibits the catalytic activities of the pro-apoptotic factors caspase-9 and ASK1, and activates the survival factors NFκB, CREB, and eNOS. This figure provides a summary of most reported PKB targets in survival signalling but does not intend to imply that all PKB-mediated survival events happen under the same conditions in every cell-type.

(48, 49). The kinase that is in the PKB immunoprecipitates and that phosphorylates serine 133 could be either PKB itself or $p90^{Rsk}$, a protein kinase previously shown to promote survival via serine 133 phosphorylation of CREB (50). Interestingly, as mentioned in the previous chapter, PDK1 is an activator of $p90^{Rsk}$ and hence the two proteins might be in the complex with PKB and precipitate with anti-PKB antibodies. It therefore remains to be established that PKB is the kinase that transduces the survival signal via CREB.

PKB can activate NFκB. This transcription factor is sequestered in the cytoplasm by proteins of the IκB family and can be released by the kinase IKK that phosphorylates IκB and thereby targets it for ubiquitin-mediated degradation (reviewed in (51)). Direct phosphorylation of the α subunit of this IKK on threonine 23 by PKB results in NFκB activation and a subsequent survival signal (table 1 and figure 1) (52, 53). In support of a role for PKB in the transduction of a survival signal via NFκB is a study that shows that mice carrying the gagPKB transgene in T-cells display both enhanced NFκB activity, bcl-X_L expression and protection from apoptosis (54).

In addition to the regulation of the CREB and NFκB transcription factors, PKB can increase survival by enhancing the production of the second messenger nitric oxide (NO) through direct activation of eNOS (table 1 and figure 1) (55, 56). eNOS is responsible for the maintenance of blood vessels by regulating blood pressure, angiogenesis and vascular remodelling, and can be activated by shear stress as well as by the vascular endothelial growth factor (VEGF). When activated by these two factors PKB phosphorylates eNOS on threonine 1177 thereby increasing NO production thus contributing to endothelial cell-survival (55, 56, 57). Besides through the regulation of eNOS activity, PKB seems also more generally involved in angiogenesis, since VEGF activates PKB which in turn regulates the expression and activity of the hypoxia-inducible factor (HIF) that activates gene expression of the VEGF gene itself (58, 59, 60).

The ASK1 kinase is a mitogen-activated protein kinase kinase (MKK) that activates the stress-induced c-jun N-terminal kinase (JNK) as well as p38 kinases. It thereby participates in the induction of apoptosis caused by a variety of toxic agents (61). PKB phosphorylates ASK1 on serine 83 leading to inhibition of the kinase and inhibition of JNK/p38-mediated apoptosis (table 1 and figure 1) (62).

This summary of potential PKB targets and PKB-regulated processes shows that PKB can act as a survival factor in a large number of cell types by regulating a variety of proteins that are somehow involved in the control of apoptosis. What is, however, the evidence for the involvement of PKB's anti-apoptotic function in cellular transformation? Despite observations that high PKB activity in tumor cell-lines is often found associated with increased protection from apoptotic signals, sufficient formal proof for an essential function of PKB in survival signalling in tumor cells is lacking. Most of the studies concerning PKB and survival have used constitutively active PKB to overcome an apoptotic signal (see for example (63, 64)) and/or a kinase-dead version of PKB (PKB-KD) as a dominant negative mutant to inhibit a survival signal (see for example (40)). However, kinase-dead PKB does not inhibit endogenous PKB activation by growth factors as shown by van Weeren et al, whereas a number of other PKB mutants do (PKBcaax, PKB-AA and PKB-AAA) (15, 65, 66). Only a few reports to date have shown, using either of these bonafide dominant-negative mutants, that PKB can be involved in anti-apoptotic signalling in certain situations. Applying calcium at low concentrations to certain cultured neurons elicits a survival signal and using PKB-AA it was shown that calcium-

induced survival in these cells depends on PKB activity (67). In addition, matrix detachment-induced apoptosis (anoikis) by overexpression of ILK is reverted by co-expression of PKB-AAA (68). Finally, fibroblasts from mice in which the gene for the PTEN tumor suppressor was knocked out show increased sensitivity to apoptotic stimuli and this can be reverted by expression of PKB-AAA (7).

Because of the apparent implications on cellular transformation, extensive research on PKB-mediated survival has been performed in PTEN-negative tumor cell-lines. Several of such cell-lines die upon PTEN re-expression and additional overexpression of an active form of PKB counteracts this (69, 70, 71). Nevertheless, the reversal of tumor formation by increasing apoptosis through expression of a dominant-negative mutant of PKB in PTEN-negative cell-lines has never been shown. Moreover, recent studies suggest that PKB-mediated tumor formation induced by loss of PTEN function might actually primarily be due to an effect on proliferation rather than survival (72, 73). This is in agreement with other recent findings that inhibition of interleukin-3 (IL3)-regulated PI(3)K function by conditional expression of dominant-negative PI(3)K (Δp85) in Ba/F3 cells mainly affects proliferation but not apoptosis, despite the fact that these cells die rapidly upon IL3 withdrawal (74).

PKB and the control of cell proliferation

One of the first studies to describe an involvement of PI(3)K in mitogenic signalling showed that micro-injection of neutralizing antibodies to the p110α catalytic subunit of PI(3)K blocks the initiation of DNA synthesis by PDGF- and EGF-receptor signalling in NIH-3T3 cells (75). Another study showed that conditional activation of PI(3)K in serum-deprived cells can induce cell-cycle re-entry and DNA synthesis (76). Furthermore, the mast cell c-Kit receptor transduces mitogenic but not anti-apoptotic signals through the PI(3)K pathway (77), and reduction of PDK1 protein levels by an antisense oligonucleotide approach inhibits $p70^{S6k}$ and PKB activities, which results in a decrease in cell doublings and an increase in apoptosis (78). Also, small cell lung cancer cells have constitutively activated PI(3)K which mediates anchorage-independent growth of these cells presumably via PKB and $p70^{S6k}$ (79). Subsequently, the PI(3)K/PKB pathway was shown to decrease expression of the $p27^{kip1}$ cell-cycle inhibitor and to increase expression of the D-type G1-phase cyclin, possibly via inhibition of GSK3 (figure 2) (80, 81, 82). GSK3 was shown to phosphorylate cyclin D1 on threonine 286 resulting in both decreased stability and a redistribution of cyclin D1 from the nucleus to the cytoplasm (81, 82).

Other targets for PKB in the control of cellular proliferation may include the cell-cycle inhibitor $p21^{waf1/cip1}$ via direct inhibition of the Raf kinase in human breast cancer cells (83), and the proto-oncogene c-myc (5). Importantly, embryos of *mPTEN* knockout mice display regions of increased proliferation (7), and re-expression of PTEN in PTEN-negative tumor cells results in an increase in protein levels of the $p27^{kip1}$ cell-cycle inhibitor and a concomitant G1 cell-cycle arrest (6, 11, 84, 85, 86). In these cells neither $p21^{waf1/cip1}$ nor cyclin D1 protein levels were affected. This suggested that the control of $p27^{kip1}$ protein levels might play a major role in the regulation of the G1/S transition by the PI(3)K pathway in PTEN-negative tumor cells. Nevertheless, an important question remained unanswered: What are the targets of PI(3)K signalling that can mediate cell-cycle progression through the regulation of the $p27^{kip1}$ protein? Recently, those targets were identified and shown to be three PKB-regulated Forkhead transcription factors.

Forkhead transcription factors in PKB signal transduction

The superfamily of Forkhead transcription factors consists of approximately ninety members with orthologues expressed in an array of species ranging from yeast to man (reviewed in (87)). All members of the family show high sequence homology within their DNA-binding 'winged-helix' domain to the fork head protein of *Drosophila melanogaster*, which is involved in the formation of terminal structures in the early fly embryo (88). The prototype of Forkheads in mammals are the α, β and γ isoforms of the hepatic nuclear factor 3 (HNF3). HNF3 is a liver-enriched transcription factor that was identified as an activator of liver-specific gene expression in rat (89).

A subset of Forkhead transcription factors has been associated with tumorigenesis. The Forkhead gene *qin* of the ASV-31 virus is responsible for the transforming activity of the virus and is closely related to the telencephalon-specific brain factor-1 (BF-1), a Forkhead transcription factor of which knockout mice show severe cerebral hemisphere abnormalities (90, 91). Furthermore, in acute lymphoid leukemias (ALL), the vast majority of chromosomal translocations that cause the ALL disrupt the gene for the mixed-lineage leukemia (MLL) transcription factor (92). The proteins resulting from such chromosomal breaks are fusion proteins consisting of the DNA binding domain of MLL fused to the transactivation domain of another transcription factor. Two of those other transcription factors are the Forkhead genes *AFX* and *AF6q21* (93, 94). A similar event occurs in rhabdomyosarcomas. Here, a chromosomal translocation results in a fusion between the PAX3 DNA-binding domain and the transactivation domain of the Forkhead protein FKHR (95).

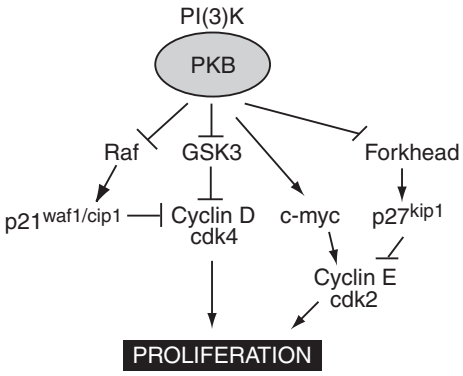

Figure 2. *Targets of PKB in the control of cell-cycle progression.* PKB can decrease the expression of the cell-cycle inhibitors $p27^{kip1}$ and $p21^{waf1/cip1}$ via inactivation of the Forkhead transcription factors AFX, FKHR and FKHR-L1, and Raf, respectively. Additional contributions of PKB to cell-cycle progression include activation of cdk4 via an increase in cyclin D1 protein levels and nuclear localization. Finally, PKB can increase protein levels of c-myc.

Initial evidence for the control of Forkhead-mediated transcription by the PI(3)K pathway came from studies performed on the nematode *Caenorhabditis elegans*. Two groups independently reported a pathway in the worm that controls lifespan and dauer formation, a developmental stage of the animal by which it ensures survival of adverse conditions by lowering its metabolism and closing its mouth and anus. This way, it can live up to ten times longer than a normal adult. When conditions turn for the better, the worm goes back into the development program and lives a normal fifteen day adult life (reviewed in (96)). Genes that regulate this dauer formation are called *daf* genes.

Two studies showed that the pathway controlling dauer formation is regulated by pheromones that activate the DAF-16 protein (97, 98). DAF-16 in turn is negatively regulated by DAF-2 via AGE-1 (also known as DAF-23). Surprisingly, DAF-2 is an InsR-like protein, AGE-1 is PI(3)K-like and DAF-16 turned out to be a transcription factor of the Forkhead family with highest homology to mammalian AFX, FKHR and FKHR-L1 (figure 3) (93, 97, 98, 99, 100, 101, 102). Soon thereafter it was shown in

mammalian systems that insulin- and IGF-I-activated PKB directly phosphorylates the DAF-16-like Forkheads on two or three residues (T24, S253 and S316 in FKHR) thereby inactivating them (103, 104, 105, 106). Subsequently, also a PKB-like (AKT1, AKT2), a PDK1-like (PDK1) and a PTEN-like (DAF-18) protein were placed in the *C. elegans* dauer formation pathway, suggesting a signalling route from the InsR to Forkheads that is fully conserved between worm and man (107, 108, 109).

In mammalian cells PKB regulates Forkhead activity by excluding them from the nucleus (figure 4). For human AFX, FKHR-L1 and FKHR and for mouse FKHR1 it was shown that phosphorylation by PKB upon insulin-, IGF-I-, serum- or EGF-treatment of cells relocates the transcription factors from the nucleus to the cytoplasm ((103, 110, 111, 112) and chapter 4). Obviously, this results in the inhibition of gene transcription of Forkhead target genes, as shown by reporter assays using either an IGFBP-1 promoter, an IRE from the Fas ligand (FasL) promoter or a recently identified optimal DAF-16 binding element (DBE) (103, 104, 106, 113).

The mechanism as to how PKB-mediated phosphorylation of the Forkheads causes their relocalization to the cytoplasm is poorly understood. One report uses progressive C-terminal truncations to identify a region in mouse FKHR1 required for nuclear export. In that region, near a PKB phosphorylation site, lies a sequence that conforms to a classical nuclear export signal (NES) and this NES might be functionally modified by phosphorylation of that PKB site (figure 4) (110). Alternatively, Forkheads might be excluded from the nucleus by cytoplasmic retention due to phosphorylation-dependent binding of Forkheads to 14-3-3ζ proteins ((103) and chapter 4). The formation of a Forkhead/14-3-3 complex as observed for FKHR-L1 and AFX could, however, have a function different from cytoplasmic retention. The 14-3-3 protein Rad24, for instance, binds the mitotic regulator Cdc25 in the nucleus and thereby provides Cdc25 with a NES (114). Since the intrinsic NES-like sequence of AFX does not seem to function as a NES (see discussion of

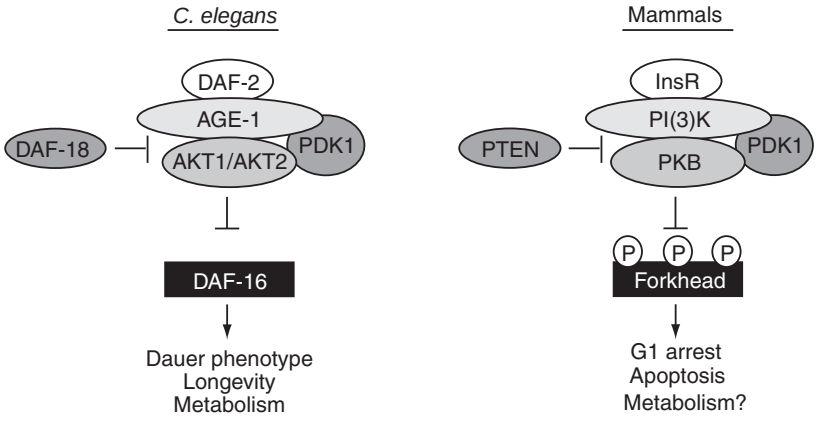

Figure 3. *PI(3)K-mediated inhibition of Forkhead transcription factors is conserved between C. elegans and mammals.* In both nematode and mammalian cells, the insulin receptor (DAF-2) activates PKB (AKT1/AKT2) via PI(3)K (AGE-1) and PDK1 (PDK1). Conversely, PTEN (DAF-18) inactivates the pathway. Activation of PKB (AKT1/AKT2) leads to inactivation of Forkhead transcription factors (DAF-16), at least in mammalian systems via direct phosphorylation by PKB. These Forkheads control dauer formation, lifespan and metabolism in *C. elegans*, and cell-cycle progression, apoptosis and metabolism in mammals. See text for further details.

chapter 4) and since it has not been shown that Forkheads complex with 14-3-3 in the cytoplasm, the possibility remains that 14-3-3 proteins, like Rad24, are required for nuclear export of the Forkheads. Finally, phosphorylation of the Forkheads can inactivate a nuclear localization signal (NLS) (chapter 4). AFX continuously shuttles between the nucleus and the cytoplasm and PKB-mediated phosphorylation on serine 193 inactivates a NLS that surrounds that residue (chapter 4). Subsequently, the steady state localization of AFX shifts from predominantly nuclear to predominantly cytoplasmic (figure 4).

The exact mechanism as to how insulin and IGF-I inhibit the Forkheads might be more complex than portrayed above. AFX, for example, is phosphorylated by an as yet unidentified Ras/Ral-dependent but PI(3)K-independent kinase and this phosphorylation contributes to insulin-mediated inhibition of AFX (106). Furthermore, it was recently argued that the IGF-I-regulated T24 kinase of FKHR is not PKB and that S315 in FKHR-L1 is preferentially phosphorylated by the PI(3)K-dependent serum- and glucocorticoid-inducible kinase (SGK) (figure 5) (115, 116). How all these kinases contribute to the insulin- and IGF-I-induced inhibition of the Forkheads still remains to be elucidated, but possibly Forkhead-specific and growth factor-specific elements are involved as was suggested for T24 phosphorylation of FKHR (115).

A role for Forkheads in PKB-mediated cellular transformation?

Despite the complex regulatory mechanism of insulin- and IGF-I-mediated control of the Forkheads, one thing is clear: All three transcription factors are efficiently inhibited by PKB-dependent phosphorylation. In this way, PKB decreases expression of the genes encoding FasL, Bim and $p27^{kip1}$ that cause apoptosis, especially in haematopoietic cells (figure 1) (103, 111, 117, 118, 119). Jurkat T-cells expressing a triple mutant of FKHR-L1 that cannot be phosphorylated by PKB and therefore cannot be inhibited by PKB show a twofold increase in apoptosis compared to control (103). This induction of cell death by FKHR-L1 depends on the presence of a functional receptor for FasL, the Fas protein, since Jurkat cells expressing a mutant of Fas that is unable to respond to FasL do not die upon expression of the active Forkhead. Besides FasL, FKHR-L1 has been shown to regulate the expression of Bim, a pro-apoptotic member of the Bcl2 family which is expressed in haematopoietic cells (120, 121). The mouse pre-B cell line Ba/F3 requires IL-3 for its

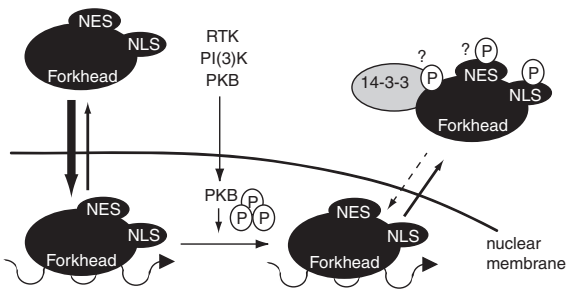

Figure 4. *PKB-mediated nuclear exclusion of Forkhead transcription factors.* In quiescent cells (left), steady-state localization of Forkheads is nuclear, although the Forkhead can still shuttle between nucleus and cytoplasm. Growth factor treatment of cells activates PKB, which results in phosphorylation of the Forkheads. The steady state localization of phosphorylated Forkheads is then cytoplamic which is caused by functional inactivation of a NLS located around the phosphorylated serine in the DNA binding domain (serine 193 in AFX, chapter 4) and/or cytoplasmic retention due to 14-3-3 binding and/or activation of a NES, although the latter two mechanisms have yet to be proven.

survival and this is mediated by the PI(3)K/PKB pathway. IL-3 withdrawal causes apoptosis which coincides with an activation of the Forkhead FKHR-L1 and a subsequent increase in the expression of the gene for Bim (118). Interestingly, in the same system the cell-cycle inhibitor $p27^{kip1}$ is upregulated upon Forkhead activation and this was shown to contribute to cell death induced by cytokine withdrawal of mouse bone marrow cells (119). As FKHR-L1, the other two Forkheads can also induce apoptosis. FKHR overexpression results in the death of 293T-cells and AFX overexpression causes apoptosis in CHO-K1 cells (111, 117). Nevertheless, proper proof for the involvement of any of the three Forkhead transcription factors in the induction of apoptosis by inactivation of the PI(3)K/PKB pathway has not been provided, since no study has yet used an inhibitor of the Forkheads to reduce apoptosis upon, for instance, cytokine withdrawal.

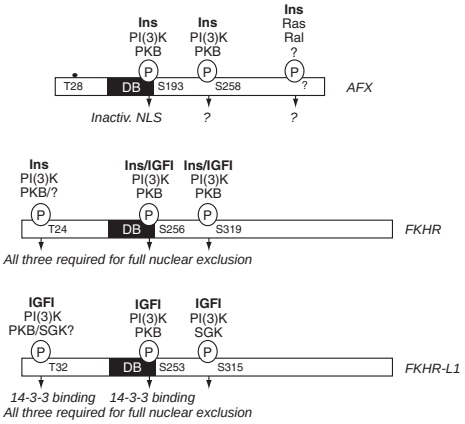

Figure 5. *Forkhead-kinases and their effects on Forkhead function.* Insulin-activated PKB phosphorylates AFX on serines 193 (S193) and 258 (S258), and phosphorylation of S193 results in the functional inactivation of a NLS causing nuclear exclusion. Neither the kinase for the Ral-dependent site nor the site itself is known, and the effects of the phosphorylation of S258 and the Ral-dependent residue on AFX function are unknown. Although PKB can phosphorylate FKHR on all three residues in a PKB consensus site, T24 is possibly not phosphorylated by PKB upon insulin treatment of cells and possibly not phosphorylated at all upon IGF-I-treatment. Phosphorylation of S256 is required for full inhibition of FKHR, but all three residues seem to participate in nuclear exclusion. FKHR-L1 is phosphorylated by at least two kinases: PKB and SGK. Although both kinases can phosphorylate all three residues in a PKB consensus site upon IGF-I-treatment of cells, PKB preferentially phosphorylates S253 whereas SGK prefers S315. Both kinases can act on T32, but whether PKB or SGK is the *in vivo* T32-kinase remains to be investigated. As for FKHR, all three residues seem to be required for nuclear exclusion, but only T32 and S253 bind 14-3-3 proteins. DB; DNA binding domain.

In addition to their role in apoptosis, a surprising new function was recently described for the Forkheads. Overexpression of AFX, FKHR and FKHR-L1 in a variety of cell-lines, including Ras-transformed and PTEN-negative cells, results in a G1 cell-cycle arrest (116, 122, 123). In two of those studies it was shown that this is caused by an increase in the expression of the $p27^{kip1}$ cell-cycle inhibitor, although other cell-cycle regulatory targets for the Forkheads are likely to exist (122, 123). Notably, activation of PKB can revert the cell-cycle arrest imposed by Forkhead expression and a putative dominant-negative Forkhead mutant can partially rescue the inhibition of cyclin E/cdk2 kinase activity by an inhibitor of PI(3)K (123). Therefore, PKB contributes to cell-cycle progression by inhibiting these Forkheads thus reducing $p27^{kip1}$ expression.

Altogether, this raises the exciting possibility that tumors that contain oncogenic Ras or that carry PTEN mutations have aquired the capacity of enhanced proliferation and/or survival, at least in part by inhibiting the DAF-16-like Forkhead transcription factors (figure 3). Indeed, PTEN-negative renal and prostate carcinoma cell-lines show increased Forkhead phosphorylation and an increased ratio of cytoplasmic versus nuclear localization compared to renal and prostate carcinoma cell-lines that are PTEN-positive (122). Importantly, overexpression of Forkheads in such PTEN-negative cells inhibits proliferation by causing

either apoptosis (PTEN-negative prostate carcinoma) or cell-cycle arrest (PTEN-negative renal carcinoma and glioma; Ki-RasL61-transformed Rat1 fibroblasts) (122, 123), further emphasizing the putative importance of Forkhead inactivation for the enhanced proliferation of certain types of tumor cells.

Concluding remarks

The PI(3)K/PKB pathway participates in oncogenesis at multiple levels. Not only does it influence metabolism and translation, it also antagonizes apoptotic signals and overcomes blocks imposed on cell-cycle progression. In this respect, the recently identified PKB substrates AFX, FKHR and FKHR-L1 are of particular interest, for they can regulate the expression of cell-cycle inhibitory genes but also of pro-apoptotic and metabolic genes (124). These Forkhead transcription factors may be key players in tumor formation caused by uncontrolled activation of the PI(3)K pathway.

The entire signalling cascade from the InsR to Forkheads is conserved between humans and *C. elegans*, and in the nematode, DAF-16 regulates longevity but not apoptosis. It is of interest to note that the cell-cycle arrest induced by Forkhead ativation in mammalian cells does not precede apoptosis but rather represents an exit from the cell-cycle (unpublished observations, chapter 6). With respect to the contribution of Forkheads to cellular transformation, this might imply that Forkhead inactivation is a prerequisite for re-entry into the cell-cycle of certain types of tumor cells.

The gene targets of DAF-16 that have been identified to date and that mediate the effect of DAF-16 on *C. elegans* lifespan are cytosolic catalase (*ctl-1*) and manganese superoxide dismutase (*sod-3*) (125, 126). Possibly also in humans free radical-scavenging enzymes are targets of AFX, FKHR and FKHR-L1 and might contribute to the regulation of apoptosis, cell-cycle progression and tumor formation by PI(3)K/PKB, and conversely DAF-16 in *C. elegans* might regulate the expression of a cell-cycle inhibitor. Preliminary experiments from our laboratory in fact suggest that indeed the DAF-16-like Forkheads cause enhanced protection form oxidative damage in human cells by increasing protein levels of manganese superoxide dismutase (unpublished observations, chapter 7).

In conclusion, the RTK/PI(3)K/PKB/Forkhead pathway seems a very good candidate for providing critical oncogenic signals to cells and identifying all gene targets of the Forkheads might prove to be important in understanding the transformed phenotype of, for instance, PTEN-negative tumor cells.

Outline of this thesis

Four years ago, a role for PKB in the regulation of the expression of genes that contribute to processes like cell-cycle control and glycogen metabolism was apparent (5, 12). However, no PKB-controlled transcription factors that could explain the observed effects of PKB on gene transcription had been found. We set out to identify substrates of PKB involved in transcriptional regulation.

In *chapter 3*, we describe the identification of the AFX Forkhead transcription factor as a bonafide PKB effector in insulin signalling. This study was inspired by previous observations in *C. elegans* that showed control of the DAF-16 Forkhead transcription factor by an InsR/PI(3)K-like pathway. We confirm and extend those observations by showing that PKB directly phosphorylates AFX on two serine residues causing its inhibition. In addition, we provide evidence that Ras-mediated activation of Ral cooperates with PKB in insulin-mediated inhibition of AFX.

In *chapter 4*, we illustrate in detail how AFX is inhibited upon phosphorylation by PKB.

We show that in the absence of PKB activity, the steady-state of AFX localization is nuclear but the protein is actively shuttled between the cytoplasm and the nucleus. Phosphorylation by PKB on one of the two designated serines causes masking of a NLS in AFX leading to cytoplasmic retention and thus inhibition of the Forkhead.

In *chapter 5*, we identify a transcriptional target for the AFX-like Forkheads. Transcription of the gene for the $p27^{kip1}$ cell-cycle inhibitor is shown to be increased upon Forkhead expression, leading to inhibition of cell proliferation of many cell-types including tumor-derived cell lines lacking PTEN activity. Activation of PKB and Ras might thus contribute to cellular proliferation via the inhibition of Forkheads.

Chapter 6 deals with the different responses of distinct cell-types to Forkhead activity. We show that non-haematopoietic cell-types do not undergo FKHR-L1-induced apoptosis as haematopoietic cells do, but rather enter G0 and remain quiescent. In addition, we identify a second putative gene target for the Forkheads, namely the p130 pocket protein, a pRb-like protein known to be involved in the maintenance of quiescence.

Finally, in *chapter 7* we show that induction of quiescence by Forkheads is coupled to enhanced protection from oxidative damage via direct promoter control of the gene for manganese superoxide dismutase (MnSOD). In this chapter we pose that this coupling is required for survival of the quiescent cells. In conclusion, we have uncovered the Forkhead transcription factor AFX as part of the PI(3)K/PKB and Ras/Ral signalling cascades both proposed to be involved in cellular transformation. Moreover, we show that AFX can regulate the expression of the genes for $p27^{kip1}$, p130 and MnSOD, providing new insights into why these pathways contribute to tumor formation. The possible consequences of these findings are discussed in *chapter 8.*

References

1. C. Jimenez, et al., Identification and characterization of a new oncogene derived from the regulatory subunit of phosphoinositide 3-kinase. *Embo J* **17**, 743-53 (1998).
2. L. R. Borlado, et al., Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation in vivo. *Faseb J* **14**, 895-903 (2000).
3. H. W. Chang, et al., Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. *Science* **276**, 1848-50 (1997).
4. Y. Y. Ma, et al., PIK3CA as an oncogene in cervical cancer. *Oncogene* **19**, 2739-44 (2000).
5. T. Skorski, et al., Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. *Embo J* **16**, 6151-61 (1997).
6. D. M. Li, H. Sun, PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. *Proc Natl Acad Sci U S A* **95**, 15406-11 (1998).
7. V. Stambolic, et al., Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39 (1998).
8. L. C. Cantley, B. G. Neel, New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc Natl Acad Sci U S A* **96**, 4240-5 (1999).
9. M. Aoki, O. Batista, A. Bellacosa, P. Tsichlis, P. K. Vogt, The akt kinase: molecular determinants of oncogenicity. *Proc Natl Acad Sci U S A* **95**, 14950-5 (1998).
10. A. M. Mirza, A. D. Kohn, R. A. Roth, M. McMahon, Oncogenic transformation of cells by a conditionally active form of the protein kinase Akt/PKB. *Cell Growth Differ* **11**, 279-92 (2000).
11. J. R. Graff, et al., Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. *J Biol Chem* **275**, 24500-5 (2000).
12. A. D. Kohn, et al., Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J Biol Chem* **273**, 11937-43 (1998).
13. A. Barthel, et al., Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. *J Biol Chem* **274**, 20281-6 (1999).
14. D. A. Cross, D. R. Alessi, P. Cohen, M. Andjelkovich, B. A. Hemmings, Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **378**, 785-9 (1995).
15. P. C. van Weeren, K. M. de Bruyn, A. M. de Vries-Smits, J. van Lint, B. M. Burgering, 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 (1998).

16. J. Deprez, D. Vertommen, D. R. Alessi, L. Hue, M. H. Rider, 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 (1997).
17. L. Bertrand, et al., 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 (1999).
18. T. Kitamura, et al., Insulin-induced phosphorylation and activation of cyclic nucleotide phosphodiesterase 3B by the serine-threonine kinase Akt. *Mol Cell Biol* **19**, 6286-96 (1999).
19. S. B. Cichy, et al., Protein kinase B/Akt mediates effects of insulin on hepatic insulin- like growth factor-binding protein-1 gene expression through a conserved insulin response sequence. *J Biol Chem* **273**, 6482-7 (1998).
20. J. Liao, A. Barthel, K. Nakatani, R. A. Roth, Activation of protein kinase B/Akt is sufficient to repress the glucocorticoid and cAMP induction of phosphoenolpyruvate carboxykinase gene. *J Biol Chem* **273**, 27320-4 (1998).
21. D. Wang, H. S. Sul, Insulin stimulation of the fatty acid synthase promoter is mediated by the phosphatidylinositol 3-kinase pathway. Involvement of protein kinase B/Akt. *J Biol Chem* **273**, 25420-6 (1998).
22. D. Schmoll, et al., Regulation of Glucose-6-phosphatase Gene Expression by Protein Kinase Balpha and the Forkhead Transcription Factor FKHR. *J Biol Chem* **275**, 36324-36333 (2000).
23. T. A. Smith, Facilitative glucose transporter expression in human cancer tissue. *Br J Biomed Sci* **56**, 285-92 (1999).
24. P. Polakis, Wnt signaling and cancer. *Genes Dev* **14**, 1837-51 (2000).
25. H. Aberle, A. Bauer, J. Stappert, A. Kispert, R. Kemler, beta-catenin is a target for the ubiquitin-proteasome pathway. *Embo J* **16**, 3797-804 (1997).
26. M. Bienz, H. Clevers, Linking colorectal cancer to Wnt signaling. *Cell* **103**, 311-20 (2000).
27. T. C. He, et al., Identification of c-MYC as a target of the APC pathway. *Science* **281**, 1509-12 (1998).
28. O. Tetsu, F. McCormick, Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature* **398**, 422-6 (1999).
29. V. W. Ding, R. H. Chen, F. McCormick, Differential regulation of glycogen synthase kinase 3beta by insulin and wnt signaling *J Biol Chem* **275**, 32475-81 (2000).
30. R. P. de Groot, J. Auwerx, M. Bourouis, P. Sassone-Corsi, Negative regulation of Jun/AP-1: conserved function of glycogen synthase kinase 3 and the Drosophila kinase shaggy. *Oncogene* **8**, 841-7 (1993).
31. A. C. Gingras, S. G. Kennedy, M. A. O'Leary, N. Sonenberg, N. Hay, 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev* **12**, 502-13 (1998).
32. B. M. Burgering, P. J. Coffer, Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602 (1995).
33. A. Sekulic, et al., A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. *Cancer Res* **60**, 3504-13 (2000).
34. A. Dufner, M. Andjelkovic, B. M. Burgering, B. A. Hemmings, G. Thomas, Protein kinase B localization and activation differentially affect S6 kinase 1 activity and eukaryotic translation initiation factor 4E-binding protein 1 phosphorylation. *Mol Cell Biol* **19**, 4525-34 (1999).
35. M. Aoki, E. Blazek, P. K. Vogt, A role of the kinase mTOR in cellular transformation induced by the oncoproteins P3k and Akt. *Proc Natl Acad Sci U S A* **98**, 136-141 (2001).
36. A. Lazaris-Karatzas, K. S. Montine, N. Sonenberg, Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature* **345**, 544-7 (1990).
37. D. Rousseau, A. C. Gingras, A. Pause, N. Sonenberg, The eIF4E-binding proteins 1 and 2 are negative regulators of cell growth. *Oncogene* **13**, 2415-20 (1996).
38. R. Yao, G. M. Cooper, Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science* **267**, 2003-6 (1995).
39. R. Yao, G. M. Cooper, Growth factor-dependent survival of rodent fibroblasts requires phosphatidylinositol 3-kinase but is independent of pp70S6K activity. *Oncogene* **13**, 343-51 (1996).
40. H. Dudek, et al., Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* **275**, 661-5 (1997).
41. E. Yang, et al., Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* **80**, 285-91 (1995).
42. S. R. Datta, et al., Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231-41 (1997).
43. J. Zha, H. Harada, E. Yang, J. Jockel, S. J. Korsmeyer, Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87**, 619-28 (1996).
44. S. Kitada, et al., Expression and location of pro-apoptotic Bcl-2 family protein BAD in normal human tissues and tumor cell lines. *Am J Pathol* **152**, 51-61 (1998).
45. J. Downward, How BAD phosphorylation is good for survival. *Nat Cell Biol* **1**, E33-5 (1999).
46. M. H. Cardone, et al., Regulation of cell death protease

caspase-9 by phosphorylation. *Science* **282**, 1318-21 (1998).
47. J. Rodriquez, H. H. Chen, S. C. Lin, Caspase phosphorylation, cell death, and species variability. *Science* **287**, 1363 (2000).
48. K. Du, M. Montminy, CREB is a regulatory target for the protein kinase Akt/PKB. *J Biol Chem* **273**, 32377-9 (1998).
49. S. Pugazhenthi, et al., Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J Biol Chem* **275**, 10761-6 (2000).
50. A. Bonni, et al., Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* **286**, 1358-62 (1999).
51. M. J. May, S. Ghosh, Rel/NF-kappa B and I kappa B proteins: an overview. *Semin Cancer Biol* **8**, 63-73 (1997).
52. O. N. Ozes, et al., NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* **401**, 82-5 (1999).
53. J. A. Romashkova, S. S. Makarov, NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* **401**, 86-90 (1999).
54. R. G. Jones, et al., Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo. *J Exp Med* **191**, 1721-34 (2000).
55. S. Dimmeler, et al., Activation of nitric oxide synthase in endothelial cells by Akt- dependent phosphorylation. *Nature* **399**, 601-5 (1999).
56. D. Fulton, et al., Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**, 597-601 (1999).
57. S. Dimmeler, A. M. Zeiher, Nitric oxide-an endothelial cell survival factor. *Cell Death Differ* **6**, 964-8 (1999).
58. N. M. Mazure, E. Y. Chen, K. R. Laderoute, A. J. Giaccia, Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood* **90**, 3322-31 (1997).
59. B. H. Jiang, J. Z. Zheng, M. Aoki, P. K. Vogt, Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc Natl Acad Sci U S A* **97**, 1749-53 (2000).
60. H. Zhong, et al., Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* **60**, 1541-5 (2000).
61. H. Ichijo, et al., Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* **275**, 90-4 (1997).
62. A. H. Kim, G. Khursigara, X. Sun, T. F. Franke, M. V. Chao, Akt Phosphorylates and Negatively Regulates Apoptosis Signal-Regulating Kinase 1. *Mol Cell Biol* **21**, 893-901 (2001).
63. A. Khwaja, P. Rodriguez-Viciana, S. Wennstrom, P. H. Warne, J. Downward, Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *Embo J* **16**, 2783-93 657-8501, Japan. (1997).
64. A. Kauffmann-Zeh, et al., Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385**, 544-8 (1997).
65. T. Kitamura, et al., Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Mol Cell Biol* **18**, 3708-17 (1998).
66. Q. Wang, et al., Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol Cell Biol* **19**, 4008-18 (1999).
67. S. Yano, H. Tokumitsu, T. R. Soderling, Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. *Nature* **396**, 584-7 (1998).
68. S. Attwell, C. Roskelley, S. Dedhar, The integrin-linked kinase (ILK) suppresses anoikis. *Oncogene* **19**, 3811-5 (2000).
69. M. A. Davies, et al., Adenoviral transgene expression of MMAC/PTEN in human glioma cells inhibits Akt activation and induces anoikis. *Cancer Res* **58**, 5285-90 (1998).
70. J. Li, et al., The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. *Cancer Res* **58**, 5667-72 (1998).
71. W. Wick, F. B. Furnari, U. Naumann, W. K. Cavenee, M. Weller, PTEN gene transfer in human malignant glioma: sensitization to irradiation and CD95L-induced apoptosis. *Oncogene* **18**, 3936-43 (1999).
72. M. A. Davies, et al., Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN. *Cancer Res* **59**, 2551-6 (1999).
73. S. Ramaswamy, et al., Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc Natl Acad Sci U S A* **96**, 2110-5 (1999).
74. B. L. Craddock, E. A. Orchiston, H. J. Hinton, M. J. Welham, Dissociation of apoptosis from proliferation, protein kinase B activation, and BAD phosphorylation in interleukin-3-mediated phosphoinositide 3-kinase signaling. *J Biol Chem* **274**, 10633-40 (1999).
75. S. Roche, M. Koegl, S. A. Courtneidge, The phosphatidylinositol 3-kinase alpha is required for DNA synthesis induced by some, but not all, growth factors. *Proc Natl Acad Sci U S A* **91**, 9185-9 (1994).
76. A. Klippel, et al., Activation of phosphatidylinositol 3-kinase is sufficient for cell cycle entry and promotes cellular changes characteristic of oncogenic

transformation. *Mol Cell Biol* **18**, 5699-711 (1998).
77. H. Serve, et al., Differential roles of PI3-kinase and Kit tyrosine 821 in Kit receptor-mediated proliferation, survival and cell adhesion in mast cells. *Embo J* **14**, 473-83 (1995).
78. P. Flynn, M. Wongdagger, M. Zavar, N. M. Dean, D. Stokoe, Inhibition of PDK-1 activity causes a reduction in cell proliferation and survival. *Curr Biol* **10**, 1439-1442 (2000).
79. S. M. Moore, et al., The presence of a constitutively active phosphoinositide 3-kinase in small cell lung cancer cells mediates anchorage-independent proliferation via a protein kinase B and p70s6k-dependent pathway. *Cancer Res* **58**, 5239-47 (1998).
80. P. Brennan, et al., Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity* **7**, 679-89 (1997).
81. J. A. Diehl, M. Cheng, M. F. Roussel, C. J. Sherr, Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes Dev* **12**, 3499-511 (1998).
82. R. C. Muise-Helmericks, et al., Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. *J Biol Chem* **273**, 29864-72 (1998).
83. S. Zimmermann, K. Moelling, Phosphorylation and regulation of Raf by Akt (protein kinase B). *Science* **286**, 1741-4 (1999).
84. I. W. Cheney, S. T. Neuteboom, M. T. Vaillancourt, M. Ramachandra, R. Bookstein, Adenovirus-mediated gene transfer of MMAC1/PTEN to glioblastoma cells inhibits S phase entry by the recruitment of p27Kip1 into cyclin E/CDK2 complexes. *Cancer Res* **59**, 2318-23 (1999).
85. Y. Lu, et al., The PTEN/MMAC1/TEP tumor suppressor gene decreases cell growth and induces apoptosis and anoikis in breast cancer cells. *Oncogene* **18**, 7034-45 (1999).
86. H. Sun, et al., PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. *Proc Natl Acad Sci U S A* **96**, 6199-204 (1999).
87. E. Kaufmann, W. Knochel, Five years on the wings of fork head. *Mech Dev* **57**, 3-20 (1996).
88. D. Weigel, G. Jurgens, F. Kuttner, E. Seifert, H. Jackle, The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. *Cell* **57**, 645-58 (1989).
89. R. H. Costa, D. R. Grayson, J. E. Darnell, Jr., Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and alpha 1-antitrypsin genes. *Mol Cell Biol* **9**, 1415-25 (1989).
90. S. Xuan, et al., Winged helix transcription factor BF-1 is essential for the development of the cerebral hemispheres. *Neuron* **14**, 1141-52 (1995).
91. J. Li, H. Thurm, H. W. Chang, J. S. Iacovoni, P. K. Vogt, Oncogenic transformation induced by the Qin protein is correlated with transcriptional repression. *Proc Natl Acad Sci U S A* **94**, 10885-8 (1997).
92. N. R. McCabe, et al., Cloning of cDNAs of the MLL gene that detect DNA rearrangements and altered RNA transcripts in human leukemic cells with 11q23 translocations. *Proc Natl Acad Sci U S A* **89**, 11794-8 (1992).
93. A. Borkhardt, et al., 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 (1997).
94. J. Hillion, M. Le Coniat, P. Jonveaux, R. Berger, O. A. Bernard, AF6q21, a novel partner of the MLL gene in t(6;11)(q21;q23), defines a forkhead transcriptional factor subfamily. *Blood* **90**, 3714-9 (1997).
95. J. E. Sublett, I. S. Jeon, D. N. Shapiro, The alveolar rhabdomyosarcoma PAX3/FKHR fusion protein is a transcriptional activator. *Oncogene* **11**, 545-52 (1995).
96. J. H. Thomas, Chemosensory regulation of development in C. elegans. *Bioessays* **15**, 791-7 (1993).
97. K. Lin, J. B. Dorman, A. Rodan, C. Kenyon, daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science* **278**, 1319-22 (1997).
98. S. Ogg, et al., The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature* **389**, 994-9 (1997).
99. N. Galili, et al., Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. *Nat Genet* **5**, 230-5 (1993).
100. J. Z. Morris, H. A. Tissenbaum, G. Ruvkun, A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. *Nature* **382**, 536-9 (1996).
101. K. D. Kimura, H. A. Tissenbaum, Y. Liu, G. Ruvkun, daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. *Science* **277**, 942-6 (1997).
102. M. J. Anderson, C. S. Viars, S. Czekay, W. K. Cavenee, K. C. Arden, Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. *Genomics* **47**, 187-99 (1998).
103. A. Brunet, et al., Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-68 (1999).
104. S. Guo, et al., Phosphorylation of Serine 256 by Protein Kinase B Disrupts Transactivation by FKHR and Mediates Effects of Insulin on Insulin-like Growth Factor-binding Protein-1 Promoter Activity through a Conserved Insulin Response Sequence. *J Biol Chem* **274**, 17184-17192 (1999).
105. G. Rena, S. Guo, S. C. Cichy, T. G. Unterman, P. Cohen, Phosphorylation of the Transcription Factor Forkhead Family Member FKHR by Protein Kinase B. *J Biol Chem* **274**, 17179-17183 (1999).

106. G. J. Kops, et al., Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**, 630-4 (1999).
107. S. Ogg, G. Ruvkun, The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. *Mol Cell* **2**, 887-93 (1998).
108. S. Paradis, G. Ruvkun, 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 (1998).
109. S. Paradis, M. Ailion, A. Toker, J. H. Thomas, G. Ruvkun, A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans. *Genes Dev* **13**, 1438-1452 (1999).
110. W. H. Biggs, 3rd, J. Meisenhelder, T. Hunter, W. K. Cavenee, K. C. Arden, Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A* **96**, 7421-7426 (1999).
111. H. Takaishi, et al., Regulation of nuclear translocation of forkhead transcription factor AFX by protein kinase B. *Proc Natl Acad Sci U S A* **96**, 11836-41 (1999).
112. J. G. Jackson, J. I. Kreisberg, A. P. Koterba, D. Yee, M. G. Brattain, Phosphorylation and nuclear exclusion of the forkhead transcription factor FKHR after epidermal growth factor treatment in human breast cancer cells. *Oncogene* **19**, 4574-81 (2000).
113. T. Furuyama, T. Nakazawa, I. Nakano, N. Mori, Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* **349**, 629-634 (2000).
114. A. Lopez-Girona, B. Furnari, O. Mondesert, P. Russell, Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature* **397**, 172-5 (1999).
115. J. Nakae, V. Barr, D. Accili, Differential regulation of gene expression by insulin and IGF-1 receptors correlates with phosphorylation of a single amino acid residue in the forkhead transcription factor FKHR. *Embo J* **19**, 989-96 (2000).
116. A. Brunet, et al., Protein Kinase SGK Mediates Survival Signals by Phosphorylating the Forkhead Transcription Factor FKHRL1 (FOXO3a). *Mol Cell Biol* **21**, 952-965 (2001).
117. E. D. Tang, G. Nu#ez, F. G. Barr, K. L. Guan, Negative Regulation of the Forkhead Transcription Factor FKHR by Akt. *J Biol Chem* **274**, 16741-16746 (1999).
118. P. F. Dijkers, R. H. Medemadagger, J. J. Lammers, L. Koenderman, P. J. Coffer, Expression of the pro-apoptotic bcl-2 family member bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* **10**, 1201-4 (2000).
119. P. F. Dijkers, et al., Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol Cell Biol* **20**, 9138-48 (2000).
120. L. O'Connor, et al., Bim: a novel member of the Bcl-2 family that promotes apoptosis. *Embo J* **17**, 384-95 (1998).
121. P. Bouillet, et al., Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science* **286**, 1735-8 (1999).
122. N. Nakamura, et al., Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. *Mol Cell Biol* **20**, 8969-82 (2000).
123. R. H. Medema, G. J. Kops, J. L. Bos, B. M. Burgering, AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782-7 (2000).
124. J. E. Ayala, et al., Conservation of an insulin response unit between mouse and human glucose-6-phosphatase catalytic subunit gene promoters: transcription factor FKHR binds the insulin response sequence. *Diabetes* **48**, 1885-9 (1999).
125. Y. Honda, S. Honda, The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. *Faseb J* **13**, 1385-93 (1999).
126. J. Taub, et al., A cytosolic catalase is needed to extend adult lifespan in C. elegans daf-C and clk-1 mutants. *Nature* **399**, 162-6 (1999).
127. D. R. Alessi, F. B. Caudwell, M. Andjelkovic, B. A. Hemmings, P. Cohen, Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett* **399**, 333-8 (1996).
128. S. S. Kang, T. Kwon, D. Y. Kwon, S. I. Do, Akt protein kinase enhances human telomerase activity through phosphorylation of telomerase reverse transcriptase subunit. *J Biol Chem* **274**, 13085-90 (1999).
129. T. Kwon, D. Y. Kwon, J. Chun, J. H. Kim, S. S. Kang, Akt protein kinase inhibits Rac1-GTP binding through phosphorylation at serine 71 of Rac1. *J Biol Chem* **275**, 423-8 (2000).
130. S. Altiok, et al., Heregulin induces phosphorylation of BRCA1 through phosphatidylinositol 3-Kinase/AKT in breast cancer cells. *J Biol Chem* **274**, 32274-8 (1999).
131. K. Paz, et al., Phosphorylation of insulin receptor substrate-1 (IRS-1) by protein kinase B positively regulates IRS-1 function. *J Biol Chem* **274**, 28816-22 (1999).

CHAPTER 3

Direct control of the Forkhead transcription factor AFX by protein kinase B

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Direct control of the Forkhead transcription factor AFX by protein kinase B

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The phosphatidylinositol-3-OH-kinase (PI(3)K) effector protein kinase B (1, 2) regulates certain insulin-responsive genes (3, 4), but the transcription factors regulated by protein kinase B have yet to be identified. Genetic analysis in *Caenorhabditis elegans* has shown that the Forkhead transcription factor *daf*-16 is regulated by a pathway consisting of insulin-receptor-like *daf*-2; and PI(3)K-like *age*-1 (5, 6, 7, 8). Here we show that protein kinase B phosphorylates AFX, a human orthologue of *daf*-16 (5, 6, 9), both *in vitro* and *in vivo*. Inhibition of endogenous PI(3)K and protein kinase B activity prevents protein kinase B-dependent phosphorylation of AFX and reveals residual protein kinase B-independent phosphorylation that requires Ras signalling towards the Ral GTPase. In addition, phosphorylation of AFX by protein kinase B inhibits its transcriptional activity. Together, these results delineate a pathway for PI(3)K-dependent signalling to the nucleus.

To test for potential regulation of the *daf*-16 orthologue AFX (5, 6, 9) in mammalian cells, we labelled A14 cells transiently expressing haemagglutinin-epitope-tagged AFX (HA-AFX) with ^{32}P-orthophosphate and treated them with insulin. HA-AFX underwent a rapid and sustained increase in phosphorylation following insulin treatment (figure 1a). Treatment of A14 cells with epidermal growth factor and of Rat1 cells with platelet-derived growth factor also increased phosphorylation of HA-AFX (data not shown). Phospho-amino-acid analysis of immunoprecipitated AFX revealed that AFX was phosphorylated on serine and threonine residues (figure 1b). To investigate whether PI(3)K and protein kinase B (PKB) were involved in insulin-induced phosphorylation of AFX, we co-expressed HA-AFX with active forms of PI(3)K or PKB. Constitutively active, but not inactive, forms of PI(3)K or PKB induced a strong increase in HA-AFX phosphorylation (figure 1c).

AFX contains three putative PKB phosphorylation sites (10) (T28, S193 and S258; figure 2a) that are conserved between AFX and *daf*-16. As PKB activation *in vivo* was sufficient to increase HA-AFX phosphorylation, we investigated whether AFX could be phosphorylated by PKB *in vitro*. As shown in figure 2b, both immunoprecipitated HA-AFX (right panel) and a bacterially expressed fusion with glutathione-*S*-transferase (GST-AFX; left panel) were phosphorylated by purified active baculo-PKB (11). Mutating any one of the three putative PKB phosphorylation sites did not produce a marked decrease in phosphorylation by PKB, suggesting that at least two sites were

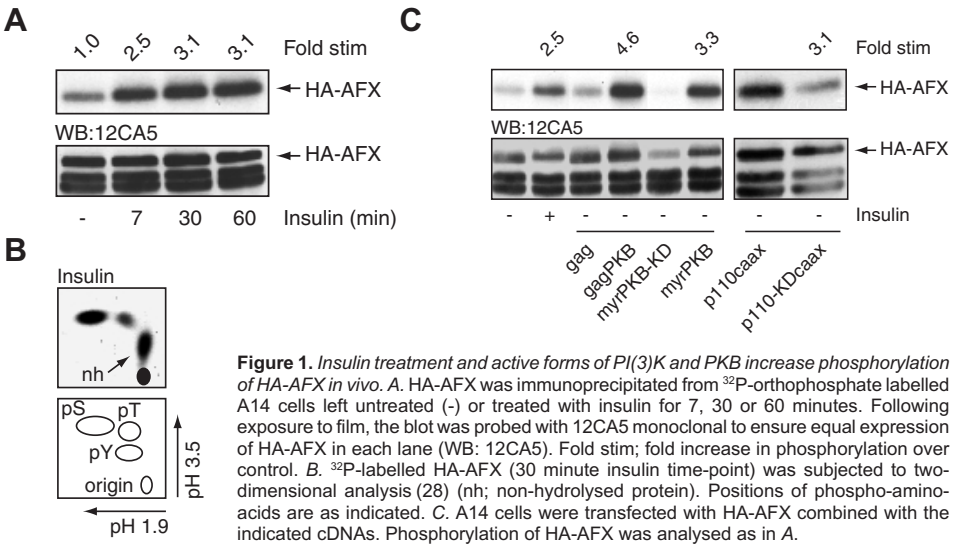

Figure 1. *Insulin treatment and active forms of PI(3)K and PKB increase phosphorylation of HA-AFX in vivo. A.* HA-AFX was immunoprecipitated from ^{32}P-orthophosphate labelled A14 cells left untreated (-) or treated with insulin for 7, 30 or 60 minutes. Following exposure to film, the blot was probed with 12CA5 monoclonal to ensure equal expression of HA-AFX in each lane (WB: 12CA5). Fold stim; fold increase in phosphorylation over control. *B.* ^{32}P-labelled HA-AFX (30 minute insulin time-point) was subjected to two-dimensional analysis (28) (nh; non-hydrolysed protein). Positions of phospho-amino-acids are as indicated. *C.* A14 cells were transfected with HA-AFX combined with the indicated cDNAs. Phosphorylation of HA-AFX was analysed as in *A*.

phosphorylated by PKB (figure 2b). Tryptic peptide mapping of *in vitro* phosphorylated wild-type HA-AFX revealed three reproducibly radiolabelled peptides designated 1, 2 and 3 (figure 2c). Peptides 1 and 2 contained S258 and S193, respectively, because peptide maps of the *in vitro* phosphorylated S258A and S193A mutants no longer displayed ^{32}P-incorporation into the corresponding peptides (figure 2c). Susceptibility of peptide 1, but not 2, to secondary thermolysin digestion, manual Edman degradation (release of ^{32}P in the third cycle) and phospho-amino-acid analysis of eluted peptides 1 and 2 (serine phosphorylation only) supported this conclusion (data not shown). The phosphorylation of peptide 3 was not due to phosphorylation of T28, as the T28A mutation did not abolish phosphorylation of peptide 3. Therefore, PKB can phosphorylate proteins on residues not lying within the consensus sequence for PKB, albeit at a low stoichiometry (figure 2c). Substitution of either S193 or S258 to a threonine residue (S193T and S258T, respectively), proved that these two specific residues are phosphorylated by PKB, because these mutations conferred phosphorylation of a threonine residue on HA-AFX whereas only serines residues were phosphorylated in wild-type HA-AFX (figure 2d). Peptide mapping of *in vivo* phosphorylated HA-AFX showed that the same 3 peptides are phosphorylated, as well as one additional peptide designated peptide 4 (figure 2e). Phosphorylation of peptides 1 and 2 was induced when cells were treated with insulin or when active PKB (gagPKB) was co-expressed (figure 2e). Peptide map analysis of the S193A and S258A mutants showed that these two residues were also phosphorylated *in vivo* following insulin treatment (figure 2f). From these results, we conclude that PKB predominantly phosphorylates S193 and S258, both *in vitro* and *in vivo*.

Three observations suggest that there is an insulin-induced, PKB-independent pathway acting on AFX. First, *in vitro,* PKB induces only serine phosphorylation. Second, *in vivo,* one PKB-independent peptide is phosphorylated (peptide 4, figure 2e and 2f). Finally, both

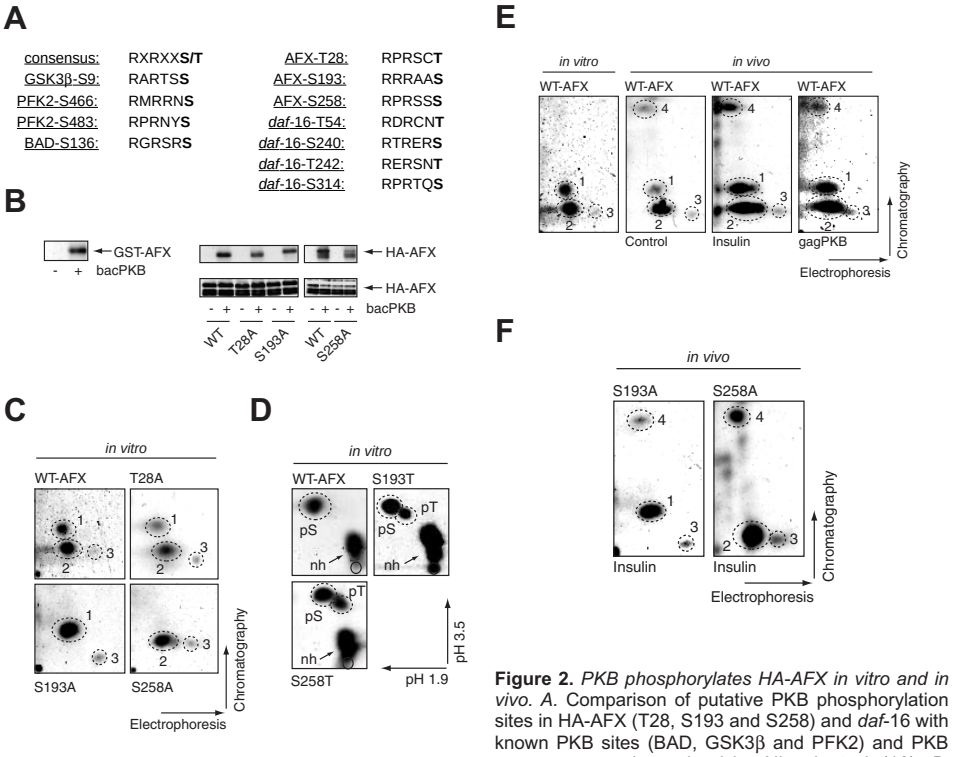

Figure 2. *PKB phosphorylates HA-AFX in vitro and in vivo. A.* Comparison of putative PKB phosphorylation sites in HA-AFX (T28, S193 and S258) and *daf*-16 with known PKB sites (BAD, GSK3β and PFK2) and PKB consensus as determined by Allessi *et al*. (10). *B.* Immunoprecipitated wild-type HA-AFX and PKB-site mutants of HA-AFX (T28A, S193A and S258A) or purified bacterially expressed GST-AFX were incubated in the presence (+) or absence (-) of 2 ml of purified active baculo-PKB (bacPKB). *C.* HA-AFX phosphorylated *in vitro* by PKB as in *B* was processed for two-dimensional phospho-peptide mapping as described (29). Positions of the three resolved peptides are indicated by numbers. *D.* HA-AFX, S193T and S258T phosphorylated *in vitro* by PKB as in *B* were processed for two-dimensional phospho-amino-acid analysis as in figure 1b. nh; non-hydrolysed protein. *E.* HA-AFX phosphorylated *in vivo* following insulin treament of A14 cells or by co-expression with gagPKB were isolated as in figure 1a, and analysed as described in *C*. *F.* S193A and S258A phosphorylated *in vivo* following insulin treament of A14 were isolated as in figure 1a, and analysed as described in *C*.

complete inhibition of PI(3)K (and thus PKB, data not shown and (1)) by pretreatment of A14 cells with wortmannin (12) (figure 3a) or LY294002 ((12); data not shown), and inhibition of endogenous PKB activity by expressing a dominant-negative PKB (PKBcaax) (11) resulted in a pronounced but incomplete inhibition of insulin-induced HA-AFX phosphorylation (figure 3a). Treatment of A14 cells with insulin activates many signalling cascades, including those elicited by activation of Ras (13). Expression of an active mutant of Ras (RasV12) (14) induced an insulin-independent phosphorylation of HA-AFX (figure 3b), in keeping with the ability of oncogenic Ras to induce PI(3)K-dependent signalling (15). Furthermore, inhibiting Ras activation by expressing a dominant-negative Ras (RasN17) (14), which does not inhibit PKB activation (data not shown and (1)), partially inhibited HA-AFX phosphorylation induced by insulin (figure 3b). Simultaneous blocking of Ras and PI(3)K using RasN17 and wortmannin completely blocked insulin-induced phosphorylation of HA-AFX

(figure 3c). As well as PI(3)K, Ras activates the RalGEF/Ral and the Raf/MEK/MAPK pathways (16, 17). Expression of active Rlf (Rlfcaax), a guanylyl-exchange factor for Ral (18), resulted in phosphorylation of HA-AFX, whereas active Raf (19) or a catalytically inactive form of Rlf had no effect (figure 3d). Dominant-negative RalN28 (20) completely abolished the Rlfcaax-induced phosphorylation of HA-AFX, whereas continuous inhibition of PI(3)K using wortmannin had no effect (figure 3e). Combined inhibition of both Ral and PI(3)K using RalN28 and wortmannin completely blocked insulin-induced phosphorylation of HA-AFX (figure 3f).

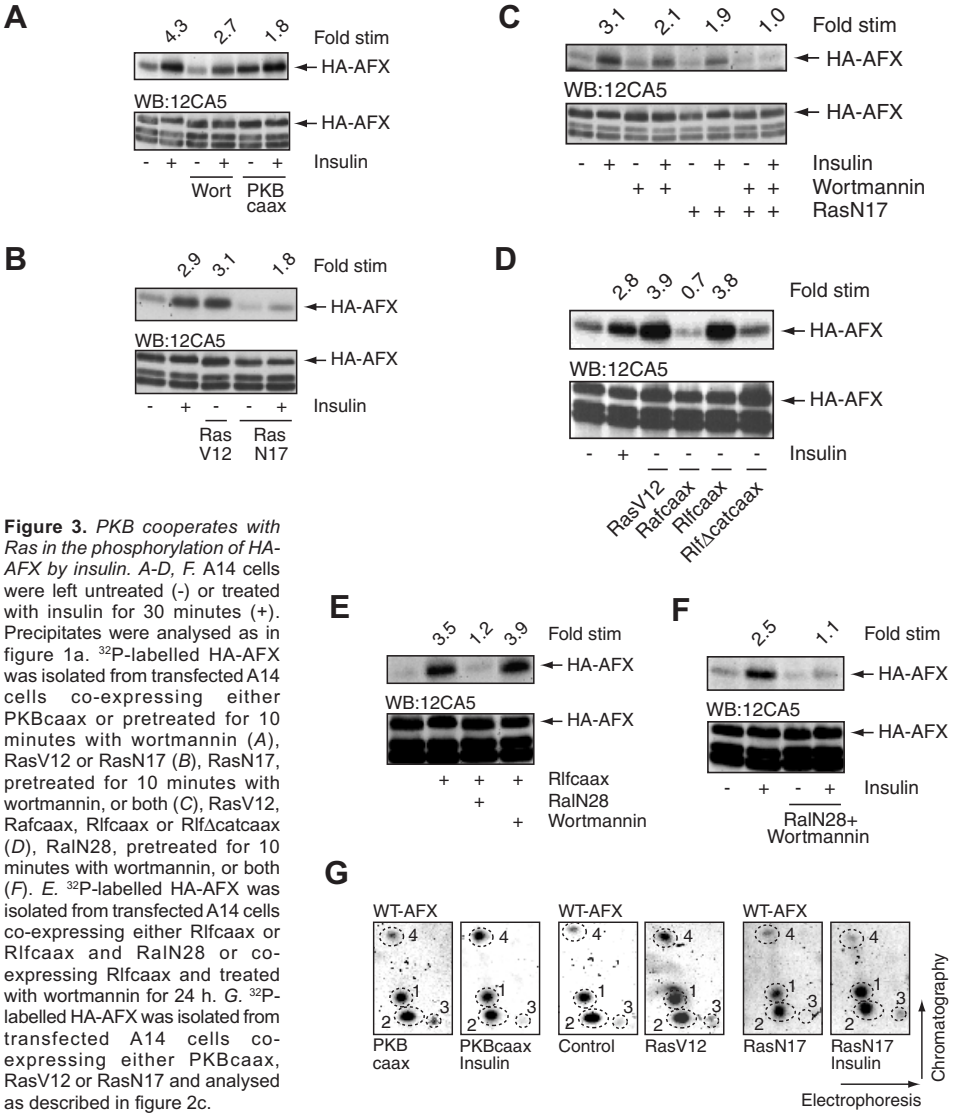

Figure 3. *PKB cooperates with Ras in the phosphorylation of HA-AFX by insulin. A-D, F.* A14 cells were left untreated (-) or treated with insulin for 30 minutes (+). Precipitates were analysed as in figure 1a. ^{32}P-labelled HA-AFX was isolated from transfected A14 cells co-expressing either PKBcaax or pretreated for 10 minutes with wortmannin (*A*), RasV12 or RasN17 (*B*), RasN17, pretreated for 10 minutes with wortmannin, or both (*C*), RasV12, Rafcaax, Rlfcaax or RlfΔcatcaax (*D*), RalN28, pretreated for 10 minutes with wortmannin, or both (*F*). *E.* ^{32}P-labelled HA-AFX was isolated from transfected A14 cells co-expressing either Rlfcaax or Rlfcaax and RalN28 or co-expressing Rlfcaax and treated with wortmannin for 24 h. *G.* ^{32}P-labelled HA-AFX was isolated from transfected A14 cells co-expressing either PKBcaax, RasV12 or RasN17 and analysed as described in figure 2c.

These findings were corroborated by phosphopeptide analysis. Dominant-negative PKBcaax abolished insulin-induced phosphorylation of peptides 1 and 2, but not 4. In contrast, RasV12 increased the phosphorylation of peptide 4, but RasN17 abolished insulin-induced phosphorylation of peptide 4, but not of 1 and 2 (figure 3g). Hence, Ras signalling through Ral is a strong candidate for mediating PKB-independent phosphorylation of AFX by insulin.

Members of the Forkhead family, to which AFX belongs, have been reported to bind to T(G/A)TTT motif-containing insulin-response elements (IRE), which inhibit the transcription of certain genes by insulin (21). To investigate whether AFX can also bind such IREs, we fused the AFX DNA-binding domain (DB) carboxy terminal to GST, creating GST-AFX-DB. Purified GST-AFX-DB and GST-AFX could bind specifically to a radiolabelled oligonucleotide containing the IRE of the insulin-like growth factor-binding protein-1 (IGFBP-1) promoter, but not to an oligonucleotide containing a mutated IRE (Am2Bm2) (22) defective in insulin responsiveness (figure 4a). To examine whether AFX can regulate transcription of the IGFBP-1 gene, we analysed the activity of a CAT reporter gene under the control of the IGFBP-1 promoter (1205-CAT) (22). HA-AFX induced a pronounced increase in CAT activity (figure 4b). The effect of HA-AFX depended on an intact IRE, since the Am2Bm2 mutant blocked the effects of HA-AFX (figure 4b). Insulin treatment or co-expression of either gagPKB, RasV12 or Rlfcaax inhibited the activity of the IGFBP-1 promoter (figure 4c). Transcriptional repression by insulin was regulated by the same pathways which induced AFX phosphorylation. Thus, only simultaneous blocking of Ras-mediated signalling to Ral (RasN17, RalN28) and PI(3)K-dependent signalling to PKB (wortmannin) could fully inhibit the insulin-induced repression of transcription (figure 4c). To determine whether phosphorylation of AFX by PKB was involved in the ability of AFX to regulate IGFBP-1 promoter activity, we examined the effect of a double mutant of HA-AFX lacking both PKB sites (HA-SASA). gagPKB had no effect on IGFBP-1 promoter activity when HA-SASA was expressed. In contrast, insulin was only slightly less able to inhibit expression of the CAT gene (figure 4c). This effect was probably due to insulin-induced Ras/Ral signalling, as RasV12 still inhibited transcription induced by HA-SASA and blocking only Ras (Ras N17) or Ral (RalN28), but not PI(3)K (wortmannin), prevented insulin from inhibiting AFX activity (figure 4c).

To investigate the underlying mechanism of insulin-induced inhibition of AFX, we constructed a fusion protein of full length AFX with the Gal4 DNA-binding domain (Gal4-FL). Expression of Gal4-FL with a Gal4-luciferase reporter construct (23) increased luciferase gene expression. Again, this transactivation was inhibited by insulin and involved both PKB and Ral (figure 4d). To determine which region of AFX was involved in the insulin-induced repression, we created Gal4 fusion proteins containing either the amino terminal or the C-terminal part of AFX (Gal4-N and Gal4-C, respectively). We found that the C-terminal part of AFX acted as a strong transcriptional transactivating domain. Transcription by Gal4-C, however, was not repressed by insulin (figure 4d). This, and the observation that insulin could repress activity of Gal4-FL, may indicate that the full-length AFX protein is required for inhibition by insulin.

Insulin induces the phosphorylation of AFX by way of both PI(3)K/PKB and a Ras/Ral signalling pathway, PKB phosphorylates AFX predominantly on two residues, S193 and S258, and this phosphorylation by PKB can regulate the transcriptional activity of AFX (figure 4e).

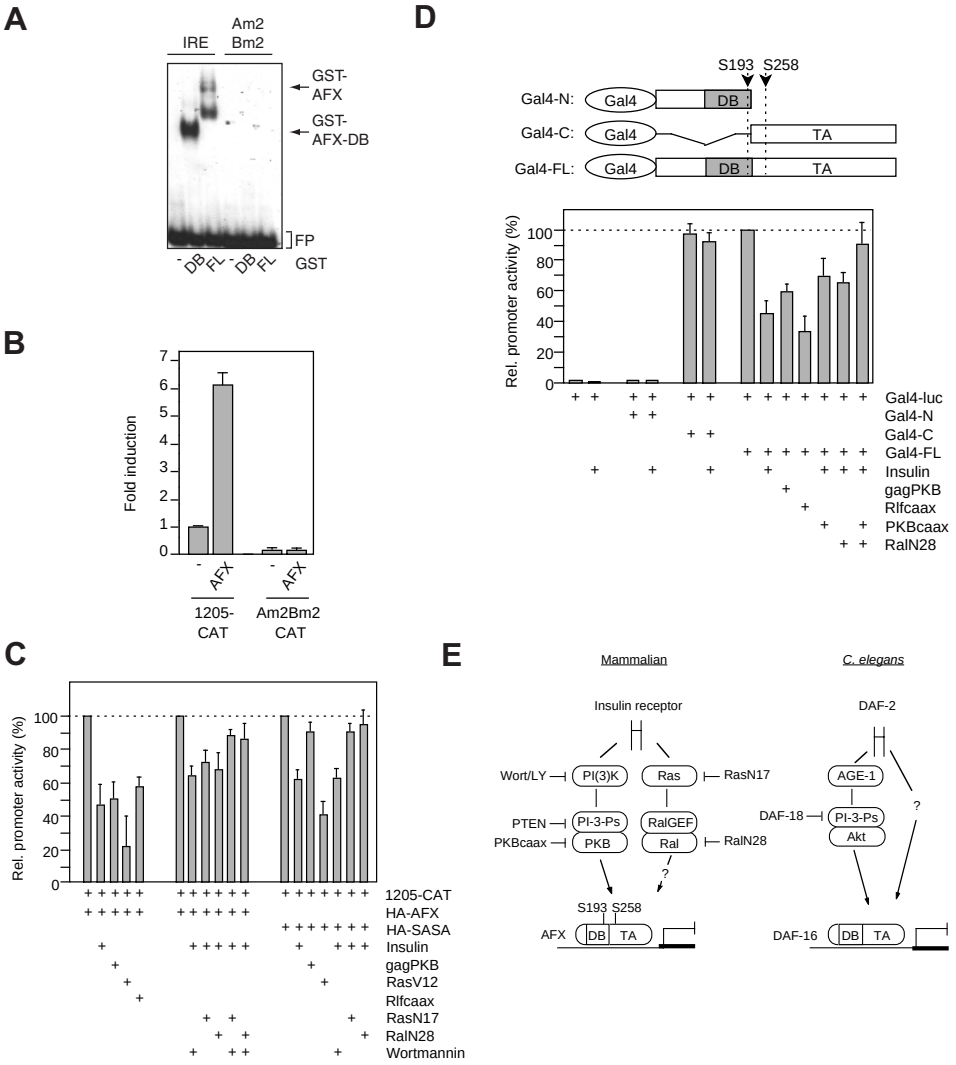

Figure 4. *Phosphorylation of HA-AFX by PKB regulates transcriptional activity. A.* GST protein alone (-), GST-AFX-DB (DB) or GST-AFX (FL) were incubated with ^{32}P-labelled oligonucleotides encompassing the IRE or a mutated IRE (Am2Bm2) of the IGFBP-1 promoter. Faster migrating complexes in the GST-AFX lane represent GST-AFX breakdown products. FP; free probe. *B.* A14 cells were transfected with the 1205-CAT reporter construct or the Am2Bm2-CAT reporter and co-transfected with (AFX) or without (-) wild-type HA-AFX. *C.* A14 cells were transiently transfected with the 1205-CAT reporter together with HA-AFX or HA-SASA either in the absence or presence of gagPKB, RasV12, Rlfcaax, RasN17 or RalN28. Treatment with insulin was for 16 h. Pretreatment with wortmannin was for 10 min. *D.* A14 cells were transiently transfected with the Gal4-luc reporter construct together with either Gal4-N, Gal4-C or Gal4-FL in combination with either gagPKB, Rlfcaax, PKBcaax or RalN28. Treatment with insulin was for 7 h. *B-D.* Controls are set at 100%. Data are obtained from three independent experiments. *E.* A model for insulin-mediated inhibition of AFX-dependent transcription. Left, the insulin-induced pathways leading to phosphorylation and inactivation of AFX as described in the text. Inhibitors are indicated by blunted arrows. Right, pathways similar to those depicted in the left panel have been identified in *C. elegans*. DB; DNA-binding domain, TA; transactivation domain.

Although it is unlikely, we cannot formally exclude that, *in vivo*, a kinase with PKB-like specificity activated by PKB acts between PKB and AFX. Currently, no kinase acting downstream of Ral is known. Identification of the non-PKB target site on AFX will probably facilitate the isolation of such a kinase. Based on the use of a range of inhibitors and activators, MAP-kinase kinase (MEK), $p90^{Rsk}$, $p70^{S6k}$, protein-kinase A, protein-kinase C enzymes sensitive to 12-*D*-tetradecanoyl phorbol-13-acetate (TPA), p38/HOG1, glycogen synthase kinase 3 and calmodulin kinase II do not seem to be involved in the phosphorylation of this PKB-independent target site (unpublished observations). Although activated PKB translocates to the nucleus, the physiological consequences of this translocation were unknown (24). Our data indicate that phosphorylation of the AFX transcription factor is one of the consequences of PKB relocalization. Our results confirm and extend the genetic data obtained in *C. elegans*. Complementation studies in *C. elegans* have suggested that a PI(3)K (*age*-1)-independent route from the insulin receptor (*daf*-2) may lead to inactivation of AFX (*daf*-16), but the components of this route have yet to be identified (figure 4e and (25)). To our knowledge, a role for Ras in the regulation of *daf*-16 in *C. elegans* has not been proposed. Our data identify the AFX transcription factor as an integration point for Ras and PI(3)K signalling, and as the first nuclear target of PI(3)K/PKB-dependent signalling.

Note added in proof. Recently, Brunet *et al*. reported that the AFX-related transcription factor FKHR-L1 is also a substrate for PKB (Brunet, A. *et al*. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. (*Cell* **96**, 857-868; 1999))

Methods

Cells and transfections. Insulin receptor overexpressing mouse NIH3T3 cells (A14) were grown as described (1). Insulin was added at 1 µg/ml and wortmannin (100 nM; Sigma) or LY294002 (10 µM; Sigma) was added 10 minutes before insulin. Transfections were carried out using the $CaPO_4$ method. Generally, 1 µg of the designated plasmid was added to 7 X 10^5 cells. Amount of transfected DNA were equalized using pBluescipt KSII+.

Cloning and plasmids. The 5' region of the AFX gene was obtained by PCR of cDNA obtained from human leukaemic THP-1 cells. The 3' oligonucleotide (5'-AGCAGCTTGCTGC TGCTATCCATGGAG-3') contained the NcoI site located at position +578 of the *AFX* gene. The 5' oligonucleotide (5'-CACAGAAGGCCGTCGCGATCATAGAC-3') created a NruI site at position +26 of the *AFX* gene. Restriction sites are underlined. The 3' region of *AFX* was obtained from pUC-AFX1#3 (26). The NruI/NcoI fragment of the PCR product was ligated to the blunted NcoI/HindIII fragment of pUC-AFX1#3 and the resulting NruI/HindIII fragment was blunted and inserted into SmaI-cut pMT2HA. pMT2HA-AFXT28A, S193A, S258A, S193A/S258A, S193T and S258T were generated by site-directed mutagenesis of the pMT2HA-AFX cDNA using the following forward primers and subsequent complementary reverse primers: T28A (5'-CGCTCCT GCGCCTGGCCCC-3'), S193A (5'-GCCGGGCCGCCGCCA TGGATA-3'), S258A (5'-CACGAAGCAGTGCAAATGCCA -3'), S193T (5'-CCGCCGCCGGGCCGCCACCATGGATAG CAGCAGC-3'), and S258T (5'-CCGTCCACGAAGCAGTA CAAATGCCAGCAGTGTC-3'). Basepair changes are underlined. The double mutant was created by mutating the S193A cDNA using the S258A primers. pRP261-AFX-DBD (GST-AFX-DB) was generated by site-directed mutagenesis of pMT2HA-AFX to create a KpnI site at position +249 and a XbaI site combined with an in-frame stop codon at position +623 of the *AFX* gene, using the following forward primers and subsequent complementary reverse primers: KpnI (5'-C G G A A T C C T G G G G G C GGTACC A G GTCCTCGGAAGGG-3') and XbaI (5'-GCCGCAGTAAAG CCC*TCTAGA*AGAAACCATCTG TGC-3'). Restriction sites are underlined and stop codon is in italic. Next, the KpnI/XbaI fragment was ligated into KpnI/XbaI cut pRP261. GST-AFX was created by ligating a SalI/XhoI fragment from pMT2HA-AFX into SalI cut dephosphorylated pRP261. Gal4-N was created by ligating a blunted SalI/NdeI fragment of pMT2HA-AFX into XmaI cut and blunted pSG424. Gal4-C was created by ligating a blunted NcoI/EcoRI fragment of pMT2HA-AFX into SmaI cut pSG424. Gal4-FL was created by ligating a blunted SalI/EcoRI fragment of pMT2HA-AFX into XmaI cut and blunted pSG424. All generated constructs were verified

by automated sequencing.

The following plasmids have been described: pSG5-gag and pSG5-gagPKB (1), p110caax and p110-KDcaax (27). pcDNA3-myrPKB and pcDNA3-myrPKB-KD were obtained from D. Stokoe.

^{32}P-orthophosphate labelling. *In vivo* labelling of A14 cells was performed as described (1).

Immunoprecipitations and western blotting. Immunoprecipitations using the mouse monoclonal 12CA5 antibody and western blot analysis were performed under standard conditions and as described (1).

***In vitro* kinase assay.** *In vitro* kinase assay using active baculo-PKB was performed as described (11). The relative activity of 1 µl purified active PKB was calculated to be 1 pmol PO_4 per min, using a peptide containing S258 of AFX as a substrate.

Phospho-amino-acid analysis and tryptic peptide mapping. ^{32}P-orthophosphate labelled HA-AFX was immunoprecipitated from A14 cells, electroforesed and immobilized on PVDF membrane (Immobilon). Protein was cut from the membrane and treated as described previously for phospho-amino-acid analysis (28) or peptide mapping (29). Sequence-grade trypsin was obtained from Boehringer-Mannheim.

Electrophoretic mobility shift assay (EMSA). GST-AFX-DB and GST-AFX were purified using standard GST-fusion-protein-purification protocol (22). Bandshift assay was performed using a standard protocol (22) and using the following oligonucleotides and subsequent complementary oligonucleotides: IGFBP-1 (5'-CACTAGCAAAACAAACTTATTTTGAACAC-3'), Am2Bm2 (5'-CACTAGCAACCATGACCATGGTTGAACAC-3').

Chloramphenicol-acetyl transferase assay. CAT assays were done as described (30).

Luciferase assay. Luciferase assays were done as described (23).

Acknowledgements

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References

1. B. M. Burgering, P. J. Coffer, Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602 (1995).
2. T. F. Franke, et al., The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**, 727-36 (1995).
3. S. B. Cichy, et al., Protein kinase B/Akt mediates effects of insulin on hepatic insulin- like growth factor-binding protein-1 gene expression through a conserved insulin response sequence. *J Biol Chem* **273**, 6482-7 (1998).
4. D. Wang, H. S. Sul, Insulin stimulation of the fatty acid synthase promoter is mediated by the phosphatidylinositol 3-kinase pathway. Involvement of protein kinase B/Akt. *J Biol Chem* **273**, 25420-6 (1998).
5. S. Ogg, et al., The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature* **389**, 994-9 (1997).
6. K. Lin, J. B. Dorman, A. Rodan, C. Kenyon, daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science* **278**, 1319-22 (1997).
7. K. D. Kimura, H. A. Tissenbaum, Y. Liu, G. Ruvkun, daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. *Science* **277**, 942-6 (1997).
8. J. Z. Morris, H. A. Tissenbaum, G. Ruvkun, A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in Caenorhabditis elegans. *Nature* **382**, 536-9 (1996).
9. A. Borkhardt, et al., 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 (1997).
10. D. R. Alessi, F. B. Caudwell, M. Andjelkovic, B. A. Hemmings, P. Cohen, Molecular basis for the substrate specificity of protein kinase B; comparison with MAPKAP kinase-1 and p70 S6 kinase. *FEBS Lett* **399**, 333-8 (1996).
11. P. C. van Weeren, K. M. de Bruyn, A. M. de Vries-Smits, J. van Lint, B. M. Burgering, 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 (1998).
12. B. Cheatham, et al., Phosphatidylinositol 3-kinase activation is required for insulin stimulation of pp70 S6 kinase, DNA synthesis, and glucose transporter translocation. *Mol Cell Biol* **14**, 4902-11. (1994).
13. G. J. Pronk, J. L. Bos, The role of p21ras in receptor tyrosine kinase signalling. *Biochim Biophys Acta* **1198**, 131-47. (1994).
14. R. H. Medema, R. Wubbolts, J. L. Bos, Two dominant inhibitory mutants of p21ras interfere with insulin-induced gene expression. *Mol Cell Biol* **11**, 5963-7. (1991).
15. A. Kauffmann-Zeh, et al., Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385**, 544-8 (1997).
16. T. Urano, R. Emkey, L. A. Feig, Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. *Embo J* **15**, 810-6. (1996).
17. B. M. Burgering, J. L. Bos, Regulation of Ras-

mediated signalling: more than one way to skin a cat. *Trends Biochem Sci* **20**, 18-22. (1995).
18. R. M. Wolthuis, N. D. de Ruiter, R. H. Cool, J. L. Bos, Stimulation of gene induction and cell growth by the Ras effector Rlf. *Embo J* **16**, 6748-61 (1997).
19. S. J. Leevers, H. F. Paterson, C. J. Marshall, Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature* **369**, 411-4. (1994).
20. R. M. Wolthuis, et al., RalGDS-like factor (Rlf) is a novel Ras and Rap 1A-associating protein. *Oncogene* **13**, 353-62. (1996).
21. T. G. Unterman, et al., Hepatocyte nuclear factor-3 (HNF-3) binds to the insulin response sequence in the IGF binding protein-1 (IGFBP-1) promoter and enhances promoter function. *Biochem Biophys Res Commun* **203**, 1835-41. (1994).
22. R. M. O'Brien, et al., Hepatic nuclear factor 3- and hormone-regulated expression of the phosphoenolpyruvate carboxykinase and insulin-like growth factor-binding protein 1 genes. *Mol Cell Biol* **15**, 1747-58. (1995).
23. M. C. Duyndam, H. van Dam, A. J. van der Eb, A. Zantema, The CR1 and CR3 domains of the adenovirus type 5 E1A proteins can independently mediate activation of ATF-2. *J Virol* **70**, 5852-9. (1996).
24. M. Andjelkovic, et al., Role of translocation in the activation and function of protein kinase B. *J Biol Chem* **272**, 31515-24 (1997).
25. S. Paradis, G. Ruvkun, 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 (1998).
26. U. Peters, G. Haberhausen, M. Kostrzewa, D. Nolte, U. Muller, AFX1 and p54nrb: fine mapping, genomic structure, and exclusion as candidate genes of X-linked dystonia parkinsonism. *Hum Genet* **100**, 569-72 ;|. (1997).
27. S. A. Didichenko, B. Tilton, B. A. Hemmings, K. Ballmer-Hofer, M. Thelen, Constitutive activation of protein kinase B and phosphorylation of p47phox by a membrane-targeted phosphoinositide 3-kinase. *Curr Biol* **6**, 1271-8 (1996).
28. W. J. Boyle, P. van der Geer, T. Hunter, Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. *Methods Enzymol* **201**, 110-49. (1991).
29. K. X. Luo, T. R. Hurley, B. M. Sefton, Cyanogen bromide cleavage and proteolytic peptide mapping of proteins immobilized to membranes. *Methods Enzymol* **201**, 149-52. (1991).
30. R. P. de Groot, W. Kruijer, Transcriptional activation by TGF beta 1 mediated by the dyad symmetry element (DSE) and the TPA responsive element (TRE). *Biochem Biophys Res Commun* **168**, 1074-81. (1990).

CHAPTER 4

Inhibition of nuclear import by protein kinase B/Akt regulates the subcellular distribution and activity of the Forkhead transcription factor AFX

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Inhibition of nuclear import by protein kinase B/Akt regulates the subcellular distribution and activity of the Forkhead transcription factor AFX

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AFX belongs to a subfamily of Forkhead transcription factors that are phosphorylated by protein kinase B/Akt (PKB). Phosphorylation inhibits the transcriptional activity of AFX and changes the steady-state localization of the protein from the nucleus to the cytoplasm. Our goal was threefold: to identify the cellular compartment in which PKB phosphorylates AFX, to determine whether the nuclear localization of AFX plays a role in regulating its transcriptional activity, and to elucidate the mechanism by which phosphorylation alters the localization of AFX. We now show that phosphorylation of AFX by PKB occurs in the nucleus. In addition, nuclear export mediated by the export receptor Crm1 is required for the inhibition of AFX transcriptional activity. Both phosphorylated and unphosphorylated AFX, however, bind Crm1 and can be exported from the nucleus. These results suggest that export is unregulated and that phosphorylation by PKB is not required for the nuclear export of AFX. We show that AFX enters the nucleus by an active, Ran-dependent mechanism. Amino acids 180-221 of AFX comprise a non-classical NLS. Serine 193, contained within this atypical NLS, is a PKB-dependent phosphorylation site on AFX. Addition of a negative charge at serine 193 by mutating the residue to glutamate reduces nuclear accumulation. PKB-mediated phosphorylation of AFX, therefore, attenuates the import of the transcription factor, which shifts the localization of the protein from the nucleus to the cytoplasm and results in the inhibition of AFX transcriptional activity.

Introduction

PKB promotes cell survival in many different cell types (1, 2, 3, 4). Following its initial cloning (5, 6), PKB was isolated as the transforming gene of v-Akt, hence the name c-Akt and its classification as a proto-oncogene (7, 8). Activation of PKB requires the lipid PI(3,4,5)P_3 (9) and phosphorylation by an upstream kinase, PDK1 (10, 11, 12). PIP_3 is produced by PI(3)K in response to signals from extracellular growth factors (for a review see (13)). PKB has been shown to phosphorylate and regulate the activity of transcription factors in response to survival factors. Genetic studies of *C. elegans* have demonstrated that the PKB signal transduction pathway inhibits the activity of the *daf-16* geneproduct, a Forkhead

transcription factor that regulates longevity (14). There are three human orthologues of *daf-16*, AFX (15), FKHR (16), and FKHR-L1 (17), that were first identified as chromosomal breakpoints in human tumors.

AFX is phosphorylated by PKB in response to insulin and serum at two sites: serine 193 (S193) and serine 258 (S258) (18). Phosphorylation of these residues by PKB leads to both inhibition of the transcriptional activity of AFX and cytoplasmic retention/nuclear exclusion of the protein. Withdrawal of serum or insulin results in AFX dephosphorylation, nuclear localization, and target gene activation. In the absence of survival factors, Forkhead family members have been shown to induce the transcription of pro-apoptotic genes such as *FasL* (19) and *Bim* (20), thus triggering a cascade of events that lead to apoptosis. In addition, overexpression of AFX blocks cell-cycle progression at G1 by a mechanism that is independent of functional retinoblastoma protein, but dependent on the cell-cycle inhibitor $p27^{kip1}$ (21). Dysregulation of Forkhead proteins may therefore comprise an important step in oncogenic transformation by both inhibiting apoptosis and promoting progression through the cell-cycle.

Efficient regulated nuclear localization of transcription factors in response to extracellular signals is essential for their function (22). For example, in unstressed cells, p53 continuously shuttles into and out of the nucleus and its subcellular distribution varies throughout the cell-cycle. In response to certain stresses, however, p53 relocalizes to the nucleus to promote gene transcription. In some p53-related tumors there is a defect in p53 localization (23, 24). In these cells, p53 is constitutively cytoplasmic due to an increase in the export rate of the protein. As a result, p53 cannot accumulate in the nucleus to carry out its normal activities. This defect leads to unregulated cellular proliferation. Likewise, the transcriptional activity of Forkhead family members may be regulated not only by phosphorylation, but also by changes in their subcellular localization.

Many proteins are transported constitutively into and out of the nucleus by members of the β-importin family of nuclear transport receptors (25, 26). These receptors recognize specific localization signals within their cargoes and their association with these signal sequences is controlled by the small GTPase Ran. The regulators of Ran are distributed asymmetrically within the cell: the RanGTPase activating protein (RanGAP) is cytoplasmic (27), whereas the Ran exchange factor (RanGEF) is nuclear (28). Therefore, most of the Ran present in the nucleus is predicted to be GTP-bound while cytoplasmic Ran is predicted to be GDP-bound. The directionality of transport depends on this RanGTP gradient. Unloading of cargo from an import receptor in the nucleus is triggered by the binding of RanGTP to the receptor (29, 30, 31). Conversely, an export receptor can only load its cargo in the presence of RanGTP. Release of export cargo into the cytoplasm occurs by the hydrolysis of RanGTP to RanGDP, stimulated by RanGAP and other required cofactors (32).

In contrast to constitutive transport, regulated transport occurs only in response to specific cellular signals, such as phosphorylation (33). For example, MAPKAP kinase-2 is a nuclear protein in unstimulated cells. In response to specific extracellular signals, it is phosphorylated, binds p38 stress-activated protein kinase, and is only then exported from the nucleus (34). Regulated transport mechanisms play critical roles in augmenting or attenuating signalling information, by controlling the cellular localization of proteins. Many proteins that have regulated transport mechanisms are shuttling proteins that move continuously between the cytoplasm and nucleus.

Thus, their steady-state localization is determined by the relative rate constants for import and export. Shuttling coordinates nuclear and cytoplasmic events by providing a rapid and reversible method to regulate a nuclear and/or cytoplasmic activity. Nuclear transport often works in tandem with other mechanisms to regulate the function of proteins. Some transcription factors require phosphorylation in addition to changes in nuclear localization to completely regulate their activity. This redundancy increases the strength of cellular switches and provides additional ways to integrate environmental cues with cellular signals. Finally, proteins that alter their cellular localization in response to extracellular signals need a mechanism for turning that signal off when it is no longer required. Protein degradation, dephosphorylation, and nuclear transport are all mechanisms used to terminate activity. Although the regulated transport mechanisms for several yeast proteins have been identified, including those for Pho4 (35, 36) and Mig1 (37, 38), few regulated transport mechanisms have been described for mammalian proteins.

Our goal, therefore, was to elucidate how PKB-dependent phosphorylation and the resulting cellular relocalization of AFX alter its transcriptional activity. In this study, we show that PKB phosphorylation of AFX in the nucleus followed by Crm1-dependent nuclear export is required to inhibit the transcriptional activity of the Forkhead family member. Although phosphorylation by PKB does not significantly alter AFX export from the nucleus, it appears that nuclear import of phosphorylated AFX is regulated. Amino acids 180-221 of AFX are necessary and sufficient for nuclear import, but encode a non-classical NLS. Addition of a negative charge at S193 by mutating the residue to a glutamate inhibits nuclear import. Therefore, phosphorylation of AFX by PKB likely reduces the nuclear import rate of AFX. This results in a shift in the steady-state localization of AFX from the nucleus to the cytoplasm, thereby inhibiting its transcriptional activity.

Methods

Plasmids and recombinant proteins. pMT2HA-AFX, pMT2HA-SASA, pMT2HA-A3, pSG5-gagPKB, p1205-luc, and pCMV-lacZ have been described (18, 21). pMT2HA-AFX (Δ198-216) was constructed using PCR-based mutagenesis. To construct the vectors encoding the fusions of GFP3 to the C-termini of various AFX sequences, pKGFP3 was used. To create pKGFP3, GFP-GFP was PCR-amplified and inserted as a BglII-BglII fragment into pKGFP. For pKGFP3-AFX(180-197), pKGFP3-AFX(198-221), and pKGFP3-AFX(180-221), the indicated residues of AFX were PCR-amplified and ligated into pKGFP3 as XbaI-XbaI fragments. Site-directed mutagenesis of residues within pKGFP3-AFX(180-221) to produce pKGFP3-AFX(180-221)I-V, pKGFP3-AFX(180-221)S193A, and pKGFP3-AFX(180-221)S193E was carried out using the Quikchange mutagenesis kit (Stratagene) according to the manufacturer's recommendations. All mutations were verified by sequencing. pQE60-Crm1 was a gift from Iain Mattaj (EMBL, Heidelberg) (39). Crm-His_6 was purified using Talon beads (Clontech) as described (32). The addition of 14 mM β-mercaptoethanol was required to retain the stability of the protein. pQE32-RanQ69L was a gift from Dirk Görlich (University of Heidelberg). His_6-RanQ69L was prepared as described (31).

Cell Culture. Insulin-receptor overexpressing NIH3T3 cells (A14) were grown as described (9). All other cells were passaged in Dulbecco's modified eagle medium (supplemented with 5% fetal calf serum (v/v) and penicillin/streptomycin). Baby hamster kidney cells (BHK21), human embryonic kidney cells (HEK293), and HeLa cells were cultured in a humidified, 37°C/5% CO_2 incubator. tsBN2 cells were grown at 33.5°C. Where indicated, the tsBN2 cells were temperature-shifted to 39.5°C for 3 h after the addition of 50 μM cycloheximide. Leptomycin B (LMB) used in some cell-based assays was a gift from Barbara Wolff (Novartis).

Transfections. HA-tagged AFX constructs were transfected into A14, BHK21, HEK293, HeLa, and tsBN2 cells by the calcium phosphate method. At 24 h posttransfection, the transfection medium was replaced with fresh medium and the cells were incubated at 37°C for an additional 24 h. Transfected HEK293 cells were harvested for immunoprecipitations at 48 h posttransfection. Transfected A14 cells were serum-starved overnight where indicated. For immunofluorescence, A14 cells

were either untreated, treated with 10 μM LY294002 (Calbiochem) for 10 min, or treated with 2 ng/ml LMB for 30 min prior to the addition of 1 μg/ml insulin. The cells were then incubated with insulin for an additional 30 min before fixation. Transfected tsBN2, HeLa, and BHK21 cells were plated on poly-L-lysine-coated coverslips. Transfected tsBN2 and HeLa cells, where indicated, were serum-starved for 1.5 h prior to fixation. BHK21 cells were transfected with all pKGFP3 constructs using Effectene transfection reagent (Qiagen), according to the manufacturer's instructions. These cells were processed 20 h after transfection. After the indicated treatments, the transfected cells were fixed and permeabilized with 4% paraformaldehyde/ 2% sucrose in phosphate-buffered saline (PBS; 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 (pH 7.4)) and ice-cold methanol as described previously (40, 41, 42). Fixed cells were then prepared for analysis by fluorescence microscopy.

Fluorescence microscopy. After fixation and permeabilization, cells expressing GFP fusions were incubated with 4',6-diamidino-2-phenylindole (DAPI; 10 ng/ml) to stain the nuclei, then the coverslips were mounted on glass slides using GelMount (Biomeda). Images of the cells were captured using a 60x water-immersion objective lens on a Nikon inverted microscope equipped with a Hamamatsu CCD camera. All immunofluorescence data were obtained and processed using Openlab (Improvision) and Adobe Photoshop software. Images for each set of experiments were obtained using the same camera settings. The relative nuclear and cytoplasmic fluorescence levels of the GFP3 constructs were measured using Openlab (Improvision). Nuclear fluorescence was calculated as a percentage of the total cellular fluorescence (N/(N+C)). All fluorescence measurements were corrected for background fluorescence levels. Each data point represents the mean fluorescence obtained from 12 randomly chosen cells. Error is expressed as the standard deviation of the mean. All cells expressing HA-tagged AFX constructs were blocked in 10% BSA-PBS at RT; incubated with 12CA5 Mab (2 μg/ml) and Texas Red-conjugated donkey anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, 1:1500 dilution) both in 3% BSA; stained with DAPI; and mounted and viewed as described above. Cells expressing gag-PKB were detected using anti-gag and anti-PKB antisera (9).

Immunodetection. BHK21 cells expressing GFP3 constructs were lysed with an equal volume of boiling Laemmli sample buffer. Equal volumes of lysate (10 μl) were then analyzed by SDS-PAGE and immunoblotting for GFP3 with polyclonal anti-GFP (Molecular Probes, 1:1000) and HRP-conjugated anti-rabbit secondary antibody (Jackson Laboratories, 1:20,000). Proteins were revealed by chemiluminescence (Kirkegaard & Perry Laboratories). Subcellular fractionation of insulin-treated (1 μg/ml) A14 cells was carried out according to published protocols (43, 44). Anti-phosphoT32 FKHR-L1, anti-c-cbl, and anti-RNA pol II were gifts from Anne Brunet (Harvard University), Kris Reedquist (UMC, Utrecht), and Marc Timmers (UMC,Utrecht), respectively. Anti-phosphoS193 AFX and anti-phosphoS473 PKB were obtained from New England Biolabs.

Luciferase assays. A14 cells were co-transfected with the p1205-luc reporter, pMT2HA-AFX, and pCMV-lacZ. Cells were also transfected where indicated with pSG5-gagPKB. Cells were preincubated with LMB where noted for 30 min prior to the addition of insulin. Cells were then treated with insulin for 16 hr in the absence or presence of LMB. Transcriptional activity was measured 48 hr after transfection. Luciferase and β-galactosidase measurements were performed as described (18).

Crm1-binding assays. HEK293 cells were either mock transfected or transfected with pMT2HA-AFX or pMT2HA-A3. Where indicated, cells were treated with LY294002 (10 μM) for 2 hr prior to lysis. All cells were then washed 2x with ice-cold PBS, placed on ice, and lysed by the addition of 400 μl lysis buffer (25 mM HEPES, pH 7.4, 300 mM NaCl, 1.5 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.1% Triton-X100, 1 mM okadaic acid, 1mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, and 20 μg/ml aprotinin). Lysates were cleared by centrifugation (5 min at 14,000*g*, 4°C). The lysate was used for immunoprecipitation with 12CA5 at 4°C for 1 h. Protein A-Sepharose beads were added to the samples for 1h at 4°C. Immunoprecipitates were washed 3x with buffer A (PBS, 1% NP-40, 20 mM β-glycerophosphate, 2mM sodium orthovanadate, 1 mM okadaic acid) and once with buffer B (50 mM MOPS, pH 7.5, 500 mM lithium chloride, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM okadaic acid). To release endogenous 14-3-3 protein bound to HA-AFX, immunoprecipitates were washed 3x with PBS containing 1 M $MgCl_2$ where indicated. Controls were washed 3x with PBS without added $MgCl_2$.

The immunoprecipitates were resuspended in binding buffer (20 mM HEPES, pH 7.3, 150 mM potassium acetate, 2 mM magnesium acetate, 0.1% Tween-20, 28 mM β-mercaptoethanol, 0.05% ovalbumin, 1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 1 mM okadaic acid, and 1 mM PMSF). Crm1-His_6 was added to each assay to yield a final concentration of 500 nM. His_6-RanQ69L was added as indicated to a final concentration of 3 μM. Samples were incubated for 2 h at 4°C and then were washed 3 times with binding buffer. Beads were resuspended in Laemmli sample buffer and the proteins were separated by SDS-PAGE, and immunoblotted with horseradish peroxidase (HRP)-conjugated 12CA5 (1:5000), monoclonal anti-His_6 (BabCo, 1:2000), or polyclonal anti-14-3-3β (Santa Cruz Biotechnology,

1:100), and with HRP-conjugated goat anti-mouse or anti-rabbit secondary antibody (Jackson Laboratories, 1:20,000). Proteins were revealed by chemiluminescence (Kirkegaard & Perry Laboratories).

Heterokaryon fusion assays. BHK21 cells were transfected with pMT2HA-A3. An acceptor cell line, GSN2, was a gift from Bryce Paschal (University of Virginia) (45). This stably transfected HeLa cell line expresses the nondiffusible nuclear protein, GFP-streptavidin-SV40NLS. BHK21 and GSN2 cells were co-plated onto poly-L-lysine coated coverslips overnight. Cells were treated with 50 μM cycloheximide for 30 min. The plasma membranes were then fused for 2 min with 50% polyethylene glycol (MW 8000) prewarmed to 37°C. Cells were washed 4 times with medium and were incubated at 37°C for an additional 1 h in the presence of cycloheximide. Cells were then fixed and processed for immunocytochemistry as described above.

Results

Phosphorylation of AFX by PKB triggers relocalization of AFX from the nucleus to the cytoplasm.

The subcellular distribution of Forkhead family members is altered on addition of insulin or serum to cells (19, 46, 47). This relocalization is dependent on the phosphorylation of the Forkhead protein by PKB. As a basis for subsequent experiments, we confirmed that the addition of insulin results in a change in the steady-state distribution of HA-AFX in serum-starved cells from the nucleus to the cytoplasm within 30 min (figure 1a). Mutation of PKB phosphorylation sites S193 and S258 to alanine (HA-SASA) inhibited this relocalization as did treatment with the PI(3)K inhibitor LY294002 prior to the addition of insulin (figure 1a). Additionally, the co-expression of constitutively active PKB (gagPKB) resulted in the redistribution of HA-AFX, but not HA-SASA, to the cytoplasm (figure 1b). A significant fraction of gagPKB, however, is localized to the nucleus (figure 1b). This result is consistent with previous studies that have shown gagPKB localization at the plasma membrane (40%), in the cytoplasm (30%), and within the nucleus (30%) (48, 49). These results confirm that phosphorylation of AFX by PKB results in a rapid redistribution of AFX from the nucleus to the cytoplasm.

Nuclear export subsequent to phosphorylation by PKB in the nucleus is required for the inhibition of AFX transcriptional activity.

We wanted to test the hypothesis that PKB-dependent redistribution of AFX to the cytoplasm is required to regulate the transcriptional activity of the protein. The best-characterized nuclear export pathway uses a leucine-rich NES, which is bound by the export receptor Crm1 in the presence of RanGTP in the nucleus (50, 51, 52, 53, 54). The trimeric complex is then exported to the cytoplasm and is disassembled. Crm1-dependent export can be inhibited by leptomycin B (LMB) (55), which specifically inactivates Crm1 by covalent modification of a key cysteine residue in the NES-binding region of the protein (56). We tested, therefore, whether LMB could inhibit the cytoplasmic relocalization of HA-AFX in response to insulin (figure 2a) and serum (data not shown). As shown in figure 2a, LMB completely blocked the nuclear export of AFX. Thus, nuclear export of AFX in response to insulin or serum proceeds via a Crm1-dependent pathway.

Activated PKB may enter the nucleus to phosphorylate its target proteins (57, 58), however, this view is controversial. In support of the idea that PKB translocates into the nucleus, we have observed by subcellular fractionation that A14 cells show an increase in endogenous activated PKB within the nucleus 10 minutes after the addition of insulin (figure 2b; PKB S473-P). To examine this issue within the context of Forkhead transcription factors, we used the inhibition of AFX export by LMB to establish the cellular compartment in which this protein is phosphorylated by PKB. The addition of insulin

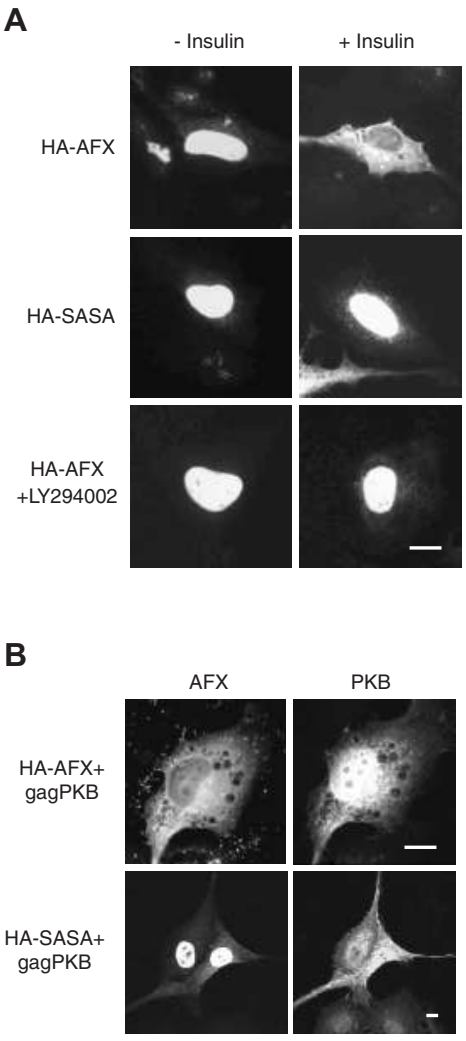

Figure 1. *PKB-dependent phosphorylation of AFX triggers relocalization of AFX from the nucleus to the cytoplasm. A.* A14 cells were transfected with pMT2HA-AFX or pMT2HA-SASA. At 24 hr post-transfection, serum was withdrawn for 18-24 hr. Insulin (1 μg/ml) was then added as indicated and these cells were incubated for 30 min. Cells treated with LY294002 (10 μM) to inhibit PI(3)K were pre-incubated for 10 min prior to the addition of insulin. Cells were fixed and then HA-AFX and HA-SASA were stained with 12CA5 and Texas red-conjugated secondary antibody. *B.* A14 cells were transfected with pMT2HA-AFX or pMT2HA-SASA and with constitutively active PKB (pSG5-gagPKB). At 48 hr post-transfection, the cells were fixed and HA-AFX and HA-SASA were stained as described in *A*. gagPKB was stained with anti-PKB antiserum and FITC-conjugated secondary antibody. Bar, 10 μm.

to serum-starved cells expressing HA-AFX resulted in phosphorylation of S193 (figure 2c). Importantly, an equivalent level of S193 phosphorylation was observed when cells were treated with LMB prior to the addition of insulin. This result suggests that PKB can translocate into the nucleus in order to phosphorylate target proteins and that PKB-dependent phosphorylation of AFX occurs in the nucleus. This conclusion is supported by our results obtained for the PKB-mediated phosphorylation of endogenous FKHR-L1 in the presence of LMB (figure 2c). FKHR-L1 is another Forkhead family member that also has a steady-state nuclear localization in serum-starved cells (19). Threonine 28 (T28) is a reported PKB-dependent phosphorylation site on FKHR-L1 (19). This site was phosphorylated by PKB to similar levels upon the addition of insulin in either the absence or presence of LMB pretreatment (figure 2c). We conclude, therefore, that Forkhead family members can be phosphorylated by PKB in the nucleus.

To test whether nuclear export is essential for the inhibitory effect of PKB on Forkhead transcriptional activity, we performed a luciferase reporter gene assay. Forkhead transcription factors can bind to and regulate the activity of the IGFBP-1 promoter *in vitro*. In co-transfection assays using a CAT-reporter under the control of the IGFBP-1 promoter, we have shown previously that Forkhead binding results in increased transcriptional activity and that insulin represses this activity (18). HA-AFX activity in serum-starved cells was normalized to 0% inhibition of transcriptional activity (i.e., the relative promoter activity is 100%). PKB-dependent phosphorylation of HA-AFX resulting from the addition of insulin or co-expression of

gagPKB inhibited the reporter gene activity by ~50% (figure 2d). Pretreatment with LMB to inhibit HA-AFX export, however, attenuated the effect of insulin to approximately 20% and abolished the effect of gagPKB on AFX activity. Since LMB does not affect the ability of PKB to phosphorylate AFX (figure 2c), the effect of LMB cannot be explained by a lack of PKB-dependent phosphorylation. Nor can it be explained by a global effect on the cellular transcription machinery, since LMB has no effect on the transcriptional activity of AFX mutants, such as HA-SASA and HA-A3 (see below), that do not relocalize to the cytoplasm on insulin treatment or on cotransfection of gagPKB (data not shown). The reduced effect of LMB observed in insulin–treated cells most likely is due to the activation of other identified signalling pathways that regulate AFX activity independent of cellular localization (18). The complete inhibition

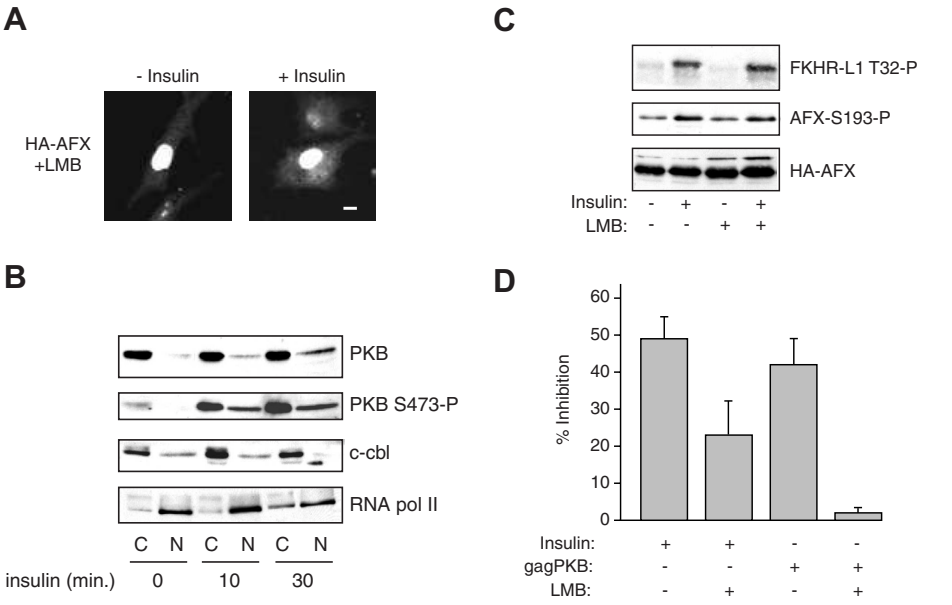

Figure 2. *Nuclear export subsequent to phosphorylation by PKB in the nucleus is required for the inhibition of AFX transcriptional activity. A*. A14 cells were transfected with pMT2HA-AFX. At 24 hr post-transfection, serum was withdrawn for 18-24 hr. The cells were treated with LMB (2 ng/ml) for 30 min to inhibit Crm1-dependent export. Then insulin (1 μg/ml) was added as indicated and the cells were incubated for an additional 30 min. Cells were fixed and then HA-AFX was stained as described previously. Bar, 10 μm. *B.* Serum-starved A14 cells were treated with insulin (1 μg/ml) and then fractionated at the indicated times. Equal amounts of nuclear and cytoplasmic lysates (20 μg) were analyzed by SDS-PAGE and Western blotting. An anti-PKB antibody was used to detect endogenous PKB protein levels. An anti-PKB S473-P antibody was used to detect activated PKB. c-cbl and RNA pol II represent cytoplasmic and nuclear protein markers, respectively. *C*. A14 cells were transfected and treated as described in *A*. prior to cell lysis. Equal amounts of cellular lysate (20 μg) were analyzed by SDS-PAGE and Western blotting. An anti-pT32 specific antibody was used to detect endogenous FKHR-L1 phosphorylated by PKB (19). Since A14 cells do not express endogenous AFX, an anti-pS193 specific antibody was used to detect HA-AFX phosphorylated by PKB. 12CA5 was used to visualize HA-AFX expression. *D*. A14 cells were co-transfected with the p1205-luc reporter, pCMV-LacZ, pMT2HA-AFX, and, where indicated, pSG5-gagPKB. Cells were preincubated with LMB where noted for 30 min prior to the addition of insulin. Cells were then treated with insulin for 16 hr in the absence or presence of LMB. Luciferase activity was measured 48 hr after transfection and luciferase levels were corrected for β-galactosidase expression. pMT2HA-AFX transfected control cells were serum-starved and the inhibition of AFX activity was normalized to 0% (i.e., 100% relative promoter activity). Data were obtained from 5 separate experiments.

observed in gagPKB cotransfected cells suggests that PKB-dependent regulation of AFX activity requires the relocalization of the protein to the cytoplasm. We therefore conclude that both PKB-mediated phosphorylation in the nucleus and nuclear export mediated by Crm1 are essential for the full inhibition of AFX transcriptional activity. These data strongly suggest that a major component of the transcriptional regulation of AFX occurs at the level of nuclear transport.

Regulation of the localization of AFX by PKB does not occur at the level of export.

There are several distinct mechanisms by which PKB-dependent phosphorylation of AFX could trigger cytoplasmic accumulation of the protein. First, PKB-dependent phosphorylation could facilitate nuclear export by promoting the binding of Crm1 to AFX. In a second model, AFX may be exported from the nucleus constitutively, but be reimported rapidly when it is unphosphorylated so that its steady-state distribution is predominantly nuclear. PKB-dependent phosphorylation would then inhibit or decrease the rate of import, leading to a redistribution to the cytoplasm. Third, both nuclear import and export of AFX could be constitutive and phosphorylation would permit cytoplasmic retention by increasing the affinity of AFX for a cytoplasmic anchor protein. Finally, phosphorylation by PKB could release AFX from a nuclear anchor.

To distinguish between these possibilities, we performed Crm1-binding assays. AFX cannot be expressed in appreciable amounts as a recombinant protein in *E.coli.* Therefore, HA-AFX and HA-A3 (HA-AFX with the following mutations: T28A, S193A, and S258A) were immunoprecipitated from mammalian cell lysates and incubated with recombinant Crm1 in the absence or presence of RanQ69L, a mutant of Ran locked in its GTP-bound conformation. RanQ69L increases the affinity of Crm1 for its export cargoes. Interestingly, both PKB-phosphorylated (HA-AFX) and unphosphorylated (HA-AFX + LY294002, and HA-A3) AFX bound to Crm1 in the presence of RanQ69L (figure 3a). These data demonstrate that binding of Crm1 to AFX is independent of phosphorylation by PKB.

To confirm that HA-AFX was phosphorylated in this experiment, we examined its interaction with 14-3-3. The 14-3-3 proteins are a family of proteins reported to bind Forkhead transcription factors in a phosphorylation-dependent manner (19). As shown in figure 3a, endogenous 14-3-3 co-immunoprecipitated with HA-AFX from cells grown in the presence of serum, thereby confirming that phosphorylation of HA-AFX by PKB had occurred. These data raised the possibility that Crm1-binding to phosphorylated AFX is indirect and is mediated by 14-3-3. It has been reported previously that 14-3-3 contains a leucine-rich NES-like motif (59). It is not clear, however, that this sequence can function as a NES, especially when binding proteins are associated with 14-3-3. To test this hypothesis, we stripped 14-3-3 proteins from AFX using 1 M $MgCl_2$. This treatment substantially decreased the amount of 14-3-3 associated with AFX, but did not reduce the amount of Crm1/RanQ69L that was bound (figure 3b). It should be noted that although 14-3-3 proteins have been reported to bind Crm1 in the presence of mammalian cell lysates (59), we have been unable to detect a direct interaction between recombinant 14-3-3ζ, Crm1, and RanQ69L (data not shown).

The observation that Crm1-binding to AFX is independent of PKB-mediated phosphorylation is supported by the results of heterokaryon fusion assays. HA-A3 has a steady-state nuclear localization, but nonetheless may actively shuttle into and out of the nucleus. Nucleocytoplasmic shuttling can be observed by the use of cell-fusion assays, in which the donor

and acceptor nuclei of the fused cells can be distinguished. Redistribution of a tagged protein from one nucleus to another can only occur if the protein exits the donor nucleus and is reimported into the acceptor nucleus. To perform heterokaryon fusion assays, we used BHK21 cells expressing HA-A3 as the donor and a reporter cell line, GSN2, that expresses green flourescent protein (GFP)-streptavidin-NLS as the acceptor. The GFP-streptavidin-NLS fusion protein which is constitutively nuclear and does not shuttle, acts as a marker for the acceptor nuclei. HA-A3 shuttling would lead to its equilibration into the nucleus of a fused GSN2 cell. On the other hand, if HA-A3 does not shuttle, no HA-A3 would be detected in the nuclei of the GSN2 cells. Figure 3c shows an AFX donor nucleus surrounded by 3 GSN2 acceptor nuclei that also stained positive for HA-A3. Therefore, even though unphosphorylated AFX is predominantly nuclear in its steady-state distribution, it is constitutively shuttling into and out of the cytoplasm. AFX phosphorylation by PKB, however, results in a shift in the steady-state distribution of AFX to the cytoplasm. Taken together these results suggest that the regulation of AFX localization in response to PKB phosphorylation occurs not at the level of nuclear export or nuclear retention, but instead at the level of nuclear import or through cytoplasmic retention of phosphorylated AFX.

AFX import into the nucleus proceeds via an active, Ran-dependent mechanism.

On account of the results described above,

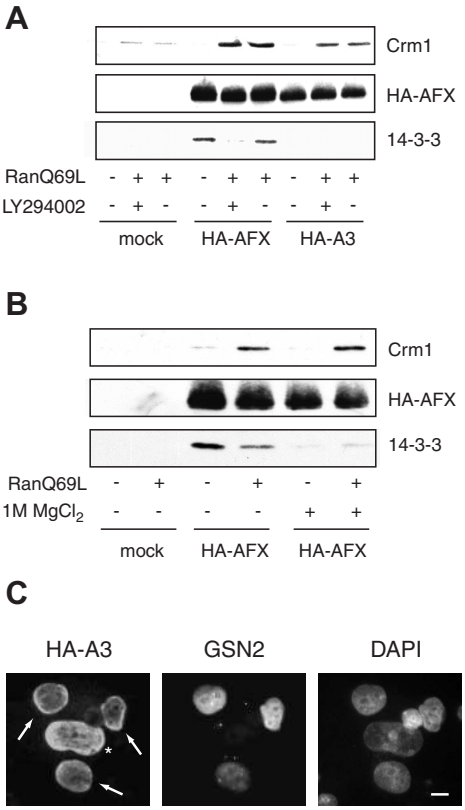

Figure 3. *Both phosphorylated and unphosphorylated AFX bind Crm1 and are exported from the nucleus. A.* HEK293 cells were transfected with either pMT2HA-AFX or pMT2HA-A3, incubated in the presence of serum for 48 hr, and where indicated, incubated with LY294002 (10 μM) for 2 hr prior to cell lysis. The HA-tagged proteins were immunoprecipitated with 12CA5 and immobilized on Protein A Sepharose. The beads were then incubated with recombinant Crm1 (500 nM) in the absence or presence of RanQ69L (3 μM). Proteins that bound to AFX were analyzed by SDS-PAGE and Western blotting. An anti-His_6 antibody was used to visualize Crm1, which possesses a C-terminal His_6 tag. Directly-conjugated HRP-12CA5 was used to assess HA-AFX and HA-A3 immunoprecipitation. An anti-14-3-3β antibody was used to detect 14-3-3 binding. *B.* HEK293 cells were transfected with pMT2HA-AFX and treated as described in *A*. Prior to incubation with Crm1 and RanQ69L, immobilized HA-AFX was washed with 1 M $MgCl_2$ where indicated to remove bound 14-3-3 proteins. Proteins that bound AFX were analyzed as described in *A*. *C.* Heterotypic cell fusions were performed between BHK21 cells transiently transfected with pMT2HA-A3 and a stably-transfected HeLa cell line expressing GFP-streptavidin-NLS (GSN2). Cell cultures were trypsinized 24 hr after transfection, mixed, allowed to adhere to coverslips overnight, and then fused using polyethylene glycol. After incubation for 1 hr with cycloheximide (50 μM), cells were fixed and stained for HA-A3 with 12CA5 and a Texas-red conjugated secondary antibody. Nuclei were visualized by staining the DNA with DAPI. Arrows indicate acceptor nuclei containing HA-A3; asterix indicates donor nucleus. Bar, 10 μm.

it was important to identify the mechanism by which AFX is imported into the nucleus. In principle, AFX could accumulate in the nucleus either by an active mechanism or by diffusion followed by nuclear retention. Most active nuclear transport pathways studied to date require energy and an intact Ran gradient across the nuclear envelope. To determine whether the nuclear accumulation of AFX observed in serum-starved cells proceeds via an active, Ran-dependent mechanism, we used tsBN2 cells. These cells have a temperature-sensitive RanGEF (RCC1) allele (60). When grown at the permissive temperature (33.5°C), tsBN2 cells have an intact Ran-gradient. However, incubation at the non-permissive temperature (39.5°C) causes collapse of the Ran-gradient and prevents Ran-dependent nuclear transport. As we have shown previously, incubation for 3 hr at 39.5°C in the presence of cycloheximide (to prevent new RanGEF synthesis) completely inhibits the import of other substrates known to utilize a classical import pathway, such as the glucocorticoid receptor (61).

To test the Ran-dependence of AFX import, we transfected tsBN2 cells with a plasmid encoding HA-AFX. In the presence of cycloheximide, cells were grown at either 33.5°C or were shifted to 39.5°C for 3 hr. The cells were then serum-starved. Immunofluorescence revealed that at the permissive temperature HA-AFX entered the nucleus on serum starvation (figure 4a; 33.5°C). In cells incubated at 39.5°C, however, HA-AFX did not accumulate in the nucleus (figure 4a; 39.5°C). We conclude, therefore, that import of AFX into the nucleus proceeds via an active, Ran-dependent mechanism.

Basic residues on both sides of S193 are necessary and sufficient for nuclear import of AFX.

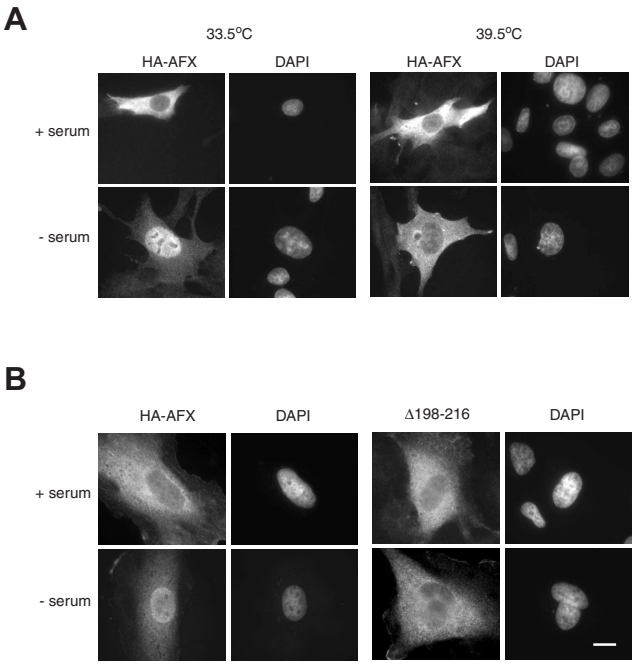

Figure 4. *AFX import into the nucleus requires a Ran-gradient and basic residues downstream from S193. A.* tsBN2 cells were transfected with pMT2HA-AFX and were incubated at 33.5∞C in the presence of serum for 48 hr. Cycloheximide (50 μM) was then added to the medium to prevent new protein synthesis and the cells were incubated at either 33.5ºC (left) or 39.5ºC (right) for 3 hr. Where indicated, the serum was then withdrawn and the cells were returned to 33.5ºC or 39.5ºC for 1.5 hr. Cells were fixed and HA-A3 was stained as described previously. *B.* HeLa cells were transfected with pMT2HA-AFX or pMT2HA-AFX(Δ198-216) and were incubated in the presence of serum for 48 hr. Serum was then withdrawn for 1.5 hr. Cells were fixed and HA-AFX and HA-AFX(Δ198-216) were stained as before. Nuclei were visualized by staining the DNA with DAPI. Bar, 10 μm.

To determine whether PKB-dependent phosphorylation regulates the nuclear import of AFX, we needed to identify the NLS within the AFX sequence. Clusters of basic residues are often predictive that a region of a protein may act as a NLS. AFX contains two clusters of basic residues flanking the PKB phosphorylation site S193. Therefore, deletion of these clusters would test whether they are required for AFX nuclear import. Deletion of residues 198-216 from HA-AFX that are C-terminal to S193 results in a protein that is unable to enter the nucleus under the same conditions of serum starvation that result in HA-AFX accumulation in the nucleus (figure 4b). Thus, this region is necessary for nuclear import of AFX. To test whether this region is sufficient to mediate import, we expressed residues 198-221 as a N-terminal fusion to GFP-GFP-GFP (AFX(198-221)-GFP3) in mammalian cells. The triple GFP construct (~80 kDa) was used to prevent passive diffusion through the nuclear pore complex (NPC), which has a diffusion limit of ~60 kDa. (These fusion proteins could not be expressed in *E. coli* because the NLS is cleaved stoichiometrically during the induction of expression, under all conditions tested.) As shown in figure 5a, AFX(198-221)-GFP3 did not accumulate in the nucleus. The basic region upstream of S193 was also not able to act as a NLS in isolation (figure 5; AFX(180-197)-GFP3). However, a construct containing both basic regions accumulated efficiently in the nucleus (AFX(180-221)-GFP3). Expression of these constructs as C-terminal fusions to GFP-GFP-GFP resulted in the same localization (data not shown). We conclude, therefore, that residues 180-221 of AFX constitute a NLS and that basic residues on both sides of S193 are required for the nuclear import of AFX.

Although the 180-221 region contains 12 lysine and arginine residues, it does not encode a classical monopartite or bipartite NLS. In addition, binding of the classical import receptors importin α1, importin α3, or importin β to this region of the protein could not be detected (data not shown). Therefore, we produced a series of mutations in AFX(180-221)-GFP3 to identify residues required for import (figure 6). Quantification of relative nuclear and cytoplasmic fluorescence levels demonstrated that mutation of arginines 188-190 to alanine had the most deleterious effect on import (figure 6, mutant II), followed by the mutation of lysines 209-211 (figure 6, mutant V). This result reinforces the requirement for basic residues both upstream and downstream of S193 for nuclear import and confirms that the sequence is an atypical NLS.

Phosphorylation of S193 reduces the rate of nuclear import.

Phosphorylation of S193 adds a negative charge to a positively charged region of AFX. This may affect the binding of AFX to its nuclear import receptor and reduce its rate of import. To test this hypothesis, we created two AFX(180-221)-GFP3 mutants; S193A, which cannot be phosphorylated, and S193E, which may mimic phosphorylation at this site. Nuclear import of S193A was comparable to wt AFX(180-221)-GFP3 (figure 7a and c). This demonstrates that mutation of S193 results in a stable fusion protein that is able to enter the nucleus. The S193E mutation, however, substantially reduced nuclear accumulation (figure 7a and c). This result suggests that PKB-dependent phosphorylation of S193 reduces the rate of nuclear import of AFX. It is important to note that these GFP3 constructs do not contain a NES. Therefore, over a 20 h transfection period even with a greatly reduced rate of import, some nuclear accumulation would be expected since the construct cannot be re-exported. Hence, a phosphorylation-dependent reduction in the import rate of AFX, combined with constitutive nuclear export would shift the steady-state distribution of the protein from the

nucleus to the cytoplasm. This mechanism is consistent with the known redistribution of AFX to the cytoplasm on addition of insulin or serum.

Discussion

Nuclear transport of the Forkhead transcription factor AFX plays a critical role in the regulation of its transcriptional activity. Phosphorylation of AFX by PKB results in a rapid change in the steady-state distribution of the protein from the nucleus to the cytoplasm. However, the mechanism by which this relocalization occurs has not been determined, although it has been proposed by others that export may be regulated by PKB, and that 14-3-3 may function as a chaperone for export, as has also been suggested for the regulation of cdc25 export (62). Based on the data presented in this paper, we propose a different model, in which nuclear import, not export, of AFX is the principal target of regulation by PKB (figure 8).

Unphosphorylated AFX is predominantly nuclear at steady-state. However, heterokaryon fusion assays (figure 3c) demonstrated that the protein is, in fact, continually shuttling between the nucleus and the cytoplasm. The rate constant for unphosphorylated AFX import, therefore, must exceed the rate constant for its export (figure 8a). Importantly, the nuclear export of AFX appears to be unaltered by PKB phosphorylation. Nuclear export of both phosphorylated and unphosphorylated AFX is likely mediated by the export receptor Crm1. Both wild-type HA-AFX and the triple-mutant HA-A3, the latter of which cannot be

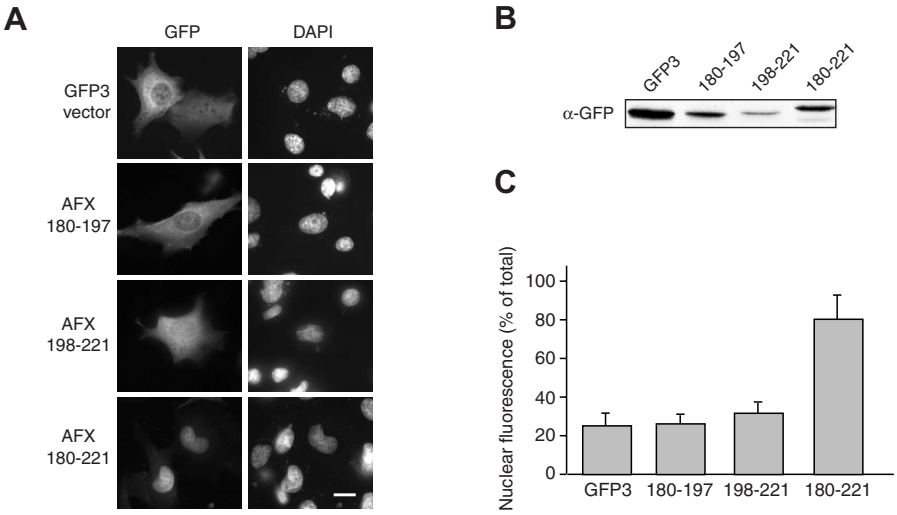

Figure 5. *Basic residues on both sides of S193 are sufficient for nuclear import.* Basic residues on either side of PKB-dependent phosphorylation site S193 were expressed as N-terminal fusions to GFP-GFP-GFP (GFP3). pKGFP3, pKGFP3-AFX(180-197), pKGFP3-AFX(198-221), and pKGFP3-AFX(180-221) were transfected into BHK21 cells. At 18 hr post-transfection, *A.* the cells were fixed and the DNA was stained with DAPI, or *B.* the cells were lysed and analyzed by SDS-PAGE (10 μl lysate/lane) and immunoblotting using an anti-GFP antibody. *C.* The relative nuclear and cytoplasmic fluorescence levels of the constructs were obtained using Openlab (Improvision). Nuclear fluorescence was calculated as a percentage of the total cellular fluorescence corrected for the background fluorescence. Each data point represents the mean fluorescence obtained from 12 randomly chosen cells. Error is expressed as the standard deviation of the mean. Bar, 10 μm.

phosphorylated by PKB, bind Crm1 in the presence of RanGTP (figure 3a). In support of the identification of Crm1 as the export receptor, we show that AFX export from the nucleus in response to insulin is blocked by the addition of LMB (figure 2a), a potent and specific inhibitor of Crm1 function.

The addition of insulin to cells results in the activation of PKB and its translocation into the nucleus where it phosphorylates Forkhead family members (figure 8b). Treatment of cells with LMB traps AFX and FKHR-L1 in the nucleus. However, both are phosphorylated in response to insulin to the same extent and at the same residues as in cells not treated with LMB (figure 2b). This result, therefore, strongly indicates that PKB enters the nucleus when activated by insulin to phosphorylate Forkhead transcription factors and other target proteins. Phosphorylation alone, however, is not sufficient to inhibit the transcriptional activity of AFX. We have shown, using transcriptional activation assays, that blockade of AFX nuclear export by the addition of LMB results in a loss of insulin-induced transcriptional control (figure 2b). Therefore, both phosphorylation by PKB and

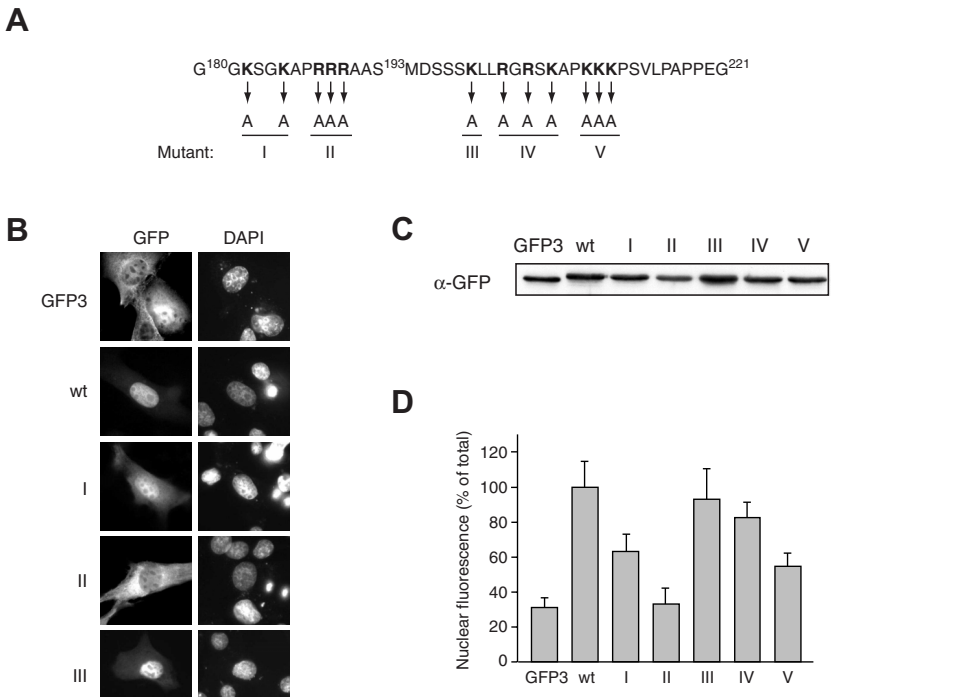

Figure 6. *Basic residues on both sides of S193 are required for nuclear import and the sequence is not a classical NLS motif. A.* There are 12 lysine and arginine residues that surround S193. Five constructs with mutations of basic residues within AFX(180-221)-GFP were made (I-V). pKGFP3, pKGFP-AFX(180-221), pKGFP3-AFX(180-221)-I, pKGFP3(180-221)-II, pKGFP3-AFX(180-221)-III, pKGFP3-AFX(180-221)-IV, and pKGFP-AFX(180-221)-V were transfected into BHK21 cells. At 18 hr post-transfection, the cells were either *B.* fixed or *C.* lysed and *D.* analyzed as described for figure 5. Bar, 10 μm.

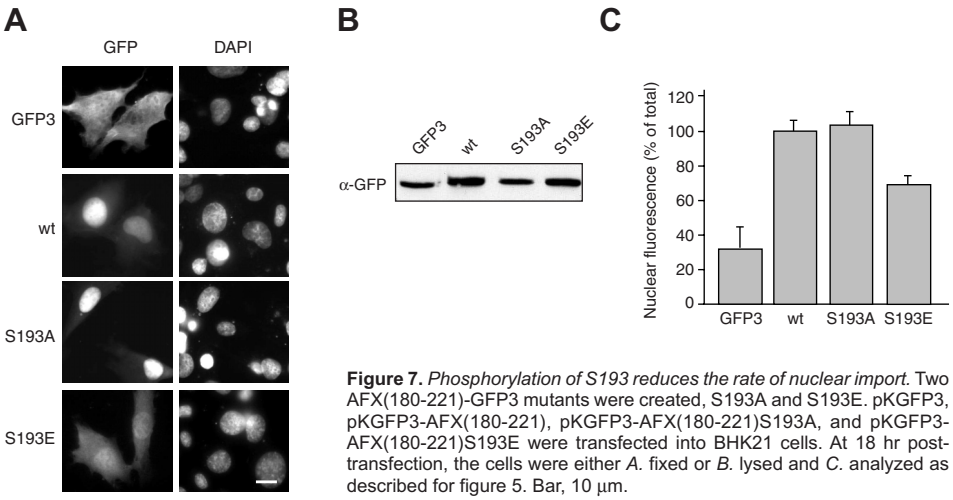

Figure 7. *Phosphorylation of S193 reduces the rate of nuclear import.* Two AFX(180-221)-GFP3 mutants were created, S193A and S193E. pKGFP3, pKGFP3-AFX(180-221), pKGFP3-AFX(180-221)S193A, and pKGFP3-AFX(180-221)S193E were transfected into BHK21 cells. At 18 hr post-transfection, the cells were either *A.* fixed or *B.* lysed and *C.* analyzed as described for figure 5. Bar, 10 μm.

nuclear export mediated by Crm1 are essential for complete inhibition of AFX transcriptional activity.

We have found that AFX enters the nucleus by an active, Ran-dependent mechanism (figure 4a) and that import requires a basic region in AFX encompassing PKB-phosphorylation site S193 (figure 5). Importantly, the addition of a negative charge at S193 substantially attenuates nuclear import (figure 7), most likely by reducing the affinity of AFX for its nuclear import receptor. We propose, therefore, that phosphorylation by PKB at S193 reduces the rate of AFX import. Since the steady-state localization of a protein is determined by its relative rate constants for import and export, the localization of AFX would shift from the nucleus to the cytoplasm (figure 8b). This alteration in the transport rate constants in response to phosphorylation by PKB, in conjunction with proposed cytoplasmic retention by binding 14-3-3 proteins (19), would result in both the efficient nuclear exclusion of AFX and the inhibition of its transcriptional activity. In conclusion, we propose that this nuclear exclusion mechanism is required to regulate the activity of AFX.

These data are consistent with the results of transcriptional activation assays reported previously for FKHR (63). The PKB phosphorylation site S256 in FKHR is analogous to S193 in AFX. Mutation of S256 to an alanine in the context of full-length FKHR abolished the ability of insulin and PKB to inhibit FKHR activity. In contrast, mutation of S256 to an aspartate resulted in a substantial inhibition of its transcriptional activity. These effects were not observed when the other two PKB sites were mutated independently. Although the localization of the S256D mutant was not assayed, our data suggest that the inhibition of FKHR activity may be due, in part, to a deficit in nuclear import.

"Classical" nuclear localization sequences are characterized by short amino acid stretches that are enriched in basic amino acids. The NLS of the large T antigen of the SV40 virus was identified by deletion analysis that resulted in mislocalization of the protein to the cytoplasm (64). It was later defined as a seven amino acid sequence (PKKKRKV) sufficient to confer nuclear localization when conjugated to a carrier protein (65). Some proteins contain similar sequences that are referred to as monopartite

NLSs. Other proteins, such as nucleoplasmin, contain bipartite NLSs that consist of two patches of basic residues separated by a ten amino acid spacer (66). Generally, proteins carrying classical or bipartite NLSs bind a cytoplasmic receptor, importin α (25, 26). Importin α associates with importin β, a protein that docks import complexes at the NPC and translocates import cargo into the nucleus. There are, however, many exceptions to this type of import and there is a large family of importins and exportins that can recognize distinct NLSs and mediate transport of different subsets of cargo. For example, ribosomal proteins (67) and histones (68) have been shown to bind directly to several different importin β family members and dock at the NPC independently of importin α. In addition, other proteins, such as hnRNP K (69) and β-catenin (70) can translocate through the NPC in the absence of any soluble factors.

We have delineated residues 180-221 of AFX as a novel type of nuclear import signal. This region contains a small portion of the DNA binding domain. Therefore, like other Forkhead family members, the DNA binding domain of AFX contributes to DNA binding and nuclear localization (71). This region of AFX contains 12 lysine and arginine residues. Although several groups of these basic residues could act as monopartite or bipartite NLSs, the information provided by mutational analysis suggests that AFX contains a non-classical NLS. Mutation of arginines 188-190 in the NLS of AFX has the greatest inhibitory effect on nuclear import followed by the mutation of lysines 209-211. These groups of basic residues are separated by

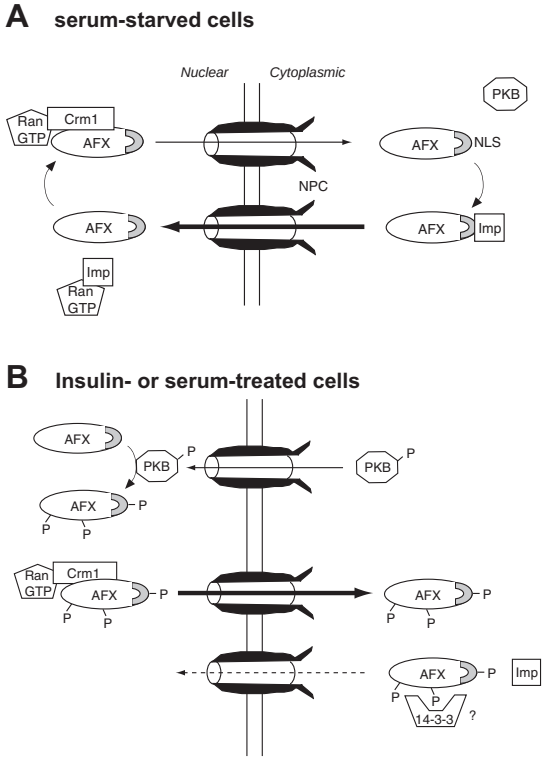

Figure 8. *Nuclear import, not export, of AFX is regulated by PKB. A.* Unphosphorylated AFX appears nuclear at steady-state, but is actually shuttling rapidly between the nucleus and the cytoplasm. Therefore, the rate of import of unphosphorylated AFX exceeds its rate of export. Nuclear export of both phosphorylated and unphosphorylated AFX are likely mediated by the exportin Crm1, since both HA-AFX and HA-A3 bind Crm1 in the presence of RanGTP. NPC, nuclear pore complex. *B.* The addition of insulin to cells activates PKB and causes it to translocate into the nucleus where it phosphorylates Forkhead family members. Phosphorylated AFX exits the nucleus by binding Crm1. Phosphorylation of AFX at S193 attenuates nuclear import, perhaps by reducing the affinity of AFX for its nuclear import receptor. Therefore, phosphorylation by PKB decreases the import rate constant without altering the export rate constant. Since the steady-state localization of a protein is determined by its relative rates of import and export, the localization of AFX would shift from the nucleus to the cytoplasm as observed. This alteration in transport rates in conjunction with proposed cytoplasmic retention by binding 14-3-3 proteins would result in exclusion of AFX from the nucleus. Since AFX requires nuclear localization to carry out transcription, this mechanism of regulated transport would inhibit the activity of AFX in response to phosphorylation by PKB.

18 amino acids and both are contained within similar sequence repeats (KAPRRR or KAPKKK). These repeats may be important for import receptor binding.

The identity of the nuclear import receptor of AFX remains to be established. Since we do not observe binding of AFX to several classical import receptors including importins α1, α3, and β, it may bind to a novel member of the importin family (which comprises >20 members in mammalian cells). Alternatively, AFX may enter the nucleus in other ways, for instance by "piggy-backing" on another protein that contains a classical NLS. The role of PKB-mediated phosphorylation and 14-3-3 binding in altering AFX import can be more thoroughly studied once the receptor is identified.

Binding of Forkhead family members to 14-3-3 proteins has been proposed to play a role in the retention of phosphorylated Forkheads in the cytoplasm (19). Phosphorylated FKHR-L1 (19) and AFX (figure 3a) both bind 14-3-3 proteins. However, we have never observed any specificity of AFX binding to a particular 14-3-3 isoform. In fact, we have observed binding to 14-3-3β, 14-3-3ε, and14-3-3ζ (data not shown). Recently, it has been proposed that 14-3-3 protein binding may impart no specific information about subcellular targeting (72). Instead, a 14-3-3 protein may obscure the NLS or NES of the protein to which it binds. In this way, 14-3-3 proteins would affect the subcellular localization of their target proteins by interfering with the binding of transport receptors. 14-3-3 binding to Forkhead family members may sterically inhibit import receptor binding and, thereby, act to prevent import.

The proposal has also been made that 14-3-3 possesses a NES that is recognized by Crm1 and participates directly in the export of binding partners through a Crm1 interaction (59). In our hands, however, 14-3-3 does not bind Crm1 directly, nor is its association with AFX necessary for the interaction of AFX with Crm1. Therefore, in this case at least, 14-3-3 isoforms appear to play no part in regulating AFX nuclear export.

What is the AFX NES? Leucine-rich regions found in several Forkhead family members conform to the consensus sequence for Crm1-dependent NES (46). This region in AFX corresponds to residues 300-308 (LELLD GLNL). Deletion of residues 298-308 results in a protein that is not properly relocalized from the nucleus to the cytoplasm on stimulation with insulin, but still binds Crm1 (unpublished data). This region, therefore, is unlikely to represents the Crm1-dependent NES, but might still contribute to the nuclear export of AFX.

Distinct chromosomal translocations in pediatric alveolar rhabdomyosarcoma and ALL involve two human forkhead genes. Alveolar rhabdomyosarcomas are associated with unique chromosomal translocations that arise from fusion of PAX3 or PAX7 to the FKHR gene (16). The PAX3-FKHR fusion protein is a more potent transcriptional activator than PAX3 alone (73) and transforms primary cells (74). Several chromosomal translocations in ALL occur at the 11q23 locus and all convey a poor prognosis that is usually associated with a high rate of treatment failure and relapse. These breakpoints affect the MLL gene (also called HRX, ALL, Htrx1) that is disrupted midway through the coding region (75, 76). A well-documented translocation arises from the fusion of MLL to the AFX gene on chromosome X (15).

Importantly, the fusions of AFX and FKHR to their breakpoint partners occur at identical amino acid positions within the Forkhead proteins. The resulting fusion proteins contain the N-terminal DNA-binding region of the fusion partner and the C-terminal transactivation domain of the forkhead protein. The MLL-AFX fusion protein expresses residues 148-501 of AFX (77). This preserves the NLS of AFX identified in this study (residues 180-

221). We have shown that deletion of residues N-terminal to S193 causes a loss of transcriptional regulation by insulin (18). In addition, potential loss of S193 phosphorylation in the context of the fusion protein would allow constitutive import into the nucleus. Therefore, both the deregulation of Forkhead activity and a constitutive nuclear localization likely contribute to the oncogenic properties of these fusion proteins.

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References

1. H. Dudek, et al., Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science* **275**, 661-5 (1997).
2. A. Kauffmann-Zeh, et al., Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385**, 544-8 (1997).
3. S. G. Kennedy, et al., The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev* **11**, 701-13 (1997).
4. Z. Songyang, D. Baltimore, L. C. Cantley, D. R. Kaplan, T. F. Franke, Interleukin 3-dependent survival by the Akt protein kinase. *Proc Natl Acad Sci U S A* **94**, 11345-50 (1997).
5. P. J. Coffer, J. R. Woodgett, 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 (1991).
6. P. F. Jones, T. Jakubowicz, F. J. Pitossi, F. Maurer, B. A. Hemmings, 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 (1991).
7. S. P. Staal, 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 (1987).
8. A. Bellacosa, J. R. Testa, S. P. Staal, P. N. Tsichlis, A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* **254**, 274-7 (1991).
9. B. M. Burgering, P. J. Coffer, Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602 (1995).
10. D. R. Alessi, et al., Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr Biol* **7**, 261-9 (1997).
11. D. Stokoe, et al., Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* **277**, 567-70 (1997).
12. K. S. Walker, et al., Activation of protein kinase B beta and gamma isoforms by insulin in vivo and by 3-phosphoinositide-dependent protein kinase-1 in vitro: comparison with protein kinase B alpha. *Biochem J* **331**, 299-308 (1998).
13. L. E. Rameh, L. C. Cantley, The role of phosphoinositide 3-kinase lipid products in cell function. *J Biol Chem* **274**, 8347-50 (1999).
14. S. Ogg, et al., The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature* **389**, 994-9 (1997).
15. A. Borkhardt, et al., 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 (1997).
16. N. Galili, et al., Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. *Nat Genet* **5**, 230-5 (1993).
17. M. J. Anderson, C. S. Viars, S. Czekay, W. K. Cavenee, K. C. Arden, Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. *Genomics* **47**, 187-99 (1998).
18. G. J. Kops, et al., Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**, 630-4 (1999).
19. A. Brunet, et al., Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-68 (1999).
20. P. F. Dijkers, R. H. Medemadagger, J. J. Lammers, L. Koenderman, P. J. Coffer, Expression of the pro-apoptotic bcl-2 family member bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* **10**, 1201-4 (2000).
21. R. H. Medema, G. J. Kops, J. L. Bos, B. M. Burgering,

AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782-7 (2000).

22. A. Komeili, E. K. O'Shea, Nuclear transport and transcription. *Curr Opin Cell Biol* **12**, 355-60 (2000).
23. U. M. Moll, M. LaQuaglia, J. Benard, G. Riou, Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. *Proc Natl Acad Sci U S A* **92**, 4407-11 (1995).
24. C. L. Schlamp, G. L. Poulsen, T. M. Nork, R. W. Nickells, Nuclear exclusion of wild-type p53 in immortalized human retinoblastoma cells. *J Natl Cancer Inst* **89**, 1530-6 (1997).
25. I. W. Mattaj, L. Englmeier, Nucleocytoplasmic transport: the soluble phase. *Annu Rev Biochem* **67**, (1998).
26. D. Gorlich, U. Kutay, Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol* **15**, 607-60 (1999).
27. F. R. Bischoff, H. Krebber, T. Kempf, I. Hermes, H. Ponstingl, Human RanGTPase-activating protein RanGAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. *Proc Natl Acad Sci U S A* **92**, 1749-53 (1995).
28. F. R. Bischoff, H. Ponstingl, Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature* **354**, 80-2 (1991).
29. D. Gorlich, N. Pante, U. Kutay, U. Aebi, F. R. Bischoff, Identification of different roles for RanGDP and RanGTP in nuclear protein import. *Embo J* **15**, 5584-94 (1996).
30. M. Rexach, G. Blobel, Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell* **83**, 683-92 (1995).
31. E. Izaurralde, U. Kutay, C. von Kobbe, I. W. Mattaj, D. Gorlich, The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *Embo J* **16**, 6535-47 (1997).
32. R. H. Kehlenbach, A. Dickmanns, A. Kehlenbach, T. Guan, L. Gerace, A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export. *J Cell Biol* **145**, 645-57 (1999).
33. A. Kaffman, E. K. O'Shea, Regulation of nuclear localization: a key to a door. *Annu Rev Cell Dev Biol* **15**, 291-339 (1999).
34. R. Ben-Levy, S. Hooper, R. Wilson, H. F. Paterson, C. J. Marshall, Nuclear export of the stress-activated protein kinase p38 mediated by its substrate MAPKAP kinase-2. *Curr Biol* **8**, 1049-57 (1998).
35. A. Kaffman, N. M. Rank, E. K. O'Shea, Phosphorylation regulates association of the transcription factor Pho4 with its import receptor Pse1/Kap121. *Genes Dev* **12**, 2673-83 (1998).
36. A. Kaffman, N. M. Rank, E. M. O'Neill, L. S. Huang, dependent expression of mammalian CRM1, a protein E. K. O'Shea, The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. *Nature* **396**, 482-6 (1998).
37. M. J. De Vit, J. A. Waddle, M. Johnston, Regulated nuclear translocation of the Mig1 glucose repressor. *Mol Biol Cell* **8**, 1603-18 (1997).
38. M. J. DeVit, M. Johnston, The nuclear exportin Msn5 is required for nuclear export of the Mig1 glucose repressor of Saccharomyces cerevisiae. *Curr Biol* **9**, 1231-41 (1999).
39. G. J. Arts, M. Fornerod, I. W. Mattaj, Identification of a nuclear export receptor for tRNA. *Curr Biol* **8**, 305-14 (1998).
40. K. Welch, J. Franke, M. Kohler, I. G. Macara, RanBP3 contains an unusual nuclear localization signal that is imported preferentially by importin-alpha3. *Mol Cell Biol* **19**, 8400-11 (1999).
41. M. E. Nemergut, I. G. Macara, Nuclear import of the ran exchange factor, RCC1, is mediated by at least two distinct mechanisms. *J Cell Biol* **149**, 835-50 (2000).
42. K. Plafker, I. G. Macara, Facilitated nucleocytoplasmic shuttling of the Ran binding protein RanBP1. *Mol Cell Biol* **20**, 3510-21 (2000).
43. K. A. Lee, A. Bindereif, M. R. Green, A small-scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. *Gene Anal Tech* **5**, 22-31 (1988).
44. K. Zerivitz, G. Akusjarvi, An improved nuclear extract preparation method. *Gene Anal Tech* **6**, 101-9 (1989).
45. B. E. Black, L. Levesque, J. M. Holaska, T. C. Wood, B. M. Paschal, Identification of an NTF2-related factor that binds Ran-GTP and regulates nuclear protein export. *Mol Cell Biol* **19**, 8616-24 (1999).
46. W. H. Biggs, 3rd, J. Meisenhelder, T. Hunter, W. K. Cavenee, K. C. Arden, Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A* **96**, 7421-7426 (1999).
47. H. Takaishi, et al., Regulation of nuclear translocation of forkhead transcription factor AFX by protein kinase B. *Proc Natl Acad Sci U S A* **96**, 11836-41 (1999).
48. N. N. Ahmed, et al., The proteins encoded by c-akt and v-akt differ in post-translational modification, subcellular localization and oncogenic potential. *Oncogene* **8**, 1957-63 (1993).
49. J. Q. Cheng, et al., Transforming activity and mitosis-related expression of the AKT2 oncogene: evidence suggesting a link between cell cycle regulation and oncogenesis. *Oncogene* **14**, 2793-801 (1997).
50. M. Fornerod, M. Ohno, M. Yoshida, I. W. Mattaj, CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**, 1051-60 (1997).
51. M. Fukuda, et al., CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390**, 308-11 (1997).
52. N. Kudo, et al., Molecular cloning and cell cycle-

involved in nuclear export of proteins. *J Biol Chem* **272**, 29742-51 (1997).

53. B. Ossareh-Nazari, F. Bachelerie, C. Dargemont, Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science* **278**, 141-4 (1997).
54. K. Stade, C. S. Ford, C. Guthrie, K. Weis, Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell* **90**, 1041-50 (1997).
55. B. Wolff, J. J. Sanglier, Y. Wang, Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem Biol* **4**, 139-47 (1997).
56. N. Kudo, et al., Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc Natl Acad Sci U S A* **96**, 9112-7 (1999).
57. M. Andjelkovic, et al., Role of translocation in the activation and function of protein kinase B. *J Biol Chem* **272**, 31515-24 (1997).
58. R. Meier, D. R. Alessi, P. Cron, M. Andjelkovic, B. A. Hemmings, Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bbeta. *J Biol Chem* **272**, 30491-7 (1997).
59. K. Rittinger, et al., Structural analysis of 14-3-3 phosphopeptide complexes identifies a dual role for the nuclear export signal of 14-3-3 in ligand binding. *Mol Cell* **4**, 153-66 (1999).
60. T. Tachibana, N. Imamoto, H. Seino, T. Nishimoto, Y. Yoneda, Loss of RCC1 leads to suppression of nuclear protein import in living cells. *J Biol Chem* **269**, 24542-5 (1994).
61. S. A. Richards, K. L. Carey, I. G. Macara, Requirement of guanosine triphosphate-bound ran for signal-mediated nuclear protein export. *Science* **276**, 1842-4 (1997).
62. S. R. Datta, A. Brunet, M. E. Greenberg, Cellular survival: a play in three Akts. *Genes Dev* **13**, 2905-27 (1999).
63. S. Guo, et al., Phosphorylation of Serine 256 by Protein Kinase B Disrupts Transactivation by FKHR and Mediates Effects of Insulin on Insulin-like Growth Factor-binding Protein-1 Promoter Activity through a Conserved Insulin Response Sequence. *J Biol Chem* **274**, 17184-17192 (1999).
64. R. E. Lanford, J. S. Butel, Construction and characterization of an SV40 mutant defective in nuclear transport of T antigen. *Cell* **37**, 801-13 (1984).
65. R. E. Lanford, P. Kanda, R. C. Kennedy, Induction of nuclear transport with a synthetic peptide homologous to the SV40 T antigen transport signal. *Cell* **46**, 575-82 (1986).
66. C. Dingwall, S. V. Sharnick, R. A. Laskey, A polypeptide domain that specifies migration of nucleoplasmin into the nucleus. *Cell* **30**, (1982).
67. S. Jakel, D. Gorlich, Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. *Embo J* **17**, 4491-502 (1998).
68. S. Jakel, et al., The importin beta/importin 7 heterodimer is a functional nuclear import receptor for histone H1. *Embo J* **18**, 2411-23 (1999).
69. W. M. Michael, P. S. Eder, G. Dreyfuss, The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein. *Embo J* **16**, 3587-98 (1997).
70. F. Yokoya, N. Imamoto, T. Tachibana, Y. Yoneda, beta-catenin can be transported into the nucleus in a Ran-unassisted manner. *Mol Biol Cell* **10**, 1119-31 (1999).
71. X. Qian, R. H. Costa, Analysis of hepatocyte nuclear factor-3 beta protein domains required for transcriptional activation and nuclear targeting. *Nucleic Acids Res* **23**, 1184-91 (1995).
72. A. J. Muslin, H. Xing, 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal* **12**, 703-709 (2000).
73. J. L. Bennicelli, R. H. Edwards, F. G. Barr, Mechanism for transcriptional gain of function resulting from chromosomal translocation in alveolar rhabdomyosarcoma. *Proc Natl Acad Sci U S A* **93**, 5455-9 (1996).
74. S. Scheidler, W. J. Fredericks, F. J. Rauscher, 3rd, F. G. Barr, P. K. Vogt, The hybrid PAX3-FKHR fusion protein of alveolar rhabdomyosarcoma transforms fibroblasts in culture. *Proc Natl Acad Sci U S A* **93**, 9805-9 (1996).
75. S. Ziemin-van der Poel, et al., Identification of a gene, MLL, that spans the breakpoint in 11q23 translocations associated with human leukemias. *Proc Natl Acad Sci U S A* **88**, 10735-9 (1991).
76. J. Corral, et al., Acute leukemias of different lineages have similar MLL gene fusions encoding related chimeric proteins resulting from chromosomal translocation. *Proc Natl Acad Sci U S A* **90**, 8538-42 (1993).
77. P. Parry, Y. Wei, G. Evans, 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 (1994).

CHAPTER 5

AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27^{kip1}

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AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through $p27^{kip1}$

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The Forkhead transcription factors AFX, FKHR and FKHR-L1 are orthologues of DAF-16, a Forkhead factor that regulates longevity in *Caenorhabditis elegans* (1, 2, 3). Here we show that overexpression of these transcription factors causes growth suppression in a variety of cell lines including a Ras-transformed cell line and a cell line lacking the tumoursuppressor PTEN. Expression of AFX blocks cell-cycle progression at phase G1, independent of functional retinoblastoma protein (pRb) but dependent on the cell-cycle inhibitor $p27^{kip1}$. Indeed, AFX transcriptionally activates $p27^{kip1}$, resulting in increased protein levels. We conclude that AFX-like proteins are involved in cell-cycle regulation and that inactivation of these proteins is an important step in oncogenic transformation.

In *C. elegans*, DAF-16 is inhibited by a pathway consisting of proteins that are orthologues of the insulin receptor, phosphatidyl-inositol-3-OH kinase (PI(3)K) and protein kinase B (PKB)/c-Akt (2, 3, 4). The same pathway exists in vertebrates, where AFX, FKHR and FKHR-L1 are directly phosphorylated and inhibited by PKB (5, 6, 7, 8, 9). Additionally, AFX-mediated transcription is negatively regulated through Ras/Ral-signalling (5). As PI(3)K/PKB and Ras signalling are both important in cell-cycle progression through G1 phase (10, 11, 12, 13), we hypothesized that the members of the AFX-family of Forkhead transcription factors might regulate aspects of G1 progression. Therefore, we have examined the effect of ectopic expression of these AFX-like proteins on cell proliferation in mammalian cells.

Transfection of A14 cells (5) with AFX, FKHR or FHKR-L1 resulted in efficient growth suppression (figure 1a). When mutants of these AFX-like proteins which lack PKB phosphorylation sites (AFX.A3, FKHR.A3 or FKHR-L1.A3) were introduced, they inhibited colony formation even more efficiently, to levels observed with the cyclin-dependent kinase (cdk)-inhibitor $p16^{ink4a}$ (figure 1a). However, a mutant of AFX lacking the DNA-binding domain (AFX.ΔDB) had no effect (figure 1a). Ectopic AFX and AFX.A3 were expressed at similar levels at 48 hr after transfection, but very little ectopic AFX or AFX.A3 could be detected in colonies that grew after 2 weeks of selection (figure 1a). AFX is regulated by PKB and Ras signalling, so we examined the consequences of expression of AFX or AFX.A3 in the PTEN-

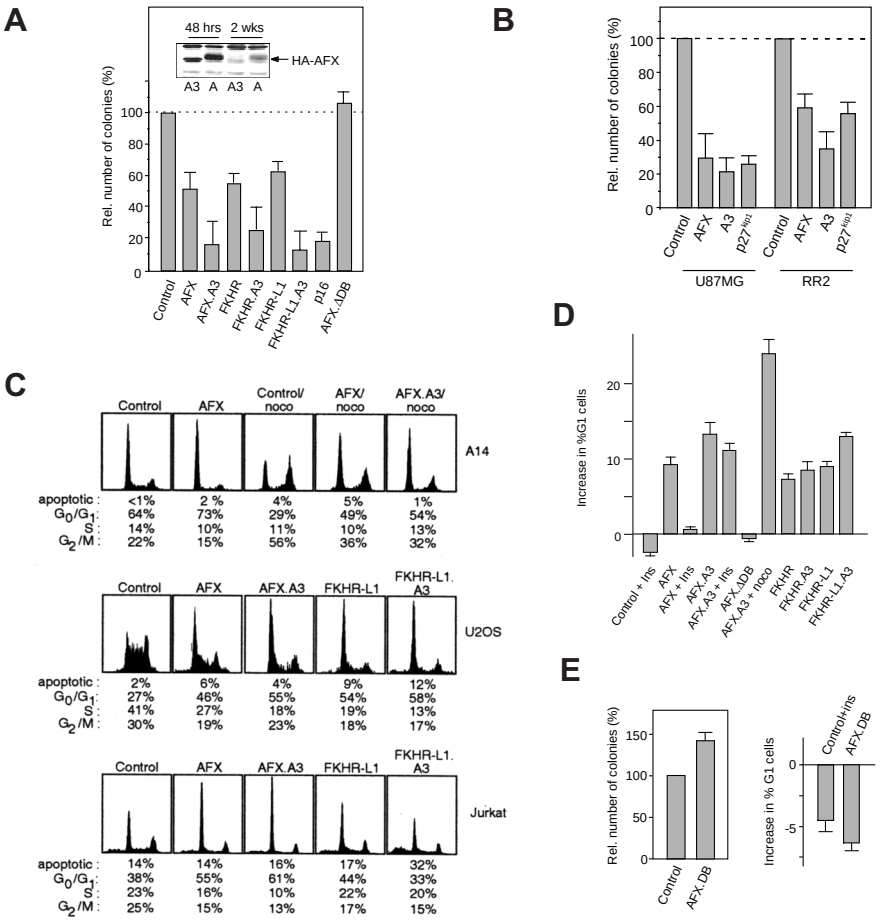

Figure 1. *Expression of AFX causes arrest at the G1 phase of the cell-cycle.* Colony formation assays in A14 cells (*A*), U87MG cells and RR2 (*B*). Cells were transfected with an expression plasmid for wild-type or mutant AFX, FKHR or FKHR-L1 in combination with pBabe-puro. Puromycin-resistant colonies were scored after two weeks of selection. Levels of ectopically expressed AFX (A) and AFX.A3 (A3) in total lysates at 48 h and 2 weeks after transfection were detected by western blot (inset). *C.* Cell-cycle profiles of transfected A14, U2OS or Jurkat cells expressing the indicated AFX/FKHR-L1 proteins or respective mutants. *D.* Absolute increase in percentage of A14 cells in the G0/G1-phase upon expression of the indicated proteins. *E.* Increase in colony formation and a decrease in G1 content in cells expressing the AFX.DB mutant. The data in *D* and *E* are the averages of three independent experiments. Where indicated cells were treated with nocodazole (noco) or insulin (ins) during the last 16 h before collection.

deficient glioblastoma cell line U87MG, which contains high levels of PKB activity (14), and in RasV12-transformed Rat-1 cell line, RR2 (15). Expression of wild-type AFX caused a marked inhibition of colony formation in these transformed cell lines (figure 1b). Growth inhibition was even more pronounced when using AFX.A3 (figure 1b). We obtained similar data with another RasV12-transformed Rat-1 cell line, RR7 (15) (not shown).

Introduction of AFX into A14 cells resulted in accumulation of cells in the G0/G1-phase of the cell-cycle (figure 1c), indicating that these cells are arrested in G1. This change in cell-cycle distribution was even more clear when cells were treated with nocodazole after transfection so that cycling cells would accumulate in the G2/M phase, unless they had been arrested in G1 (figure 1c). Expression of AFX.ΔDB had no effect on the cell-cycle distribution (figure 1d), indicating that transcriptional activity is required for G1 arrest. Insulin treatment overcame the G1 arrest induced by AFX, but had very little effect on mock-transfected cells in the presence of 10% fetal calf serum (FCS) (figure 1d). However, insulin did not overcome the AFX.A3-induced cell-cycle arrest (figure 1d). This is consistent with the finding that insulin efficiently inhibits AFX-mediated transcription (5), but transcriptional activation by AFX.A3 is only marginally affected (data not shown). Fluorescence-activated cell sorting (FACS) analysis of other cell lines, in particular tumor-derived cell lines, demonstrated that expression of AFX, AFX.A3, FKHR-L1 or FKHR-L1.A3 in U87MG, RR2, RR7 or U2OS osteosarcoma cells caused a clear G1-arrest (figure 1c and data not shown). Observation of the percentage of cells cells with less than 2N DNA content indicated only minor increases in the number of apoptotic cells. For comparison, we studied Jurkat T cells, which become apoptotic upon introduction of the FKHR-L1.A3 mutant (6). However, increased apoptosis was only seen with this particular mutant, whereas all other AFX/FKHR-L1 expression plasmids tested caused a G1-arrest (figure 1c). Thus, although apoptosis may be affected, the G1-arrest seems to be a more general response to all AFX-like proteins.

To investigate whether inhibition of endogenous AFX-like activity would affect the proliferative capacity of A14 cells, we expressed a mutant of AFX encoding only the DNA binding domain (AFX.DB). This mutant is unable to transactivate (data not shown), but is likely to compete with endogenous AFX-like proteins for binding to DNA. Interestingly, expression of AFX.DB resulted in a significant increase in colony formation (greater than 40%) and a decrease in the percentage of cells in G1, similar to that observed upon addition of insulin (figure 1e). These data show that interference with endogenous AFX-like activity results in a proliferative advantage in these cells.

To investigate the mechanism underlying the AFX-induced G1 arrest in more detail, we transfected A14 cells with myc-tagged pRb in combination with AFX or AFX.A3. Expression of AFX or AFX.A3 caused an inhibition of pRb-phosphorylation, indicating that AFX can arrest cell-cycle progression prior to the G1 restriction point (figure 2a). To analyze the expression and activity of endogenous cell-cycle regulators, we separated the transfected and untransfected A14 cells by magnetic-activated cell sorting (MACS). Exogenous AFX and AFX.A3 were detected in the transfected cells, but no protein was seen in the untransfected cells, indicating that separation was effective (figure 2b). We found that the kinase activity of cyclin E and cdk2 immunoprecipitates were significantly inhibited in cells expressing AFX or AFX.A3 (figure 2b), although cyclin E and cdk2 protein levels were not decreased (data not shown). Expression of the cdk-inhibitor $p21^{waf1/cip1}$ was unchanged upon introduction of AFX-proteins (not shown). In contrast, expression of AFX or AFX.A3 caused an increase in the protein levels of the cdk-inhibitor $p27^{kip1}$ (figure 2b), suggesting that AFX-induced upregulation of $p27^{kip1}$ is responsible for the observed inhibition of cyclin E/cdk2 complexes. These effects were not seen with the AFX.ΔDB mutant (figure 2b).

Next we examined the effect of PKB activation or inactivation on the expression of $p27^{kip1}$ and phosphorylation of endogenous AFX

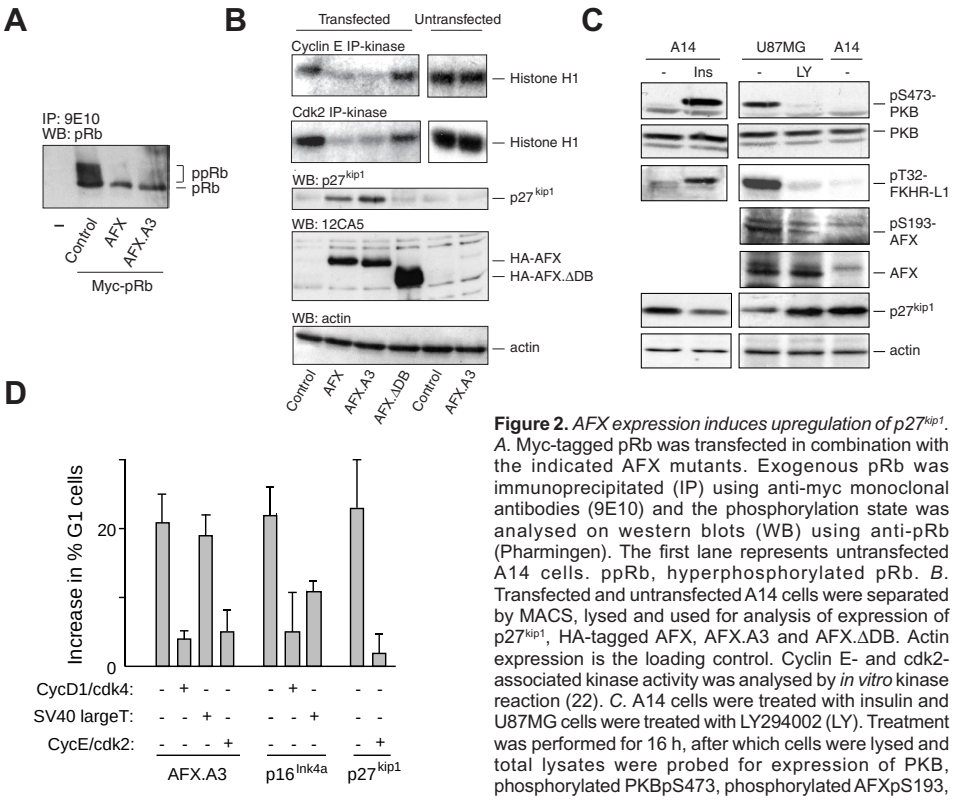

Figure 2. *AFX expression induces upregulation of p27kip1. A.* Myc-tagged pRb was transfected in combination with the indicated AFX mutants. Exogenous pRb was immunoprecipitated (IP) using anti-myc monoclonal antibodies (9E10) and the phosphorylation state was analysed on western blots (WB) using anti-pRb (Pharmingen). The first lane represents untransfected A14 cells. ppRb, hyperphosphorylated pRb. *B.* Transfected and untransfected A14 cells were separated by MACS, lysed and used for analysis of expression of p27kip1, HA-tagged AFX, AFX.A3 and AFX.ΔDB. Actin expression is the loading control. Cyclin E- and cdk2-associated kinase activity was analysed by *in vitro* kinase reaction (22). *C.* A14 cells were treated with insulin and U87MG cells were treated with LY294002 (LY). Treatment was performed for 16 h, after which cells were lysed and total lysates were probed for expression of PKB, phosphorylated PKBpS473, phosphorylated AFXpS193, phosphorylated FKHR-L1pT32, and expression of p27kip1, and actin as additional loading control. *D.* A14 cells were transfected with the indicated expression plasmids, treated with nocodazole for the last 16 h after transfection and analysed by flow cytometry. The data are the averages of three independent experiments.

and FKHR-L1. Insulin stimulation of A14 cells resulted in activation of PKB, and a reduction in p27kip1 expression (figure 2c). We were unable to detect endogenous AFX in A14 cells, but saw induction of FKHR-L1 phosphorylation in response to insulin (figure 2c). Conversely, inhibition of PKB in U87MG cells by addition of the PI(3)K inhibitor LY294002 caused a reduction in AFX- and FKHR-L1- phosphorylation and a concomitant increase in p27kip1 expression (figure 2c). Therefore, PKB-mediated phospho-rylation of AFX-like Forkhead factors is coupled with a decrease in p27kip1 levels in both of these cell lines.

We next reasoned that if induction of p27kip1 levels is responsible for the AFX-induced G1 arrest, co-expression of cyclin/cdk complexes should titrate out the induced p27kip1 and rescue cells from the cell-cycle arrest. Indeed, combinations of cyclin D/cdk4 or cyclin E/cdk2 could fully override the AFX.A3-induced cell-cycle arrest (figure 2d). In addition, we examined the effect of co-expression of the SV40 large T oncoprotein, which binds and inactivates pRb and p53. Co-expression of SV40 large T did not rescue the AFX.A3-induced G1 arrest, although it could override a p16ink4a-induced cell-cycle arrest (figure 2d).

Cells devoid of functional pRb no longer

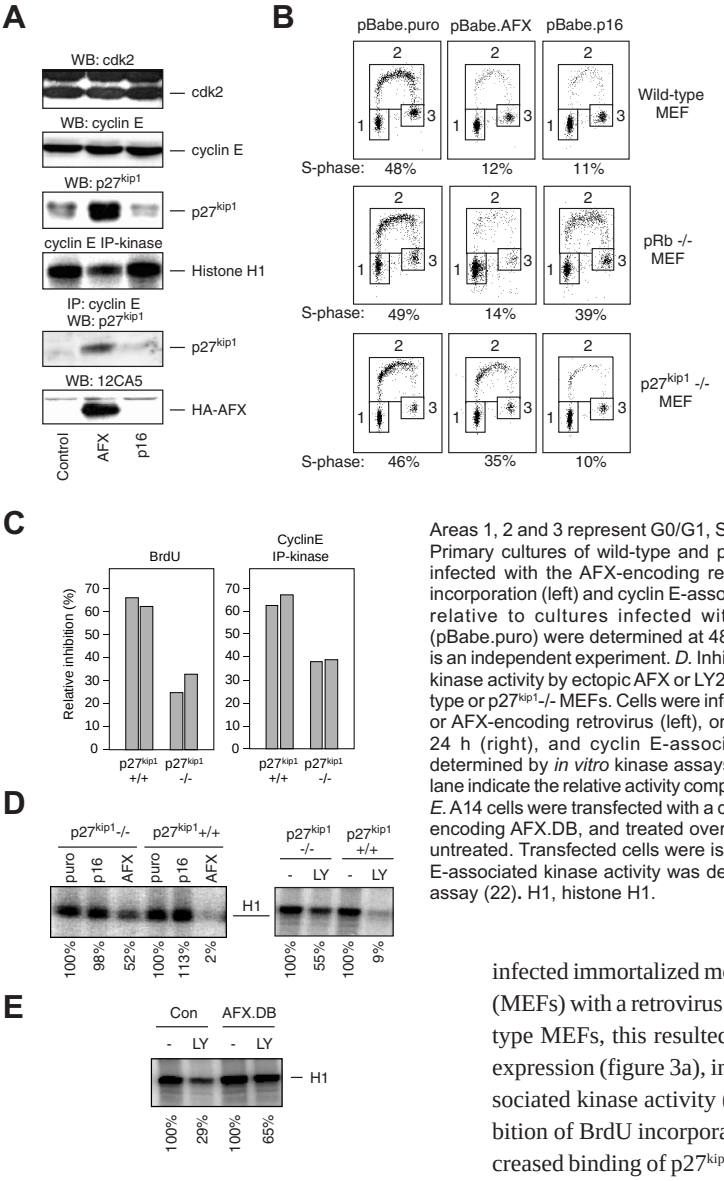

Figure 3. *AFX-induced cell-cycle arrest occurs independent of pRb but is dependent on p27kip1. A.* Expression of exogenous AFX, induction of p27kip1, expression of cyclin E and cdk2, inhibition of cyclin E-associated kinase activity and association of p27kip1 to cyclin E, in immortalized wild-type MEFs upon infection with an AFX- or p16ink4a-encoding retrovirus. *B.* Dot-plots of BrdU-fluorescence versus DNA content of immortalized wild-type, pRb-deficient, or p27kip1-deficient MEFs infected with a control virus (pBabe.puro), a retrovirus encoding AFX (pBabe. AFX) or p16 ink4a (pBabe. p16) at 48 h after infection. Areas 1, 2 and 3 represent G0/G1, S, and G2/M respectively. *C.* Primary cultures of wild-type and p27kip1-deficient MEFs were infected with the AFX-encoding retrovirus. Inhibiton of BrdU incorporation (left) and cyclin E-associated kinase activity (right) relative to cultures infected with the control retrovirus (pBabe.puro) were determined at 48 h after infection. Each bar is an independent experiment. *D.* Inhibition of cyclin E-associated kinase activity by ectopic AFX or LY294002 in immortalized wild-type or p27kip1-/- MEFs. Cells were infected with a control, p16ink4a, or AFX-encoding retrovirus (left), or treated with LY294002 for 24 h (right), and cyclin E-associated kinase activity was determined by *in vitro* kinase assays. Percentages below each lane indicate the relative activity compared to the control samples. *E.* A14 cells were transfected with a control plasmid, or a plasmid encoding AFX.DB, and treated overnight with LY294002 or left untreated. Transfected cells were isolated by MACS and cyclin E-associated kinase activity was determined by *in vitro* kinase assay (22). H1, histone H1.

require cyclin D-associated kinase activity, but do need cyclin E-associated kinase activity to initiate S phase (16). Thus, if p27kip1 mediates the AFX-induced cell-cycle block, pRb-deficient cells should arrest in response to AFX and p27kip1-deficient cells should not. To examine this, we infected immortalized mouse embryo fibroblasts (MEFs) with a retrovirus encoding AFX. In wild-type MEFs, this resulted in induction of p27kip1 expression (figure 3a), inhibition of cyclin E-associated kinase activity (figure 3a), strong inhibition of BrdU incorporation (figure 3b) and increased binding of p27kip1 in cyclin E-complexes (figure 3a). When the same retrovirus was used to infect immortalized pRb-deficient MEFs, a similar inhibition of BrdU incorporation was seen (figure 3b). More importantly, in immortalized p27kip1-deficient MEFs, inhibition of BrdU incorporation (figure 3b) and cyclin E-associated

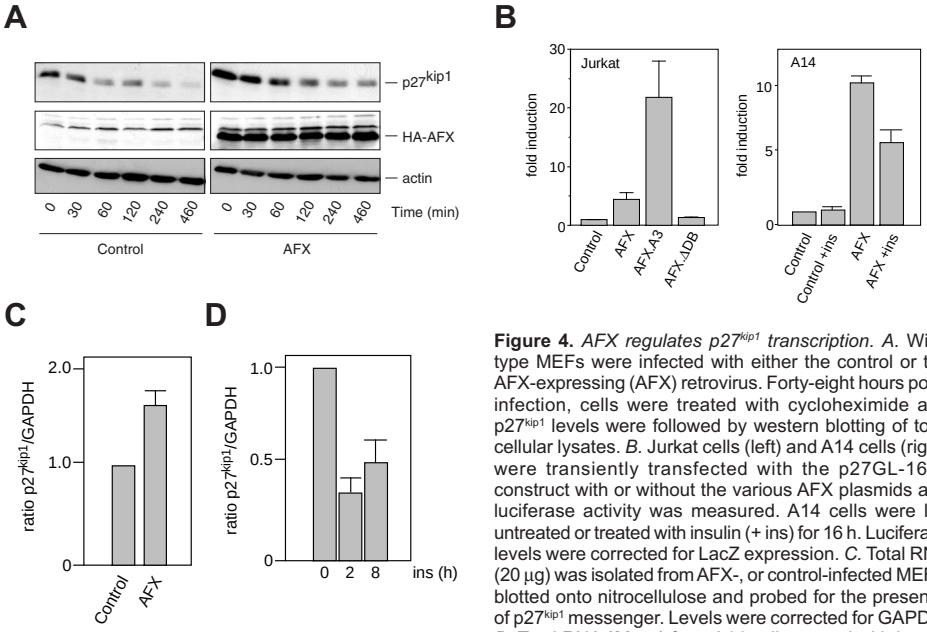

Figure 4. *AFX regulates p27kip1 transcription. A.* Wild-type MEFs were infected with either the control or the AFX-expressing (AFX) retrovirus. Forty-eight hours post-infection, cells were treated with cycloheximide and p27kip1 levels were followed by western blotting of total cellular lysates. *B.* Jurkat cells (left) and A14 cells (right) were transiently transfected with the p27GL-1609 construct with or without the various AFX plasmids and luciferase activity was measured. A14 cells were left untreated or treated with insulin (+ ins) for 16 h. Luciferase levels were corrected for LacZ expression. *C.* Total RNA (20 µg) was isolated from AFX-, or control-infected MEFs, blotted onto nitrocellulose and probed for the presence of p27kip1 messenger. Levels were corrected for GAPDH. *D.* Total RNA (20 µg) from A14 cells treated with insulin for the indicated periods was probed for p27kip1 messenger levels. Levels are corrected for GAPDH. In *B-D*, results represent the averages of three independent experiments.

kinase activity (figure 3d) by AFX were greatly reduced, although inhibition of BrdU incorporation by p16ink4a occured normally in these cells. BrdU incorporation and cyclin E-associated kinase activity were also differentially affected in primary MEFs established from wild-type and p27kip1 null mice (figure 3c), ruling out the possibility that genetic variation occurring during immortalization gave rise to the observed differences. In addition, inhibition of endogenous PI(3)K/PKB signalling by addition of LY294002 caused a very efficient inhibition of cyclin E-associated kinase activity in wild-type MEFs. This inhibitory activity was severely reduced in the p27kip1-deficient MEFs, very similar to our observations after ectopic expression of AFX (figure 3d). Moreover, LY294002-mediated inhibition of cyclin E/cdk2 activity requires endogenous AFX-like activity, as expression of the AFX.DB interfering mutant reduced LY294002-induced inhibition of cyclin E/cdk2 complexes in A14 cells (figure 3e). Thus, our data show that the inhibition of cyclin E/cdk2 complexes by AFX and the endogenous PI(3)K/PKB signalling pathway both act mainly through p27kip1. Some residual inhibition of cyclin E-associated kinase activity still appears in response to AFX in p27kip1-deficient MEFs, and therefore other cell-cycle targets of AFX besides p27kip1 may exist. Nevertheless, these data clearly show that p27kip1 is a major target of the PI(3)K/PKB/AFX signalling pathway in regulation of cellular proliferation.

To examine whether the observed increase in p27kip1 protein levels by AFX is due to a stabilization of the protein, we analyzed p27kip1 degradation in the absence or presence of AFX by western blot, using cycloheximide-treated cells (figure 4a) or a ^{35}S-methionine pulse-chase experiment (not shown). The kinetics of p27kip1 degradation were similar in both cases ($t_{1/2}$~45

min), although $p27^{kip1}$ protein levels were elevated in AFX-expressing cells (figure 4a). To study whether AFX enhances transcription of the *$p27^{kip1}$* gene, we transiently transfected Jurkat and A14 cells with a luciferase reporter construct driven by the $p27^{kip1}$ promoter, which contains multiple putative AFX binding sites (5, 17), together with the various AFX plasmids. AFX and AFX.A3 increased luciferase activity up to 20-fold (figure 4b). Insulin treatment of A14 cells expressing AFX inhibited luciferase activity by 50%, similar to the observed inhibition of insulin on other AFX-regulated promoter constructs (5, 6, 7). These experiments show that AFX can regulate $p27^{kip1}$ promoter activity. Next, we performed northern blots using RNA isolated from wild-type MEFs infected with the pBabe.AFX or pBabe.puro retroviruses. Cells expressing AFX displayed a moderate, but consistent, increase in $p27^{kip1}$ mRNA levels (figure 4c). In addition, insulin treatment of A14 results in the phosphorylation and inactivation of endogenous Forkheads (figure 1d), and a consequent reduction in $p27^{kip1}$ mRNA levels within 2 h (fig 4d).

The data presented here demonstrate that AFX is important in regulating cellular proliferation. AFX integrates signals from PI(3)K/PKB signalling as well as Ras/Ral signalling to regulate transcription of $p27^{kip1}$. Indeed, $p27^{kip1}$-/- cells are significantly less inhibited by AFX activity than their $p27^{kip1}$+/+ counterparts, showing that $p27^{kip1}$ is a major target of AFX-like Forkhead proteins. Our finding that these Forkhead transcription factors are predominantly involved in the regulation of cell-cycle progression indicates that the proclaimed role of PI(3)K/PKB signalling in tumorigenesis may need refinement. The disruption of the PI(3)K/PKB/Forkhead transcription factor pathway in tumorigenesis may override a cell-cycle block rather than bestow protection from apoptosis. In this respect, it is interesting to note that the ability to arrest in G1 is coupled with the ability to undergo apoptosis in certain cells (18, 19). This indicates that a G1 arrest may precede apoptosis, similarly to G1 arrest preceding cell differentiation.

Methods

Cloning, plasmids and cells. pBabe.AFX was created by ligating a Klenow-blunted PstI/KpnI fragment from pMT2HA-AFX (5) into EcoRI-cut and Klenow-blunted dephosphorylated pBabe.puro plasmid (20). pMT2HA-AFX.A3 was created by mutating T28, S193 and S258 to alanine using the primers as described (5). pMT2HA-AFX.ΔDB was created by deleting basepairs +313 to +537 by PCR-based mutagenesis using the primer ΔDB (5'-GAAATCAGTCATATGCAGAAGGAGGC AAGAGCGGCAAAGC-3'). pMT2HA-DB was created by ligating a KpnI/XbaI fragment from pRP261-AFX.DB (5) into KpnI/XbaI-cut pMT2HA. Myc-tagged pRb and pCMV.$p27^{kip1}$ were a gift from Dr. R. Bernards. pBabe.$p16^{ink4a}$, pCMV.CD20, pCMV.cyclin D1, pCMV.cyclin E, pCMV.cdk4, pCMV.cdk2, pCMV.largeT and pCMV.$p16^{ink4a}$ have all been described (20). pECE.FKHR-L1 and pECE.FKHR-L1.A3 were a gift from M. Greenberg (6). pAlter-MAX-FKHR and pAlter-MAX-FKHR.A3 were a gift from T. Unterman (7). p27GL-1609 was a gift from I. Touw (17). Immortalized wild-type, pRb-deficient and $p27^{kip1}$-deficient MEFs were a gift from D. Peeper (13). Early passage primary wild-type and $p27^{kip1}$-deficient MEFs were a gift from B. Scheyen.

Cell culture. A14 (NIH3T3 overexpressing human insulin receptor), U87MG, RR2, U20S, Phoenix and MEF cells were grown in DMEM with standard supplements. Jurkat cells were grown on RPMI-1640 medium. Insulin, LY294002, nocodazole or puromycin were added at a final concentration of 1 μg ml^{-1}, 10 (A14) or 20 μM (U87MG and MEFs), 250 ng ml^{-1} or 5 μg ml^{-1}, respectively.

Antibodies. The following antibodies were used: 9E10 (Pharmingen), anti-$p27^{kip1}$ (Transduction Laboratories), 12CA5 for HA-tagged proteins, anti-actin (Santa Cruz), anti-PKB (21), anti-phosphoS473 PKB (New England Biolabs), anti-phosphoT32 FKHR-L1 (gift from A. Brunet) and an anti-phosphoS193 AFX (a donation from New England Biolabs). Anti-AFX was generated by immunizing rabbits with a C-terminal peptide from AFX conjugated to KLH (Isogen).

Colony formation. Cells were transfected with the expression plasmids in combination with pBabe-puro and pCMV.CD20. Transfection efficiency of individual plates was determined at 48 hr after transfection by FACS analysis of CD20-positivity (20). Equal numbers of transfected cells were plated and cells

were then grown in the presence of 5 μg ml^{-1} puromycin for two weeks, after which colonies were scored.

Retroviral infection of MEFs. Recombinant retroviruses were obtained by transfection of the relevant retroviral construct into Phoenix cells. Conditioned medium from these cells was collected 36 hr after transfection, filtered and diluted 1:1 with fresh medium and 6 μg ml^{-1} polybreen was added. This mixture was used to infect exponentially growing MEFs by two consecutive rounds of infection. We routinely obtained 80-90% infection efficiency, as determined by puromycin selection.

Cell-cycle distribution. To analyse cell-cycle redistribution in response to ectopic expression of AFX-like proteins, cells were transfected with the appropriate expression plasmids in combination with CMV.CD20. Thirty-six hours after transfection DNA profiles of CD20-positive cells were obtained by flow cytometry (20) and analysed using ModFit software (Becton Dickinson). Retrovirally infected MEFs were pulsed with 1 μM BrdU for 30 min at 48 h after infection and analysed by bivariate flow cytometry as described (22).

Magnetic activated cell sorting (MACS). Cells transfected with pCMV.CD20 and the indicated expression plasmids were stained with anti-CD20 monoclonal antibody (DAKO) followed by staining with goat-anti-mouse microbeads (Miltenyi Biotec). Transfected (CD20-positive) and non-transfected (CD20-negative) cells were then separated on miniMACS separation units (Miltenyi Biotec) according to the supplier's protocol. Transfected (elution) and non-transfected (flow-through) fractions were obtained from each transfection and cells were lysed in the appropriate buffers. Positively selected cells were >85% CD20-positive, and <10% CD20-positive cells remained in the negatively selected fraction.

Kinase assays. MACS-separated or retrovirally infected cells were used for determination of cdk2- or cyclin E-associated kinase activity as described (22).

Northern blotting. Total RNA was extracted from wild-type MEFs infected with the indicated retroviruses using standard guanidine isothiocyanate extraction method. We electrophoresed 20 μg of RNA and blotted it onto nitrocellulose and probed for p27^{kip1} and GAPDH messenger using radiolabelled cDNA probes.

p27^{kip1} protein stability. MEFs infected with the indicated retroviruses were treated 48 h post-infection with 10 μg ml^{-1} cycloheximide for 30 minutes. Cells were collected at 0, 30, 65, 125, 240 and 460 min after cycloheximide treatment, and p27^{kip1} and actin levels were determined by western blotting of total cell lysates.

Luciferase assay. Cells were transfected with 1 μg (A14) or 5 μg (Jurkat) p27GL-1609 plasmid along with the various AFX plasmids and 1 or 5 μg pCMV-lacZ. Luciferase and lacZ measurements were performed as described (5).

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References

1. C. Kenyon, J. Chang,, E. Gensch, A. Rudner, R. Tabtiang, A C. elegans mutant that lives twice as long as wild type. *Nature* **366**, 461-4 (1993).
2. K. Lin,, J. B. Dorman, A. Rodan, C. Kenyon, daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science* **278**, 1319-22 (1997).
3. S. Ogg, et al., The Fork head transcription factor DAF-16 transduces insulin-like metabolic and ongevity signals in C. elegans. *Nature* **389**, 994-9 (1997).
4. S. Paradis, G. Ruvkun, Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals rom AGE-1 PI3 kinase to the DAF-16 transcription factor. *Genes Dev* **12**, 2488-98 (1998).
5. G.J. Kops, et al., Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**, 630-4 (1999).
6. A. Brunet, et al., Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-68 (1999).
7. S. Guo, et al., Phosphorylation of Serine 256 by Protein Kinase B Disrupts Transactivation by FKHR and Mediates Effects of Insulin on Insulin-like Growth Factor-binding Protein-1 Promoter Activity through a Conserved Insulin Response Sequence. *J Biol Chem* **274**, 17184-92 (1999).
8. G. Rena, S. Guo, S. C. Cichy, T. G. Unterman, P. Cohen, P, Phosphorylation of the Transcription Factor Forkhead Family Member FKHR by Protein Kinase B. *J Biol Chem* **274**, 17179-83 (1999).
9. E. D. Tang, G. Nuñez, F. G. Barr, K. L. Guan, Negative Regulation of the Forkhead Transcription Factor FKHR by Akt. *J Biol Chem* **274**, 16741-6 (1999).
10. F. B. Furnari, H. J. Huang, W. K. Cavenee, The phosphoinositol phosphatase activity of PTEN mediates a serum- sensitive G1 growth arrest in glioma cells. *Cancer Res* **58**, 5002-8 (1998).
11. D. M. Li, H. Sun, PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell-cycle arrest in human glioblastoma cells. *Proc Natl Acad Sci U S A* **95**, 15406-11 (1998).
12. S. Roche, M. Koegl, S. A. Courtneidge, The phosphatidylinositol 3-kinase alpha is required for DNA synthesis induced by some, but not all, growth factors. *Proc Natl Acad Sci U S A* **91**, 9185-9 (1994).
13. D. S. Peeper, et al., Ras signalling linked to the cell-

cycle machinery by the retinoblastoma protein. *Nature* **386**, 177-81 (1997).

14. D. Haas-Kogan, et al., Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN/MMAC. *Curr Biol* **8**, 1195-8 (1998).
15. B. M. Burgering, et al., Insulin stimulation of gene expression mediated by p21ras activation. *Embo J* **10**, 1103-9 (1991).
16. M. Ohtsubo, A. M. Theodoras, J. Schumacher, J. M. Roberts, M. Pagano, Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol* **15**, 2612-24 (1995).
17. T. K. Kwon, J. E. Nagel, M. A. Buchholz, A. A. Nordin, Characterization of the murine cyclin-dependent kinase inhibitor gene $p27^{kip1}$. *Gene* **180**, 113-20 (1996).
18. N. A. Lissy, et al., TCR antigen-induced cell death occurs from late G1 phase cell-cycle check point. *Immunity* **8,** 57-65 (1998).
19. G. J. Boonen, et al., Cyclin D3 regulates proliferation and apoptosis of leukemic T cell lines. *J. Biol. Chem.* **274,** 34676-82 (1999).
20. R. H. Medema, R. E. Herrera, F. Lam, R. A. Weinberg, Growth suppression by p16ink4 requires functional retinoblastoma protein. *Proc Natl Acad Sci U S A* **92**, 6289-93 (1995).
21. B. M. Burgering, P. J. Coffer, Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376,** 599-602 (1995).
22. R. H. Medema, R. Klompmaker, V. A. Smits, G. Rijksen, p21waf1 can block cells at two points in the cell cycle, but does not interfere with processive DNA-replication or stress-activated kinases. *Oncogene* **16**, 431-41 (1998).

CHAPTER

6

Control of cell-cycle exit and entry by protein kinase B: Forkhead transcription factors regulate the p130 pocket protein

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To be Submitted

Control of cell-cycle exit and entry by protein kinase B: Forkhead transcription factors regulate the p130 pocket protein

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The proto-oncogenes Ras and protein kinase B (PKB or c-akt) can promote cellular proliferation by inhibiting the Forkhead transcription factors AFX, FKHR and FKHR-L1. Inducing Forkhead activity causes an arrest in the G0/G1 phase of the cell-cycle, in part via an increase in $p27^{kip1}$ gene transcription. We set out to identify novel targets of the Forkheads that can regulate G1/S progression. Here, we show that conditional activation of FKHR-L1 causes upregulation of the p130 pocket protein, a pRb-like protein associated with quiescence and a previously suggested cdk2 inhibitor. However, the FKHR-L1-induced increase in p130 levels does not contribute to the Forkhead-induced, $p27^{kip1}$-independent cdk2 inhibition. Rather, it reflects a state of cellular quiescence as indicated by the following observations: i) the phosphorylation status of p130 shifts to a G0-specific form; ii) an increase in p130/E2F4 complexes was observed after Forkhead activation, and iii) the Forkhead-induced G0/G1 arrest is sustained in time without a marked increase in cell death, and this sustained arrest is reversible. In addition, we show that protein levels and hyperphosphorylation of p130 upon cell-cycle re-entry are controlled by the PI(3)K/PKB pathway. Together, these results indicate that Forkhead transcription factors can induce a state of cellular quiescence, even in human tumor cells, and they are the first demonstration of a complete PI(3)K-activated pathway controlling exit and entry from the cell-cycle. We propose that Forkhead inactivation by Ras and PKB signalling in quiescent cells is a crucial step in cell-cycle re-entry and contributes to the processes of transformation and regeneration.

Introduction

Mammalian cells require an extracellular proliferative signal directly after mitosis in order to keep on growing and dividing. When cells are faced with a lack of such a signal, depending on

the cell-type, the cell will either die or growth-arrest in post-mitotic G1 phase. Two of the intracellular signalling pathways that transduce proliferative signals are the Ras and PI(3)K pathways. Ras and PI(3)K can regulate various features of cell proliferation, such as cytoskeletal rearrangements, gene transcription, DNA synthesis and survival (reviewed in (1, 2)). The proto-oncogene PKB is a major target of PI(3)K signalling in the control of cell proliferation (reviewed in (3)). PKB is involved both in anti-apoptotic signalling as well as cell-cycle control. Recently, PKB was found to directly phosphorylate and inactivate a subfamily of Forkhead transcription factors, consisting of AFX, FKHR and FKHR-L1 (4, 5, 6). In addition, Ras, via the RalGEF/Ral pathway, cooperates with PKB in inhibiting AFX activity (4). Importantly, these two pathways are often found deregulated in tumor cells. Ras itself is mutated to an active form in 15% of all cancers and the negative regulator of PI(3)K signalling, the tumor suppressor PTEN, has been shown to be mutated or deleted in a wide variety of tumors (reviewed in (7, 8)).

Inactivation of the Forkhead transcription factors may play a major role in the control of cellular proliferation by the PI(3)K/PKB and Ras/Ral pathways. We and others have recently shown that all three Forkheads inhibit cell-cycle progression at the G1/S transition, at least in part by controlling transcription of the gene for the $p27^{kip1}$ cdk inhibitor (9, 10, 11). Nevertheless, a $p27^{kip1}$-independent mechanism for Forkhead-induced cell-cycle arrest is likely to exist, since AFX was still able to partly reduce activity of the cyclin E/cdk2 complex in the absence of $p27^{kip1}$ (9).

The continuation of cell proliferation at various stages of the cell-cycle involves the inactivation of at least one of three members of the retinoblastoma family of nuclear pocket proteins. The general mechanism by which this family exert its effects is by binding different members of the E2F family of transcription factors, thereby actively repressing genes required for cell-cycle progression (reviewed in (12)). The pRb/p105 protein is an essential component of the G1/S checkpoint. pRb is present at relatively constant levels throughout the cell-cycle but is hyperphosphorylated by cyclin/cdk complexes and released from E2F-1 at the G1/S transition allowing continuation through the cell-cycle (reviewed in (13)). Conversely, the p107 and pRb2/p130 proteins are regulated at the protein level as well as by phosphorylation. p107 protein levels are low during quiescence (commonly reffered to as G0) and early G1 but high during the other stages of the cell cycle. p130 protein levels, on the other hand, are low in cycling cells, but increase once cells exit the cell cycle (reviewed in (12)). The rise in p130 protein levels at the G0 stage of the cell cycle is accompanied by a change in phosphorylation of p130 from predominantly hyperphosphorylated form 3 in cycling cells to the hypophosphorylated forms 1 and 2 in G0 cells (14, 15). The high amount of hypophosphorylated p130 in G0 cells binds to the E2F-4 transcription factor which is thought to repress genes required for re-entry into early G1 phase thereby maintaining the quiescent state (16).

In addition to this well documented function for p130 is the recent observation that p130 can participate in the direct inhibition of cyclin E/cdk2 complexes (17). In that report it was shown that serum-starved MEFs from $p27^{kip1}$ knockout mice still display efficient inhibition of cyclin E/cdk2 activity, which could be accounted for by the presence of p130 in the cyclin E/cdk2 complexes. Since p130 in addition has a cyclin-interaction motif (18), it might therefore in certain cases function as a 'classical' cdk-inhibitor. Nevertheless, it remains to be established that p130 can function as a cdk-overexpression of exogenous wild-type p130

does not induce a growth arrest (19).

In an attempt to identify cell-cycle regulators besides $p27^{kip1}$ that are regulated by Forkhead transcription factors, we observed an upregulation of p130 protein levels by conditional Forkhead activation. Although p130 has been reported to be able to function as a cdk2-inhibitor, we find that in our cells the increase in p130 upon Forkhead activation does not contribute to $p27^{kip1}$-independent cdk2 inhibition. Rather, we show that the increase in p130 protein represents an entry into quiescence of a number of cell types, including human tumor cells, as supported by several additional G0 hallmarks. Furthermore, PKB signalling can drive quiescent cells back into G1 as shown by a decrease in p130 protein levels and a decrease in p130/E2F-4 complexes. These data suggest that activation of PKB and/or Ras by growth factors or by oncogenic mutations in quiescent cells can lead to a cell-cycle re-entry through the inhibition of Forkhead transcription factors.

Methods

Plasmids. pBabe-FKHR-L1.A3 was created by ligating a Klenow-blunted HindIII/BamHI fragment of pcDNA3-HA-FKHR-L1.A3 into Klenow-blunted BamHI-cut pBabe-puro. pSG5-mycBimL and pSG5-mycBimS have been described (25). pcDNA3-HA-FKHR-L1.A3-ER has been described (20). pBabe-HA-AFX has been described (9). pCMV-HA-p130 was a gift of D. Cobrinik.

Cell culture. The DL23 cell-line was created as follows: linearized pcDNA3-HA-FKHR-L1.A3-ER (20) was transfected into DLD-1 human colon carcinoma cells by electroporation. Transfectants were selected for two weeks on 300 µg/ml geneticin. Subsequently, clones were isolated and analyzed for expression of the fusion protein. The DL23 subclone was chosen for further study. Primary MEFs derived from wild-type, $p27^{kip1}$ -/-, p130 -/- and $p27^{kip1}$/p130 -/- mice were a gift of B. Scheyen. MEFs, A14, U87MG and 3T3-PKB-ER (29) cells were grown on DMEM with standard supplements. MCF-7 cells were grown on DF12 medium. DL23 and DLD-1 were grown on RPMI-1640 supplemented similarly. 4OHT (Sigma) was diluted in RPMI-1640 and added to the cells at a final concentration of 500 nM (DL23 and DLD-1) or 1 µM (3T3-PKB-ER). LY294002 was dissolved in DMSO and used at a final concentration of 10 µM.

RNA preparation and northern blotting. Total RNA was isolated from DL23 cells using the RNAZol procedure (TEL-TEST, INC). $polyA^+$-RNA was purified from 1 mg total RNA using the polyA-Tract method (Promega). Northern blotting of 2 µg mRNA was performed as described (9).

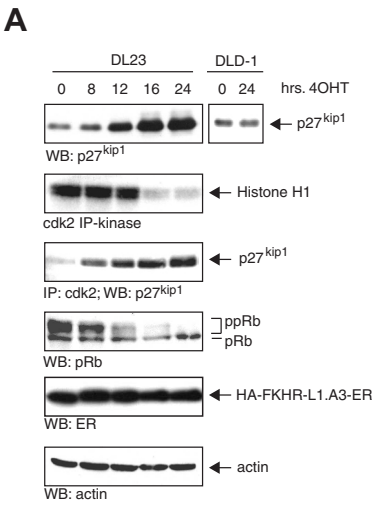
Electrophoretic mobility shift assay (EMSA). Bandshift analyses of DLD-1, DL23 and 3T3-PKB-ER cells was done as described (26).

Antibodies, immunoprecipitations and immunoblotting. Anti-p130 (C-20), anti-E2F4 (C-20), anti-ERα (MC-20), anti-cdk4 (C22), anti-cdk2 (M2), anti-p16 (C-20), anti-p21 (N-20), anti-cyclin D1 (H11), anti-cyclin E (C-19) and anti-actin (I-19) were from Sant-Cruz. Anti-pRb was from Pharmingen. Anti-$p27^{kip1}$ was from Transduction Laboratories. Anti-Bim was from Affinity Bioreagents. Anti-phosphoT32-FKHR-L1 was a gift of A. Brunet (6). Anti-TOK1 was a gift of Dr. Ariga (22). For immunoprecipitations, DL23 cells were lysed in lysis-buffer (50 mM Tris, pH 7.5, 1% Triton-X-100, 100 mM NaCl and 5 mM EDTA supplemented with NaF, aprotinin, leupeptin, benzamidin and $NaV0_4$) and the cleared supernatant was incubated in the cold with 10% protein A-sepharose and 3 µl anti-cdk2 for 3 hours. Beads were washed three times and resuspended in 1X Laemmli sample buffer. For immunoblotting of total lysates, cells were lysed in 1X Laemmli sample buffer, electrophoresed and blotted onto nitrocellulose according to standard protocol. Immunoblotting was performed by blocking the membranes in blotto (2% protifar, 0.5% BSA in PBS-Tween (PBS plus 0.1% Tween-20)) for 1 hour at room-temperature. Primary and secondary antibody incubations were in PBS-Tween overnight and for 2 hours at 4ºC, respectively, and the membrane was washed four times after each incubation. Proteins were visualized by standard enhanced chemiluminescence and autoradiography.

^{35}S-methionine pulse labeling. DLD-1 and DL23 cells were grown to sub-confluency, labeled with 100 µM ^{35}S-Methionine for 2 hours and lysed in 50 mM Tris pH 7.5/1%TX-100. Samples were corrected for amount of cells, and ^{35}S-Methionine incorporation was measured by scintillation counter.

BrdU incorporation, FACS analysis and cyclin E-associated kinase assay. BrdU incorporation, FACS analysis and cyclin E/cdk2 kinase assays were performed as described (9).

Retroviral Infections. Retroviral infections using pBabePuro, pBabe-HA-AFX and pBabePuro-HA-FKHR-L1.A3 were performed as described (9).



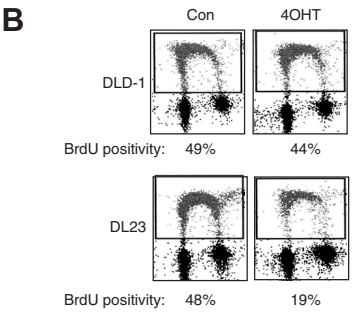


Figure 1. *Cell-cycle arrest by a conditionally active FKHR-L1. A.* DL23 and DLD-1 cells were left untreated (0) or treated with 500 nM 4OHT for 8, 12, 16 and 24 hours. Total cellular lysates were electrophoresed and blotted for presence of $p27^{kip1}$, pRb, HA-FKHR-L1.A3-ER or actin. Cell lysates were analyzed for cdk2 kinase activity by cdk2 immunoprecipitation and *in vitro* kinase reaction using histone H1 as a substrate (58). The cdk2 kinase reactions were blotted, autoradiographed and probed for $p27^{kip1}$ presence. WB: western blot. *B.* BrdU incorporation of DL23 and DLD-1 cells left untreated (con) or treated with 500 nM 4OHT for 16 hours.

Results

Forkhead-activation causes upregulation of the p130 pocket protein

To examine the effect of specific Forkhead activation on cellular proliferation, we created the DL23 cell-line, a DLD-1 human colon carcinoma subclone that stably expresses a fusion of conditionally active HA-FKHR-L1.A3 with a modified form of the estrogen receptor hormone-binding domain (HA-FKHR-L1.A3-ER) (20). This fusion is inactive untill the cells are presented with the ligand for the modified ER, 4-hydroxy-tamoxifen (4OHT) (21). Specific activation of the Forkhead transcription factor by 4OHT in the DL23 cell-line resulted in upregulation of $p27^{kip1}$ protein, hypophosphorylation of pRb, inhibition of cdk2 activity and subsequent cell-cycle arrest (figure 1), consistent with data obtained in NIH3T3 cells (9). These cell-cycle inhibitory events are specific for Forkhead activity, since the DLD-1 cell-line did not display such effects upon 4OHT addition (figure 1 and not shown). The DL23 cell-line enabled us to investigate what cell cycle parameters besides $p27^{kip1}$ might be under the control of the PI(3)K/PKB/Forkhead pathway specifically. We therefore analyzed the expression of various proteins involved in cell-cycle regulation in DL23 cells treated with 4OHT. We did not observe a Forkhead-induced change in protein levels of cdk2, cdk4, cyclin D1, cyclin E, $p16^{ink4a}$, $p21^{waf1/cip1}$ and TOK-1 (figure 2a, (9, 22)). Interestingly however, we did observe an effect on the protein amount of the p130 pocket protein. p130 protein was drastically upregulated within 16 hours of Forkhead activation, with similar kinetics as $p27^{kip1}$ upregulation (figure 2b). The effect of FKHR-L1 on p130 expression is not restricted to FKHR-L1, since infection of primary MEFs with a HA-AFX-expressing retrovirus also increases p130 protein levels (figure 2c). Moreover, p130 upregulation was independent of Forkhead-mediated regulation of $p27^{kip1}$, since a similar infection of HA-AFX in MEFs from

$p27^{kip1}$ knockout mice still caused an increase of p130 protein (figure 2c). Northern blotting revealed that after Forkhead activation p130 mRNA levels are also increased thus indicating that p130 protein levels are not solely regulated by means of protein stability (figure 2d, (23)). However, in transient reporter assays using a small (300bp) fragment of the human p130 promoter (24) we could not demonstrate regulation of p130 transcription by Forkheads (not shown). This could mean either that a Forkhead responsive element is located outside the promoter fragment that we have analyzed, that regulation is not at the level of transcription initiation, or that the regulation is indirect.

Forkhead-mediated increase in p130 protein levels does not contribute to inhibition of cyclin E-associated kinase activity

Forkhead-induced cell-cycle arrest is mediated by $p27^{kip1}$ but other cdk2-inhibitors might be involved, since AFX is still able to inhibit cylin E-associated kinase activity to some extent in $p27^{kip1}$ -/- cells (9). Considering that p130 was recently reported to inhibit cdk2 kinase activity in the absence of $p27^{kip1}$ by direct binding to the cyclin E/cdk2 complex (17), we investigated whether p130 upregulation by

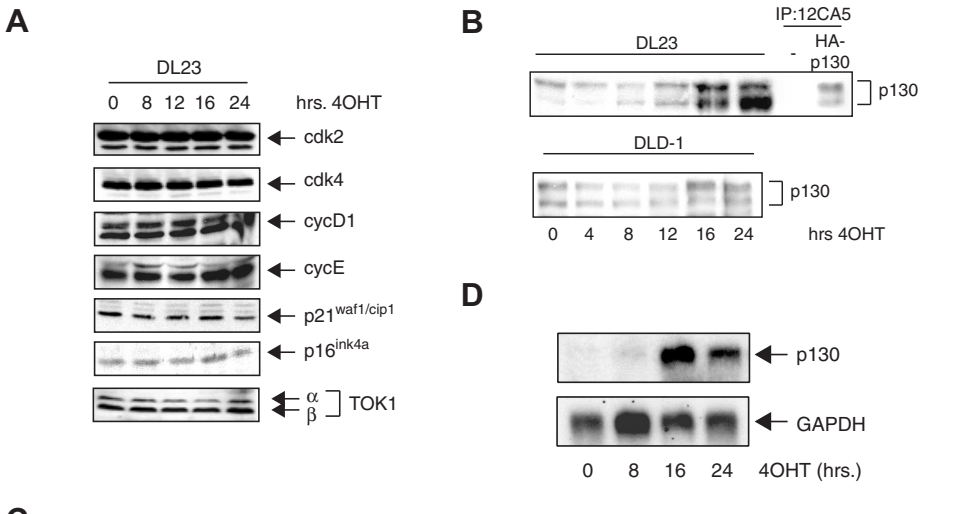
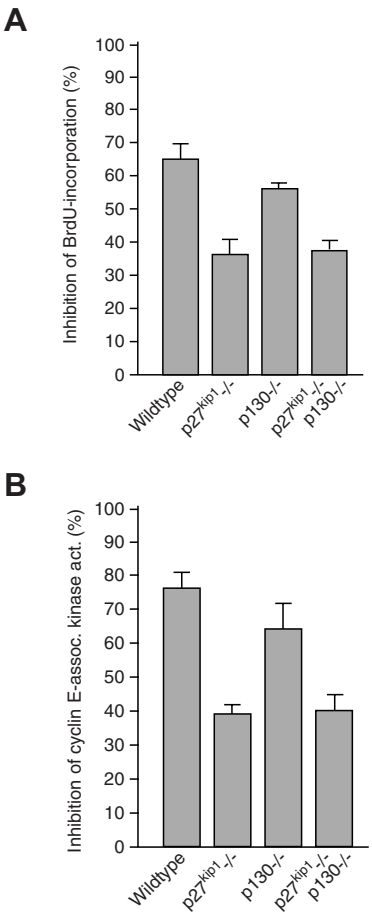


Figure 2. *FKHR-L1 upregulates p130 protein levels. A*. DL23 lysates prepared as mentioned in figure 1a were blotted for the presence of cdk2, cdk4, cyclin D1, cyclin E, $p21^{waf1/cip1}$, $p16^{ink4a}$, and TOK-1. *B*. Total cellular lysates from DLD-1 and DL23 cells treated without (0) or with 500 nM 4OHT for 4, 8, 12, 16 or 24 hours were analyzed by western blotting for presence of the p130 retinoblastoma-like protein. Anti-HA immunoprecipitations of A14 cell transiently transfected with empty vector (con) or pCMV-HA-p130 served as controls. *C*. MEFs from wildtype (wt) or $p27^{kip1}$ knockout ($p27^{kip1}$ -/-) mice were infected with a control retrovirus or a HA-AFX-containing retrovirus. Total cellular lysates were subsequently analyzed for p130, $p27^{kip1}$ and HA-AFX expression levels. *D*. 2 μg polyA$^+$-RNA from DL23 cells left untreated (0) or treated with 500 nM 4OHT for 8, 16 or 24 hours was electrophoresed and blotted onto a nylon membrane. The Northern blot was hybridized with radiolabelled probes from p130 or GAPDH cDNA.



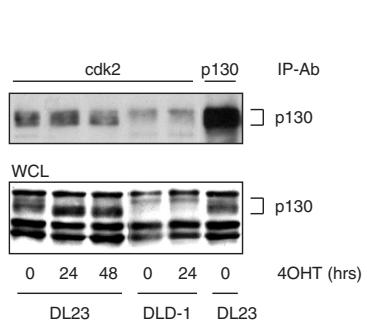


Figure 3. *Forkhead-induced upregulation of p130 does not contribute to G1 arrest.* Primary wild-type MEFs and MEFs from $p27^{kip1}$, p130, or $p27^{kip1}$/p130 knockout mice were infected with a control or FKHR-L1.A3-encoding retrovirus. 24 hours post-infection, proliferation was measured by BrdU-incorporation (*A*) and cyclin E-associated kinase activity (*B*). Relative inhibition of proliferation by FKHR-L1.A3 compared to control, as measured by these two assays, is depicted. Data represent average of at least three independent experiments. *C.* DL23 cells, treated with or without 4OHT for 24 or 48 hours, were lysed and cdk2 was immunoprecipitated. The cdk2 immunoprecipitates were subsequently analyzed for the presence of p130 by western blotting. WCL; whole cell lysate. IP-Ab; immunoprecipitating antibody.

Forkheads could contribute to the Forkhead-induced cyclin E/cdk2 inhibition and cell-cycle arrest. We infected a panel of MEFs with a FKHR-L1.A3-encoding retrovirus and analyzed cell proliferation and cyclin E-associated kinase activity. FKHR-L1.A3 induced a strong inhibition of BrdU-incorporation and cyclin E/cdk2 activity in wild-type MEFs (figure 3a and 3b, (9)). These effects were greatly reduced but not eliminated in $p27^{kip1}$ -/- cells (figure 3a and 3b, (9)). However, when compared to $p27^{kip1}$ -/- MEFs, Forkhead expression in p130-/- $p27^{kip1}$-/- cells showed no additional attenuation of cell proliferation arrest or decrease in cyclin E/cdk2 activity (figure 3a and 3b). Similar effects were seen in similar experiments using AFX instead of FKHR-L1.A3 (not shown). Finally, no increase of p130 protein in cyclin E/cdk2 complexes was observed in 4OHT-treated DL23 cells (figure 3c), as was observed for $p27^{kip1}$ (figure 1a), and as has been observed in serumstarved $p27^{kip1}$-deficients MEFs (17). These data therefore suggest that p130 does not contribute to $p27^{kip1}$-independent inhibition of cyclin E/cdk2 complexes upon Forkhead activation.

Forkhead transcription factors induce cell-cycle exit

Since the increase in p130 protein levels caused by active Forkheads does not seem to

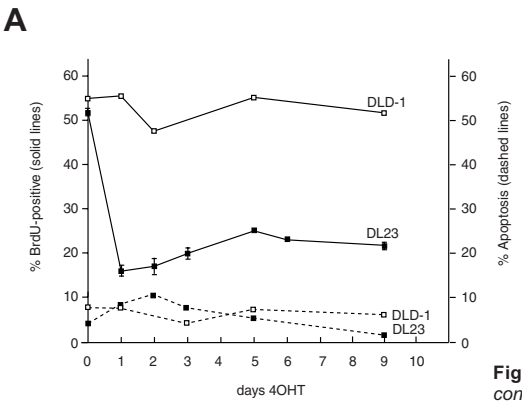


Figure 4. *Long-term activation of FKHR-L1 causes continued arrest rather than apoptosis. A*. DLD-1 and DL23 cells were treated with 500 nM 4OHT for 9 days. Samples were taken at various timepoints to measure proliferation by BrdU-incorporation (solid lines) and cell death by FACS analysis of sub-G1 population (dashed lines). *B*. DL23 cells were left untreated (0) or treated with 500 nM 4OHT for 8, 12, 16 and 24 hours. Total cellular lysates were electrophoresed and blotted for presence of Bim. As a control, lysates from COS7 cells transiently transfected with two Bim isoforms, BimS and BimL, were analyzed similarly. WB: western blot. *C*. DLD-1 and DL23 cells were left untreated (-) or treated with 500 nM 4OHT for 48 hours (+) and labelled with ^{35}S-Methionine to measure protein synthesis.

contribute to cdk2 inhibition, we questioned whether it might be indicative of a Forkhead-induced state of cellular quiescence. Several markers for the G0 phase of the cell-cycle have been described. First, p130 protein is upregulated (16), as observed after Forkhead activation (figure 2a). Second, p130 phosphorylation status changes from hyperphosphorylated form 3 to phosphorylated forms 1 and 2 (14, 15). Third, p130 associates with E2F-4, resulting in active repression of genes required for cell-cycle re-entry (16).

To more closely study the possible role of the Forkheads in the regulation of cell-cycle exit and entry, we first chose to determine the effect of prolonged Forkhead activity on cellular proliferation. To this end, we added 4OHT to the DL23 cells for a period up to 9 days, and measured sub-G1 DNA content and BrdU-incorporation to determine apoptosis and proliferative status, respectively. As seen in figure 4a, activation of FKHR-L1.A3 by 4OHT resulted in rapid, drastic and continued inhibition of BrdU- incorporation, but no great increase in the relative amount of cells in apoptosis was observed, not even after nine days. In agreement with this, no increase in protein levels of the Forkhead-regulated pro-apoptotic gene product Bim was observed (figure 4b), whereas the same approach (FKHR-L1.A3-ER expression) does result in Bim expression and apoptosis in the haematopoietic Ba/F3 mouse pre-B cell-line (25). Furthermore, protein synthesis was slowed down in cells treated with 4OHT for 48 hours, as measured by ^{35}S-methionine pulse-labelling (figure 4c). These observations suggested to us that the lack of apoptosis upon Forkhead activation correlates with an inability of these cells to upregulate pro-apoptotic genes such as

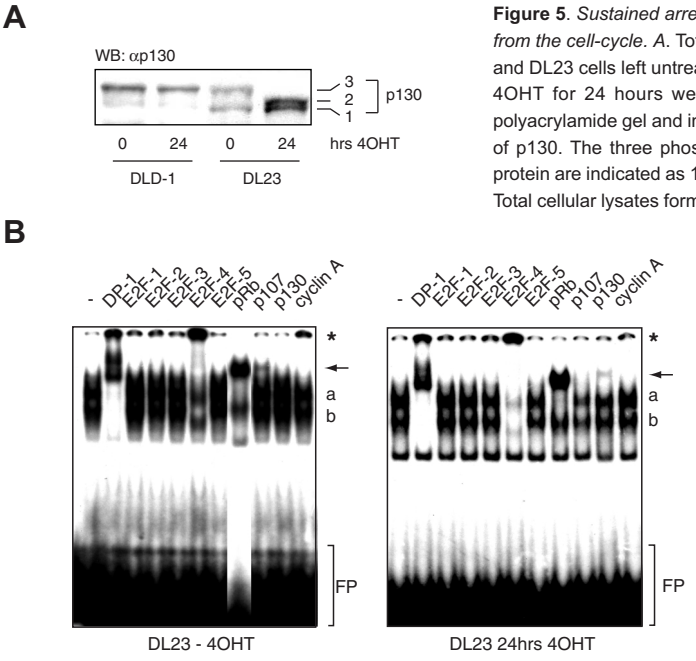


Figure 5. *Sustained arrest by FKHR-L1 represents exit from the cell-cycle. A*. Total cellular lysates from DLD-1 and DL23 cells left untreated (0) or treated with 500 nM 4OHT for 24 hours were electrophoresed on a 6% polyacrylamide gel and immunoblotted for the presence of p130. The three phosphorylated forms of the p130 protein are indicated as 1, 2 and 3. WB: western blot. *B*. Total cellular lysates form DL23 cells left untreated (left) or treated with 500 nM for 48 hours (right) were analyzed for the presence of E2F/DP-1/pocket protein complexes. Supershifting antibodies are listed on top. Arrows indicate the disappearance of p107/E2F-4 complexes in third lane from right, and the appearance of p130/E2F-4 complexes in second lane from right, upon 4OHT treatment. Asterix indicates supershifted E2F-4- or cyclin A-containing complexes. a, b, non-supershifted E2F/pocket protein complexes. FP, free probe.

Bim, and in addition that the cells are in a constitutive arrest or had entered a quiescent or senescent state.

To investigate the phosphorylation status of the p130 protein, we analyzed the 4OHT-treated DL23 cells for the different phosphorylated forms of p130. Normal cycling DL23 cells show most p130 protein hyperphosphorylated to form 3, as described for other cycling cells (figure 5a, (14, 15)). 24 hours of 4OHT treatment, however, resulted in an increase in the relative amount of p130 protein phosphorylated to forms 1 and 2 (figure 5a).

Next, we examined the functional interaction between p130 and E2F-4. Band-supershift analysis using an oligonucleotide containing a consensus E2F family binding sequence and antibodies against the various E2F and pocket proteins (26) showed that the interaction of the p130/E2F-4 complex with E2F binding sequences was increased in cells containing the activated Forkhead, whereas p107-containing complexes were lost (figure 5b). Furthermore, using an antibody against cyclin A, we observed a decrease in p107/cyclin A/E2F-4 complexes previously described to exist in S-phase (27). Taken together, these data suggest that activation of Forkhead transcription factors induces cells to exit the cell cycle and enter G0. Moreover, the data imply that the effect of the Forkheads on proliferation is sufficiently strong to cause human tumor cells to enter quiescence.

The Forkhead-induced cell-cycle exit is reversible and mediated by the PI(3)K/PKB pathway

Forkhead transcription factors were previously shown to be involved in a PI(3)K-induced senescent-like phenotype in primary MEFs through the regulation of $p27^{kip1}$ (28). In

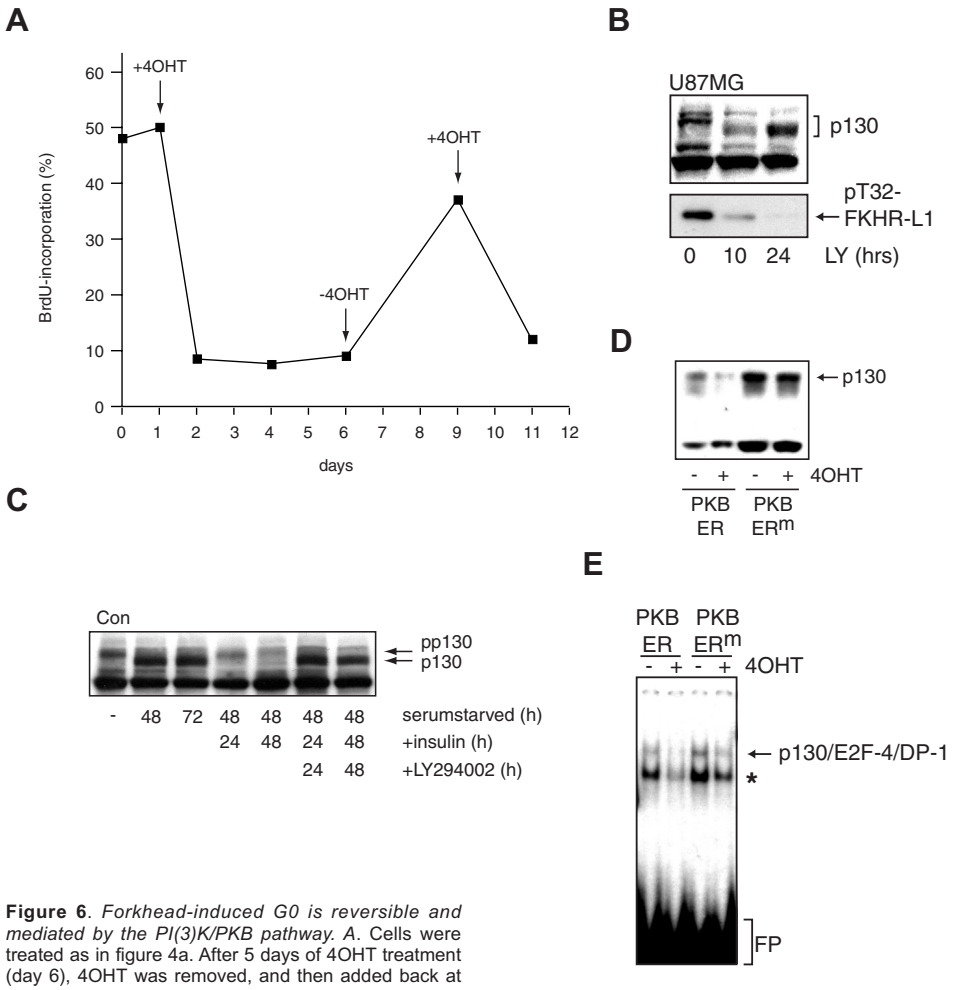


Figure 6. *Forkhead-induced G0 is reversible and mediated by the PI(3)K/PKB pathway. A*. Cells were treated as in figure 4a. After 5 days of 4OHT treatment (day 6), 4OHT was removed, and then added back at day 9. Samples were taken at days 0, 1, 2, 3, 4, 6, 9 and 11, and BrdU-incorporation was measured. *B*. U87MG cells were treated with 10 μM LY294002 (LY) for 0, 10 or 24 hours. Total cellular lysates were immunoblotted for the presence of p130 protein and T32-phosphorylated FKHR-L1. *C*. MCF-7 cells were serumstarved for 48 or 72 hours, subsequently treated with insulin for 24 or 48 hrs, with or without a 30 minute pre-incubation with LY294002. p130 protein was subsequently analyzed by western blotting. pp130; hyperphosphorylated p130. *D*. 3T3-PKB-ER (PKB-ER) and 3T3-PKB-ERmutant (PKB-ER^m) cells were serumstarved for 48 hours, then left untreated (-) or treated with 1 μM 4OHT for 14 hours. Total cellular lysates were immunoblotted for the presence of p130 protein. *D*. 3T3-PKB-ER (PKB-ER) and 3T3-PKB-ERmutant (PKB-ER^m) cells were treated as in *C* and analyzed for the presence of p130/E2F-4 complexes. Asterix indicates free E2F. FP, free probe.

these cells, the induced arrest was accompanied by p130 upregulation. We therefore wanted to know whether the FKHR-L1-induced cell-cycle exit represents quiescence or senescence. To distinguish between these two possibilities, we determined whether the Forkhead-induced cell-cycle exit is reversible. To this end, we treated DL23 cells up to 5 days with 4OHT after which the cells were grown for three days in medium without 4OHT. As seen in figure 6a, BrdU-

incorporation after 5 days of 4OHT treatment was diminished from 50 to 9 percent, but was almost completely reverted upon withdrawal of 4OHT for a subsequent 3 days. All cells had re-entered the cell-cycle, since more than 95% of the population stained BrdU-positive when given BrdU for 40 hours (not shown). The cells that re-entered the cell-cycle upon removal of 4OHT were efficiently arrested again upon re-addition of 4OHT, reflecting true reversibility. (figure 6a). This latter result demonstrates that we did not select a non-4OHT-responsive subpopulation during the initial prolonged arrest. Since one critical characteristic of senesence is irreversibility, this result demonstrates that the Forkhead-mediated cell-cycle exit indeed represents the quiescent state.

To examine whether the endogenous PI(3)K/PKB/Forkhead pathway is involved in the regulation of cell-cycle exit and entry, we investigated whether inhibition of PI(3)K could mimic the activation of Forkheads with respect to increased p130 protein levels. Indeed, treatment of the PTEN-negative glioblastoma cell-line U87MG with the PI(3)K-inhibitor LY294002 for 24 hours resulted in Forkhead dephosphorylation and a concomitant increase in p130 protein levels (figure 6b). Similarly, serum deprivation of MCF-7 cells resulted in an increase in p130 protein, which could be reverted by the re-addition of serum. However, pre-incubation with LY294002 completely blocked this, showing PI(3)K-dependence of p130 regulation in these cells (figure 6c). Next, to show that quiescence can be reverted through the PI(3)K/PKB signalling pathway, we analysed NIH3T3 cells stably expressing a myrPKB-ER fusion. In these cells PKB can be activated by adding 1 μM 4OHT (29). Previously we have shown that serum deprivation of primary fibroblasts results in an increase in p130 levels (28). Serum deprived 3T3-PKB-ER cells displayed relatively high levels of p130 protein (figure 6d). Importantly, specific activation of PKB by 4OHT treatment resulted in decreased p130 expression, whereas 4OHT had no effect on NIH3T3 cells containing a mutant fusion protein (figure 6d). In addition, p130/E2F-4 complexes accumulated as a result of serum-starvation (data not shown and (30)) and disappeared upon PKB activation (figure 6e). This strongly suggests that the PI(3)K/PKB/Forkhead pathway is involved in the regulation of cell-cycle exit and entry.

Discussion

In this study we show that signalling-independent activation of the Forkhead transcription factor FKHR-L1, which is normally controlled by the PI(3)K/PKB signalling pathway (6), results in an increase in mRNA and protein expression of the retinoblastoma-like protein p130. Increase in p130 appears not to result in inhibition of cyclin E-dependent cdk2 activity but rather reflects an exit from the cell cycle. This is indicated not only by the upregulation of p130 protein itself, but also by the change in p130 phosphorylation pattern and the increase in E2F4/p130 complex formation. Induction of cellular quiescence (G0) by growth factor deprivation also induces p130 upregulation and importantly ligand-independent activation of PKB can revert this. Although the regulation of the amount of p130 protein is generally considered to be post-translational, we observe an increase in p130 mRNA upon Forkhead activation. Interestingly, differentiating myoblasts have elevated p130 mRNA levels (31) and serum-stimulation of quiescent REF52 cells results in a decrease in p130 transcript (23), indicating that transcriptional regulation of p130 may indeed contribute to increased p130 protein levels. Forkheads may be indirect, since we have not been able to show direct control of p130 gene

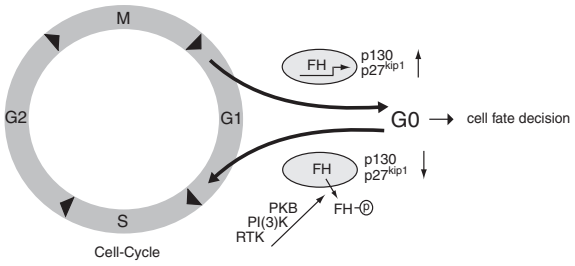


Figure 7. *Model for cell-cycle regulation by the PI(3)K/PKB/Forkhead pathway.* When cells do not receive growth factors in early G1, PKB is not activated and Forkheads remain active. This results in the upregulation of the genes for $p27^{kip1}$ and p130, leading to cell-cycle exit (in DLD-1 and NIH3T3 cells, for example) and, with the additional regulation of the Bim and FasL gene products, to apoptosis (in Jurkat and Ba/F3 cells, for example). Subsequent activation of PKB through RTKs leads to inactivation of Forkheads by nuclear exclusion and therefore cell-cycle re-entry via the downregulation of $p27^{kip1}$ and p130 proteins. See text for further details.

transcription by FKHR-L1.

Previously, we and others have shown that PKB activation results in an inhibition of Forkhead activity (4, 5, 6). Furthermore, we have provided evidence that through this pathway the levels of the cdk-inhibitor $p27^{kip1}$ can be regulated (9, 20). Together with the results presented here this suggests an elegant model in which the PI(3)K/PKB signalling pathway regulates cell-cycle arrest/exit and entry through transcriptional regulation of $p27^{kip1}$ and p130 (figure 7). Consistent with this, disruption of $p27^{kip1}$ expression or specific activation of PI(3)K can drive quiescent cells into G1/S (32, 33, 34, 35, 36). Nevertheless, it is at present unclear whether the regulation of $p27^{kip1}$ and p130 by Forkheads is sufficient to induce a cell-cycle exit. Possibly, yet more cell-cycle regulators are affected by Forkhead activity. This is supported by our findings that in MEFs deficient for both p130 and $p27^{kip1}$, Forkheads can still cause a decrease in cdk2 activity (figure 3b). Although we have tested a variety of proteins known to be involved in the regulation of cell-cycle progression in G1 phase (figure 2a), we cannot exclude that yet unidentified G1/S regulators are targets for Forkhead activity.

We show that the Forkhead-induced cell-cycle exit is reversible. This is in agreement with data obtained in *Caenorhabditis elegans*. In this nematode, the AFX/FKHR/FKHR-L1-like DAF-16 Forkhead transcription factor is regulated by a PI(3)K/PKB-like pathway and induces longevity and dauer formation (37, 38, 39). This latter effect is an exit from development at the second larval stage in adverse situations such as lack of nutrients. The dauer phenotype is reversible, allowing the worm to re-enter the developmental program when conditions turn for the better (reviewed in (40)). This suggests that also in *C. elegans*, the PI(3)K/PKB/Forkhead pathway might regulate a process that in many ways resembles quiescence rather than senescence or apoptosis.

It has been proposed that the presence of p130/E2F-4 repressor complexes on DNA has the significance of conferring reversibility to the cell-cycle exit program (12). This is potentially important in the context of oncogenic transformation and regeneration. Conceivably, quiescent tissue is forced back into the cell-cycle by activation of the PI(3)K/PKB pathway. Indeed, ligand-independent activation of PKB in quiescent cells reduces the amount of p130 protein, indicating a return to the G1 phase of the cell-cycle, and PI(3)K activated in a similar manner drives cells out of quiescence (36). This cell-cycle re-entry function of PKB might contribute to the oncogenic effect of mutations or deletions in the PTEN tumorsupressor (41,

42), especially in normally quiescent tissue such as the brain in parts of which *PTEN* genetic alterations are quite common (43, 44). Once inactivated, PTEN no longer inhibits PKB-dependent inactivation of the Forkheads, leading to inhibition of $p27^{kip1}$ and p130 gene expression and entry into the cell-cycle.

The data presented here show that specific activation of FKHR-L1 alone is sufficient to induce human tumor cells to exit the cell-cycle. Not only can Forkheads inhibit proliferation of human colon carcinoma cells, they also have the ability to arrest human leukemia cells, human glioblastoma cells, human renal carcinoma cells, and human osteosarcoma cells (9). This potent capacity of the Forkheads may indicate that proliferation during the process of oncogenic transformation cannot occur in the presence of active Forkheads. Possibly, during this transformation process, endogenous Forkhead activities have been diminished to allow the emerging tumor cell to proliferate. Indeed, human U87MG glioblastoma cells (this manuscript) and human Jurkat leukemia cells (unpublished observations) have high levels of phosphorylated and thus inactivated FKHR-L1. Furthermore, exogenous FKHR is located in the cytoplasm, and thus kept inactive, in human renal and prostate cancer cells (11). The putative importance of this pathway in oncogenesis is further supported by the findings that in many tumors $p27^{kip1}$ (reviewed in (45)) or p130 expression is low (46, 47).

Forced activation of the Forkheads that are regulated by PKB either causes cells to go into apoptosis or results in a cell-cycle exit. It is of interest to note that we only observe clear induction of apoptosis in cell-systems derived from haematopoietic cells ((6, 9, 20) and GJPLK, RHM, BMTB, unpublished observations). The cell-cycle exit appears more common and is seen in cells from various origins (fibroblasts, glioblastomas, ostesarcomas, colon carcinoma (this manuscript and (9)). In the haematopoietic cell compartment, most cells have a relatively high proliferative capacity and a concomitant limited lifespan (reviewed in (48, 49)). One can imagine that cell cycle arrest/exit in these cells in the absence of PI(3)K/PKB signalling would be deleterious and result in the activation of a default death pathway. The ability of Forkheads to induce pro-apoptotic genes such as those encoding Bim and FasL is likely to constitute part of this default death pathway. Yet the cell-types for which we observe cell-cycle arrest/exit are derived from tissues that normally display a relatively low proliferative capacity. We do not yet understand the mechanism by which the default death pathway is silenced in these cells. For instance, conditional activation of FKHR-L1 in the DL23 cell-line does not result in upregulation of Bim, whereas activation of the same construct in Ba/F3 cells (pre-B-cells) does (25). Furthermore, the DLD-1 cell-line which is the parental cell-line for the DL23 clone is not sensitive to FasL-induced apoptosis despite high levels of CD95 (50, 51). To understand more about these striking differences between the two cell-types, it will be interesting to examine what other cellular factors determine why Bim is not Forkhead-responsive in non-haematopoietic cells and why CD95 presence does not necessarily mean FasL responsiveness.

Certain differentiated tissues, such as liver, smooth and skeletal muscle, pancreas and auditory sensory epithelial have the ability of regeneration. During the process of skeletal muscle differentiation a sub-population of myoblasts, termed reserve cells, remains quiescent and confer the capacity to regenerate onto skeletal muscle (52). Recently, it was proposed that p130 is involved in defining this pool of reserve cells (31). Interestingly, that study showed that not only p130 protein but also p130 mRNA accumulates during muscle differentiation of the reserve cells (31). Since we show

in this study that p130 mRNA levels are increased upon Forkhead activation, this might suggest a role for the PI(3)K/PKB/Forkhead pathway in muscle reserve cell differentiation and muscle regeneration. A similar population (i.e. supporting-cell population) in mouse auditory sensory epithelium is kept quiescent because of $p27^{kip1}$ activity, but to date a role for p130 in this process has not been studied (34). Nevertheless, we have shown recently that $p27^{kip1}$ is under the control of Forkhead transcription factors (9, 20), suggesting the possibility that in these cells quiescence is maintained by Forkhead-dependent regulation of $p27^{kip1}$. In addition, high $p27^{kip1}$ protein levels has been been suggested to be involved in the maintenance of the quiescent state of germinal centre/memory (GC/M) B-cells (53). Finally, p130 or $p27^{kip1}$ have been implicated in liver, pancreas, and smooth muscle regeneration (54, 55, 56, 57). It is tempting to speculate that in the cells of tissues such as liver, pancreas and muscle, the PI(3)K/PKB/Forkhead pathway contributes to cell-cycle re-entry.

In conclusion, we have identified a novel function for the AFX, FKHR and FKHR-L1 Forkhead transcription factors in cell-cycle control. We have shown that these Forkheads induce cells to exit the cell-cycle and enter a state of quiescence as demonstrated by an upregulation of protein levels of the p130 retinoblastoma family member, an increase in p130/E2F-4 complexes and the reversibility of the cell-cycle arrest. This latter observation further emphasizes the importance of activation of the PI(3)K/PKB pathway in processes such as oncogenic transformation, in that it may relieve the inhibitory constraints on cell-cycle progression imposed by Forkhead-mediated regulation of $p27^{kip1}$ and p130 levels, resulting in a re-entry into the cell-cycle.

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References

1. D. A. Fruman, R. E. Meyers, L. C. Cantley, Phosphoinositide kinases. *Annu Rev Biochem* **67**, 481-507 (1998).
2. J. L. Bos, p21ras: an oncoprotein functioning in growth factor-induced signal transduction. *Eur J Cancer* **31A**, 1051-4 (1995).
3. P. J. Coffer, J. Jin, J. R. Woodgett, Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J* **335**, 1-13 (1998).
4. G. J. Kops, et al., Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**, 630-4 (1999).
5. G. Rena, S. Guo, S. C. Cichy, T. G. Unterman, P. Cohen, Phosphorylation of the Transcription Factor Forkhead Family Member FKHR by Protein Kinase B. *J Biol Chem* **274**, 17179-17183 (1999).
6. A. Brunet, et al., Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-68 (1999).
7. J. L. Bos, All in the family? New insights and questions regarding interconnectivity of Ras, Rap1 and Ral. *Embo J* **17**, 6776-82 (1998).
8. A. Di Cristofano, P. P. Pandolfi, The multiple roles of PTEN in tumor suppression. *Cell* **100**, 387-90 (2000).
9. R. H. Medema, G. J. Kops, J. L. Bos, B. M. Burgering, AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782-7 (2000).
10. A. Brunet, et al., Protein Kinase SGK Mediates Survival Signals by Phosphorylating the Forkhead Transcription Factor FKHR-L1 (FOXO3a). *Mol Cell Biol* **21**, 952-965 (2001).
11. N. Nakamura, et al., Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. *Mol Cell Biol* **20**, 8969-82 (2000).
12. X. Grana, J. Garriga, X. Mayol, Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. *Oncogene* **17**, 3365-83 (1998).
13. C. J. Sherr, Cancer cell cycles. *Science* **274**, 1672-7 (1996).
14. X. Mayol, J. Garriga, X. Grana, Cell cycle-dependent

phosphorylation of the retinoblastoma-related protein p130. *Oncogene* **11**, 801-8 (1995).
15. X. Mayol, J. Garriga, X. Grana, G1 cyclin/CDK-independent phosphorylation and accumulation of p130 during the transition from G1 to G0 lead to its association with E2F-4. *Oncogene* **13**, 237-46 (1996).
16. E. J. Smith, G. Leone, J. DeGregori, L. Jakoi, J. R. Nevins, The accumulation of an E2F-p130 transcriptional repressor distinguishes a G0 cell state from a G1 cell state. *Mol Cell Biol* **16**, 6965-76 (1996).
17. S. Coats, et al., A new pathway for mitogen-dependent cdk2 regulation uncovered in p27(Kip1)-deficient cells. *Curr Biol* **9**, 163-73 (1999).
18. L. Zhu, E. Harlow, B. D. Dynlacht, p107 uses a p21CIP1-related domain to bind cyclin/cdk2 and regulate interactions with E2F. *Genes Dev* **9**, 1740-52 (1995).
19. K. Hansen, et al., Phosphorylation-dependent and -independent functions of p130 cooperate to evoke a sustained G1 block. *Embo J* **20**, 422-32 (2001).
20. P. F. Dijkers, et al., Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol Cell Biol* **20**, 9138-48 (2000).
21. T. D. Littlewood, D. C. Hancock, P. S. Danielian, M. G. Parker, G. I. Evan, A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res* **23**, 1686-90 (1995).
22. T. Ono, et al., TOK-1, a novel p21Cip1-binding protein that cooperatively enhances p21-dependent inhibitory activity toward CDK2 kinase. *J Biol Chem* **275**, 31145-54 (2000).
23. E. J. Smith, G. Leone, J. R. Nevins, Distinct mechanisms control the accumulation of the Rb-related p107 and p130 proteins during cell growth. *Cell Growth Differ* **9**, 297-303 (1998).
24. L. Fajas, et al., A CDE/CHR-like element mediates repression of transcription of the mouse RB2 (p130) gene. *FEBS Lett* **471**, 29-33 (2000).
25. P. F. Dijkers, R. H. Medemadagger, J. J. Lammers, L. Koenderman, P. J. Coffer, Expression of the pro-apoptotic bcl-2 family member bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* **10**, 1201-4 (2000).
26. J. van der Sman, N. S. Thomas, E. W. Lam, Modulation of E2F complexes during G0 to S phase transition in human primary B-lymphocytes. *J Biol Chem* **274**, 12009-16 (1999).
27. M. Mudryj, et al., Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. *Cell* **65**, 1243-53 (1991).
28. M. Collado, et al., Inhibition of the phosphoinositide 3-kinase pathway induces a senescence-like arrest mediated by p27Kip1. *J Biol Chem* **275**, 21960-8 (2000).
29. A. D. Kohn, et al., Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J Biol Chem* **273**, 11937-43 (1998).
30. E. W. Lam, R. J. Watson, An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. *Embo J* **12**, 2705-13 (1993).
31. G. Carnac, et al., The retinoblastoma-like protein p130 is involved in the determination of reserve cells in differentiating myoblasts. *Curr Biol* **10**, 543-6 (2000).
32. M. H. Ladha, K. Y. Lee, T. M. Upton, M. F. Reed, M. E. Ewen, Regulation of exit from quiescence by p27 and cyclin D1-CDK4. *Mol Cell Biol* **18**, 6605-15 (1998).
33. N. Rivard, G. L'Allemain, J. Bartek, J. Pouyssegur, Abrogation of p27Kip1 by cDNA antisense suppresses quiescence (G0 state) in fibroblasts. *J Biol Chem* **271**, 18337-41 (1996).
34. H. Lowenheim, et al., Gene disruption of p27(Kip1) allows cell proliferation in the postnatal and adult organ of corti. *Proc Natl Acad Sci U S A* **96**, 4084-8 (1999).
35. J. McIlroy, D. Chen, C. Wjasow, T. Michaeli, J. M. Backer, Specific activation of p85-p110 phosphatidylinositol 3'-kinase stimulates DNA synthesis by ras- and p70 S6 kinase-dependent pathways. *Mol Cell Biol* **17**, 248-55 (1997).
36. A. Klippel, et al., Activation of phosphatidylinositol 3-kinase is sufficient for cell cycle entry and promotes cellular changes characteristic of oncogenic transformation. *Mol Cell Biol* **18**, 5699-711 (1998).
37. K. Lin, J. B. Dorman, A. Rodan, C. Kenyon, daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science* **278**, 1319-22 (1997).
38. S. Ogg, et al., The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature* **389**, 994-9 (1997).
39. S. Paradis, G. Ruvkun, 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 (1998).
40. J. H. Thomas, Chemosensory regulation of development in C. elegans. *Bioessays* **15**, 791-7 (1993).
41. V. Stambolic, et al., Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**, 29-39 (1998).
42. S. Ramaswamy, et al., Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc Natl Acad Sci U S A* **96**, 2110-5 (1999).
43. H. K. Ng, P. Y. Lam, The molecular genetics of central nervous system tumors. *Pathology* **30**, 196-202 (1998).
44. P. Kleihues, H. Ohgaki, Phenotype vs genotype in the evolution of astrocytic brain tumors. *Toxicol Pathol* **28**, 164-70 (2000).
45. R. V. Lloyd, et al., p27kip1: a multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. *Am J Pathol* **154**, 313-

23 (1999).
46. A. Baldi, et al., Differential expression of the retinoblastoma gene family members pRb/p105, p107, and pRb2/p130 in lung cancer. *Clin Cancer Res* **2**, 1239-45 (1996).
47. T. Susini, et al., Expression of the retinoblastoma-related gene Rb2/p130 correlates with clinical outcome in endometrial cancer. *J Clin Oncol* **16**, 1085-93 (1998).
48. J. Sprent, D. F. Tough, Lymphocyte life-span and memory. *Science* **265**, 1395-400 (1994).
49. D. F. Tough, J. Sprent, Life span of naive and memory T cells. *Stem Cells* **13**, 242-9 (1995).
50. T. Fukazawa, et al., Differential involvement of the CD95 (Fas/APO-1) receptor/ligand system on apoptosis induced by the wild-type p53 gene transfer in human cancer cells. *Oncogene* **18**, 2189-99 (1999).
51. S. Y. Han, et al., Activation of NF-kappaB determines the sensitivity of human colon cancer cells to TNFalpha-induced apoptosis. *Biol Pharm Bull* **23**, 420-6 (2000).
52. N. Yoshida, S. Yoshida, K. Koishi, K. Masuda, Y. Nabeshima, Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. *J Cell Sci* **111**, 769-79 (1998).
53. E. F. Wagner, M. Hleb, N. Hanna, S. Sharma, A pivotal role of cyclin D3 and cyclin-dependent kinase inhibitor p27 in the regulation of IL-2-, IL-4-, or IL-10-mediated human B cell proliferation. *J Immunol* **161**, 1123-31 (1998).
54. J. Morisset, J. C. Aliaga, E. L. Calvo, J. Bourassa, N. Rivard, Expression and modulation of p42/p44 MAPKs and cell cycle regulatory proteins in rat pancreas regeneration. *Am J Physiol* **277**, G953-9 (1999).
55. P. P. Claudio, et al., Adenoviral RB2/p130 gene transfer inhibits smooth muscle cell proliferation and prevents restenosis after angioplasty. *Circ Res* **85**, 1032-9 (1999).
56. B. Bouzahzah, et al., Transforming growth factor-beta1 recruits histone deacetylase 1 to a p130 repressor complex in transgenic mice in vivo. *Cancer Res* **60**, 4531-7 (2000).
57. J. H. Albrecht, et al., Involvement of p21 and p27 in the regulation of CDK activity and cell cycle progression in the regenerating liver. *Oncogene* **16**, 2141-50 (1998).
58. R. H. Medema, R. Klompmaker, V. A. Smits, G. Rijksen, p21waf1 can block cells at two points in the cell cycle, but does not interfere with processive DNA-replication or stress-activated kinases. *Oncogene* **16**, 431-41 (1998).

CHAPTER 7

Coupling of cell-cycle regulation and resistance to oxidative damage by DAF-16-like Forkhead transcription factors

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To be Submitted

Coupling of cell-cycle regulation and resistance to oxidative damage by DAF-16-like Forkhead transcription factors

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In the absence of protein kinase B (PKB or c-Akt) activity the Forkhead transcription factors AFX, FKHR and FKHR-L1, orthologues of *Caenorhabditis elegans* DAF-16, cause a G0/G1 arrest and an entry into quiescence ((1) and chapter 6). Similar to anti-apoptotic signalling in proliferating cells, which involves PKB activity, quiescent cells need to be protected from damaging agents. Here we show that FKHR-L1 protects the arrested cells from oxidative stress. Cells containing active FKHR-L1 display increased levels of manganese superoxide dismutase. FKHR-L1 directly activates the gene for manganese superoxide dismutase (*SOD2*) via a DAF-16 Binding Element, resulting in an increase in mRNA levels. Importantly, the increase in manganese superoxide dismutase is responsible for the Forkhead-induced enhancement of cellular anti-oxidant capacity. We propose that Forkhead transcription factors increase cellular lifespan by coupling a state of cellular quiescence with enhanced protection from oxidative damage.

The activity of the PI(3)K/PKB signalling pathway has been widely implicated in survival signalling (reviewed in (2)). An important component of anti-apoptotic signalling by active PKB appears to be the inhibition of the DAF-16-like Forkhead transcription factors AFX, FKHR and FKHR-L1 that regulate the pro-apoptotic genes *Bim* and *FasL* (3, 4, 5, 6). Indeed, in cell systems derived from the haematopoietic cell compartment, absence of PKB activity correlates with apoptosis partly via an increase in Bim and FasL expression (Jurkat, (3); Ba/F3, (6)). However, in many other mammalian cell types activation of the Forkheads or absence of PI(3)K/PKB signalling causes cell-cycle arrest and quiescence rather than apoptosis, at least in part through the regulation of $p27^{kip1}$ gene expression ((1, 7, 8) and chapter 6). In agreement with this, in the nematode *C. elegans* the absence of AGE-1/AKT signalling and concomitant activation of DAF-16 results in longevity, not apoptosis (9, 10, 11, 12). We set out to investigate how cells that have been driven into quiescence by the DAF-16-like Forkheads are protected from cytotoxic stress in the absence of PKB activity. Since reactive oxygen species (ROS) are a primary cause of cellular damage (reviewed in (13)), we focussed on examining the effects of

Forkhead activity on cellular anti-oxidant capacity. To this end, we stably transfected a conditionally active HA-FKHR-L1.A3-ER fusion (14) into DLD-1 human colon carcinoma cells. The HA-FKHR-L1.A3-ER fusion protein is constitutively expressed but remains inhibited unless presented with a modified ligand for the estrogen receptor (ER), 4OHT (15). Treatment of the DL23 subclone with 500 nM 4OHT for 24 hours resulted in the specific activation of FKHR-L1 as measured by previously described Forkhead activity assays, including reporter assays with the promoters for the IGFBP1 and $p27^{kip1}$ genes (figure 1a), a strong increase in $p27^{kip1}$ protein levels, and a decrease in cell proliferation (figure 1b, c) (1, 4, 14). Furthermore, protein levels of the p130 pocket protein were increased and pocket protein/E2F complexes shifted from p107/E2F-4 to p130/E2F-4 (Chapter 6). Importantly, 4OHT had no effect on the control DLD-1 cells (figure 1). To determine whether FKHR-L1.A3 activation affects cellular protection against ROS, we made use of the lipophilic C11-$BODIPY^{581/591}$ probe which is suited for quantifying lipid-oxidation in single living cells (16). Upon oxidation, the fluorescence excitation/emission maxima of the C11-$BODIPY^{581/591}$ probe shift from 581/591 to 490/510 nm, facilitating the measurement of the fraction of oxidized probe versus time. DL23 cells left untreated or treated with 4OHT for 16 hours were loaded with the C11-$BODIPY^{581/591}$ probe and after addition of 200 μM H_2O_2 as a radical inducer followed in time with dual-excitation laser scanning microscopy. It is to be noted that the C11-$BODIPY^{581/591}$ probe is not

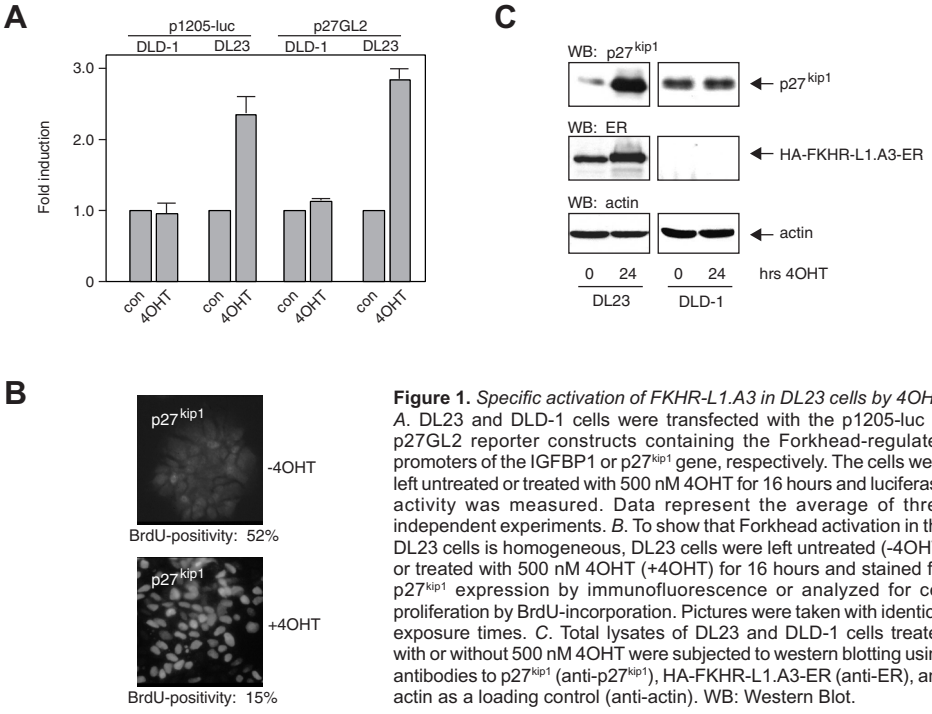

Figure 1. *Specific activation of FKHR-L1.A3 in DL23 cells by 4OHT. A.* DL23 and DLD-1 cells were transfected with the p1205-luc or p27GL2 reporter constructs containing the Forkhead-regulated promoters of the IGFBP1 or $p27^{kip1}$ gene, respectively. The cells were left untreated or treated with 500 nM 4OHT for 16 hours and luciferase activity was measured. Data represent the average of three independent experiments. *B.* To show that Forkhead activation in the DL23 cells is homogeneous, DL23 cells were left untreated (-4OHT) or treated with 500 nM 4OHT (+4OHT) for 16 hours and stained for $p27^{kip1}$ expression by immunofluorescence or analyzed for cell proliferation by BrdU-incorporation. Pictures were taken with identical exposure times. *C.* Total lysates of DL23 and DLD-1 cells treated with or without 500 nM 4OHT were subjected to western blotting using antibodies to $p27^{kip1}$ (anti-$p27^{kip1}$), HA-FKHR-L1.A3-ER (anti-ER), and actin as a loading control (anti-actin). WB: Western Blot.

oxidized by H_2O_2 itself, but by hydroxyl radicals formed in the Fenton-reaction: $H_2O_2 + O_2^{\cdot -} \rightarrow HO^{\cdot} + OH^{-} + O_2$ (catalyst: Fe^{2+}). Fifty minutes after H_2O_2 addition the total oxidized fraction of the probe was 2-3 times lower in cells containing active Forkhead compared to cells containing inactive Forkhead (figure 2a). Furthermore, the initial rate of oxidation of the probe in those cells was 4 times slower (figure 2b). Again, treatment of the DLD-1 control cell-line with 4OHT had no effect. These results demonstrate that specific activation of Forkheads increases the cellular protection against ROS.

Next we examined whether Forkheads increase cellular anti-oxidant capacity via the regulation of anti-oxidant enzymes. The increase in protection displayed in figure 2 could be due to upregulation of either catalase or superoxide dismutase, as shown by the Fenton-reaction. Superoxide dismutase was recently shown to prevent apoptosis possibly due to an inhibition of cytochrome *c* release, as induced by superoxide accumulation, from the mitochondria (17). We therefore investigated whether Forkhead activity affects protein levels of superoxide dismutase. 4OHT-treatment of the DL23 cell-line for up to 24 hours showed a gradual increase in the amount of manganese superoxide dismutase (MnSOD) protein but no change in protein levels of copper/zinc superoxide dismutase (Cu/ZnSOD) (figure 3a). The increase in MnSOD expression was not a secondary event caused by $p27^{kip1}$-induced cell-cycle arrest, since wildtype and $p27^{kip1}$ -/- MEFs showed a similar increase in MnSOD protein upon infection with a FKHR-L1.A3-expressing retrovirus (figure 3b). Similar effects were seen when we used an HA-AFX-expressing retrovirus, indicating a general effect of this Forkhead subfamily (data not shown).

To investigate whether the regulation of MnSOD protein levels by Forkheads is at the

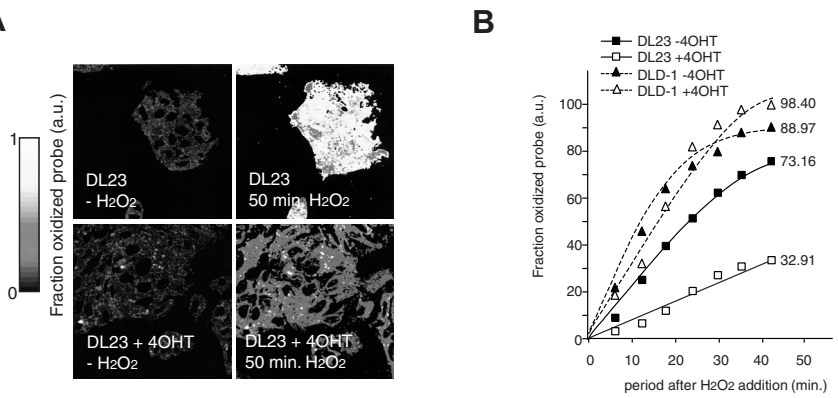

Figure 2. *Forkhead transcription factors increase cellular protection against ROS. A.* DL23 cells left untreated or treated with 500 nM 4OHT for 16 hours were loaded with the C11-BODIPY$^{581/591}$ probe for 20 minutes and subjected to 200 µM of the free-radical-inducing agent H_2O_2 for 50 minutes. Images of clusters of cells at timepoint 0 and 50 minutes after H_2O_2-addition were taken by confocal microscopy, and the fraction oxidized probe in arbitrary units (a.u.) was calculated as described in the 'Methods' section. (For full-color images, please refer to the back of the thesis: Yellow areas represent 100% oxidation, black areas represent 0% oxidation.) The circular black areas in the cell-population represent nuclei that fail to be loaded with the probe and have been ommitted from the final analysis. *B.* DL23 (solid lines) and DLD-1 (dashed lines) cells were treated as in *A*. Images were taken at timepoints 0, 8, 16, 24, 32, 40 and 48 minutes after H_2O_2-addition. Calculations of the fraction of oxidized probe per timepoint in arbitrary units (a.u.) were performed as described under 'Methods'.

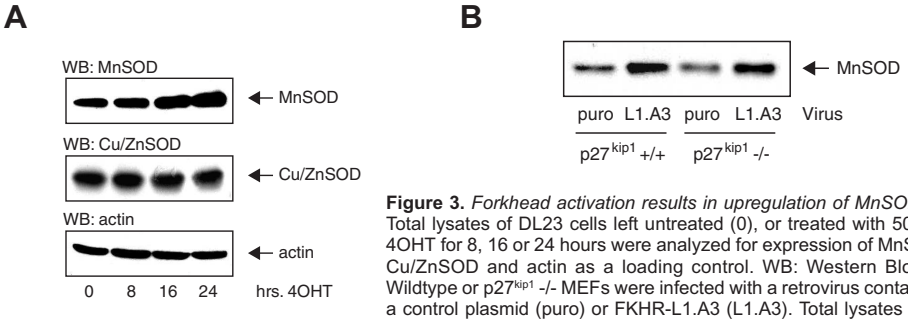

Figure 3. *Forkhead activation results in upregulation of MnSOD. A.* Total lysates of DL23 cells left untreated (0), or treated with 500nM 4OHT for 8, 16 or 24 hours were analyzed for expression of MnSOD, Cu/ZnSOD and actin as a loading control. WB: Western Blot. *B.* Wildtype or p27^{kip1} -/- MEFs were infected with a retrovirus containing a control plasmid (puro) or FKHR-L1.A3 (L1.A3). Total lysates were collected 24 hours post-infection and subjected to western blotting using an antibody against MnSOD.

level of transcription, we first examined MnSOD mRNA expression in the DL23 cell-line. As seen in figure 4a, MnSOD mRNA levels are elevated between 8-16 hours of 4OHT-treatment and a ~10-fold increase is seen after 24 hours of FKHR-L1 activation. Second, we investigated the effect of specific Forkhead activation on a 3340 basepair promoter fragment of the human *SOD2* gene for MnSOD using a luciferase reporter plasmid (pSODLUC-3340) (18).

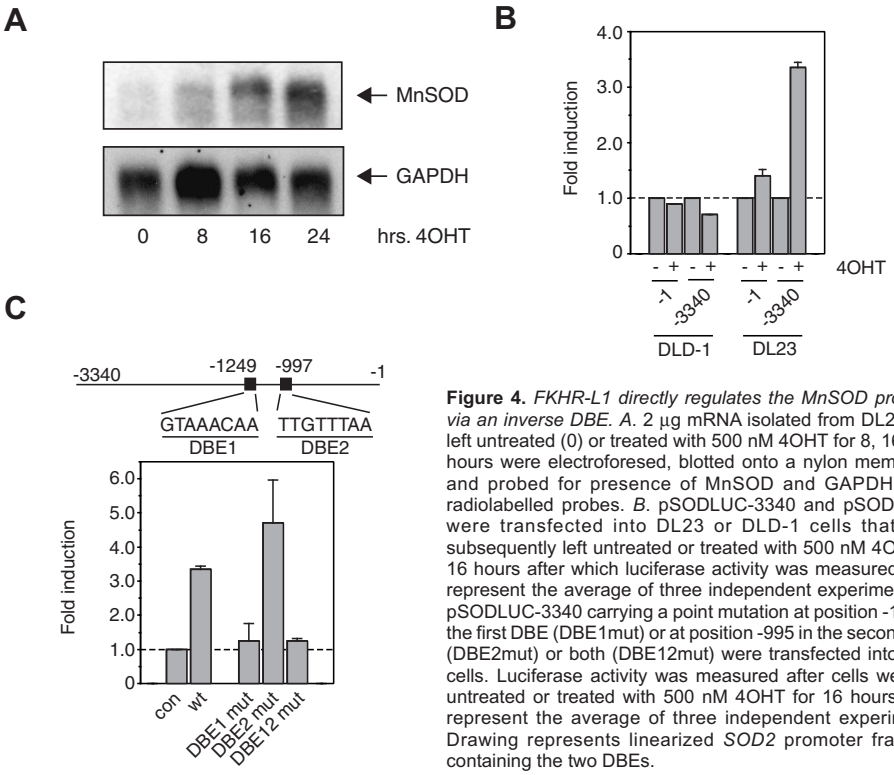

Figure 4. *FKHR-L1 directly regulates the MnSOD promoter via an inverse DBE. A.* 2 µg mRNA isolated from DL23 cells left untreated (0) or treated with 500 nM 4OHT for 8, 16 or 24 hours were electroforesed, blotted onto a nylon membrane, and probed for presence of MnSOD and GAPDH using radiolabelled probes. *B.* pSODLUC-3340 and pSODLUC-1 were transfected into DL23 or DLD-1 cells that were subsequently left untreated or treated with 500 nM 4OHT for 16 hours after which luciferase activity was measured. Data represent the average of three independent experiments. *C.* pSODLUC-3340 carrying a point mutation at position -1244 in the first DBE (DBE1mut) or at position -995 in the second DBE (DBE2mut) or both (DBE12mut) were transfected into DL23 cells. Luciferase activity was measured after cells were left untreated or treated with 500 nM 4OHT for 16 hours. Data represent the average of three independent experiments. Drawing represents linearized *SOD2* promoter fragment containing the two DBEs.

Luciferase expression controlled by this fragment was increased 3-4-fold upon cotransfection with the various Forkhead transcription factors in several cell-lines (data not shown) and upon 16 hours of 4OHT-addition to DL23 but not DLD-1 cells (figure 4b). No effect was seen on a similar reporter construct lacking the sequences upstream of the transcription startsite (pSODLUC-1) (figure 4b). Recently, the optimal DNA-binding sequence for the DAF-16-like Forkhead transcription factors has been determined (19). This DAF-16-binding element (DBE) contains the core sequence TTGTTTAC. Point mutations in the TTGTTT sequence prevent Forkhead-binding to the DBE and transactivation of a 6XDBE reporter construct. Within the human *SOD2* promoter fragment one inverse DBE at position -1249 (GTAAACAA, DBE1) and one sub-optimal DBE at position -997 (TTGTTTAA, DBE2) are found (figure 4c). To determine whether Forkhead-mediated increase in MnSOD gene expression is direct, we mutated single basepairs in the two DBEs. A single G to C substitution in the TTGTTT sequence of DBE2 had no effect on FKHR-L1-induced luciferase activity of the *SOD2* reporter construct, but a similar substitution in the AAACAA sequence of DBE1 completely abolished FKHR-L1-mediated *SOD2* gene expression (figure 4c). This clearly demonstrates that Forkhead transcription factors directly regulate MnSOD gene expression through a single inverse DBE.

MnSOD is a mitochondrial anti-oxidant enzyme that converts the free-radical-containing superoxide anion ($O_2^{\bullet -}$), generated as a byproduct of the electron-transport-chain, into hydrogenperoxide (H_2O_2). Some of the harmful effects of the superoxide anion such as lipid-oxidation and the induction of DNA breaks have been implicated in various diseases and aging (reviewed in (20)). By reducing the amount of superoxide anions, MnSOD can participate in the cellular protection against free radicals and thus contribute to the maintenance of cellular integrity. However, the hydrogenperoxide formed by MnSOD is still harmful to the cell. Therefore we investigated whether the hydrogen-peroxide-metabolizing enzyme catalase is increased in cells after Forkhead activation. Indeed, DL23 cells treated with 500 nM 4OHT displayed an increase in catalase protein with kinetics similar to MnSOD upregulation (figure 5a). This indicates that the direct regulation of MnSOD by Forkheads which results in the production of hydrogenperoxide is coupled to the regulation of catalase which converts hydrogen-peroxide to water and oxygen. Next, we inves-tigated whether the enhancement of cellular anti-oxidant capactiy by Forkheads as seen in figure 2 is due to a Forkhead-mediated increase in MnSOD gene expression. To this end we analyzed the effect of Forkhead expression on cellular stress in *Sod2* -/- MEFs (21, 22) using the C11-BODIPY$^{581/591}$ probe assay. Infection of primary MEFs from wild-type mice (*Sod2*+/+) with a retrovirus carrying FHKR-L1.A3 resulted in a marked two-fold enhancement of cellular anti-oxidant capacity compared to infection with a control virus (figure 5b). However, in primary MEFs from *Sod2* knockout mice (*Sod2*-/-) FKHR-L1.A3 expression could not protect cells from H_2O_2-induced cellular stress (figure 5b). This last results also indicates that the Forkhead-induced upregulation of catalase might be an indirect consequence of MnSOD increase, for the *Sod2*-/- cells are no longer protected. Altogether, these data clearly show that Forkhead transcription factors can decrease radical-induced oxidative damage via direct regulation of MnSOD gene expression.

In proliferating cells the activation of PKB has been associated with protection from apoptosis induced by various stimuli. Here we show that a signal equivalent to the absence of PKB activity in non-proliferating, quiescent cells

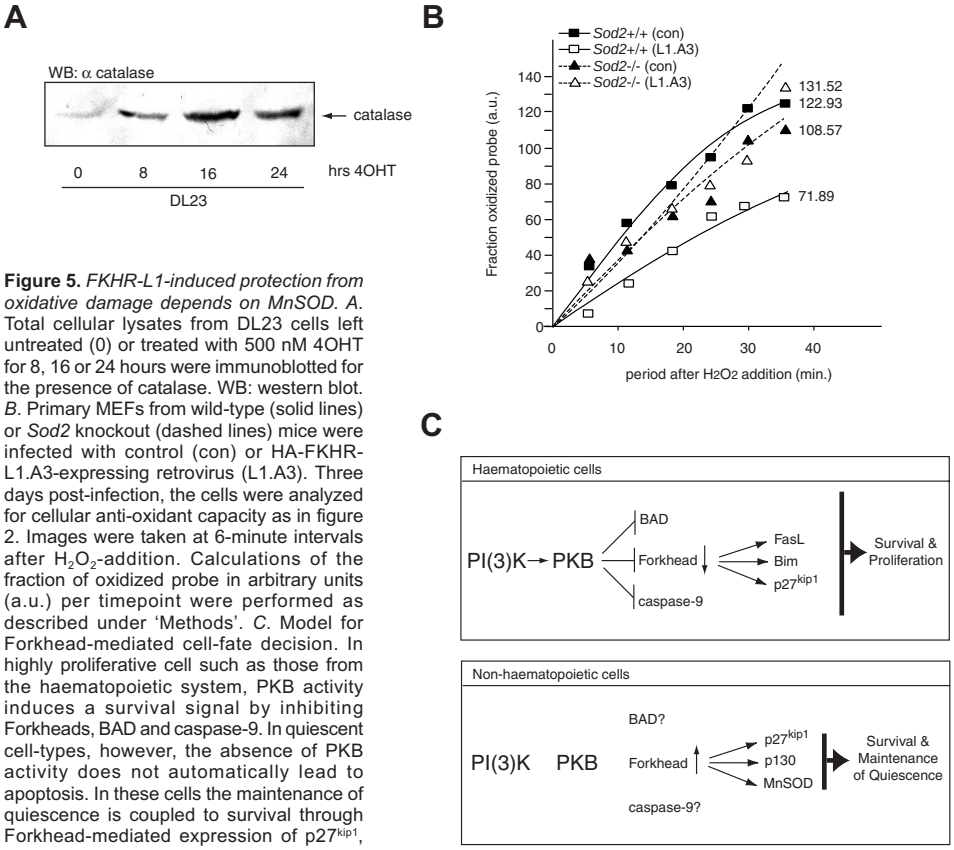

Figure 5. *FKHR-L1-induced protection from oxidative damage depends on MnSOD. A.* Total cellular lysates from DL23 cells left untreated (0) or treated with 500 nM 4OHT for 8, 16 or 24 hours were immunoblotted for the presence of catalase. WB: western blot. *B.* Primary MEFs from wild-type (solid lines) or *Sod2* knockout (dashed lines) mice were infected with control (con) or HA-FKHR-L1.A3-expressing retrovirus (L1.A3). Three days post-infection, the cells were analyzed for cellular anti-oxidant capacity as in figure 2. Images were taken at 6-minute intervals after H_2O_2-addition. Calculations of the fraction of oxidized probe in arbitrary units (a.u.) per timepoint were performed as described under 'Methods'. *C.* Model for Forkhead-mediated cell-fate decision. In highly proliferative cell such as those from the haematopoietic system, PKB activity induces a survival signal by inhibiting Forkheads, BAD and caspase-9. In quiescent cell-types, however, the absence of PKB activity does not automatically lead to apoptosis. In these cells the maintenance of quiescence is coupled to survival through Forkhead-mediated expression of $p27^{kip1}$, p130 and MnSOD.

can accomplish the same result. When challenged with incriminating stimuli such as H_2O_2, cells that contain active FKHR-L1 are protected form oxidative damage through a Forkhead-mediated increase in MnSOD gene expression. This suggests a situation where the outcome of the activity of the PI(3)K/PKB/Forkhead pathway with respect to protection from cellular damage depends on the cell-system and the cell-cycle phase the cells are in (figure 5c). In *C. elegans* the DAF-2/AKT/DAF-16 pathway regulates metabolism, dauer formation and organismal lifespan (9, 10, 11). Restoration of DAF-2/AKT/DAF-16 signalling in different cellular compartments of DAF-2 mutants has revealed that reconstitution in neuronal cells results in rescue of all three phenotypes (12). To date, the only gene targets found for DAF-16 are the free-radical-scavenging enzymes cytosolic catalase (*ctl-1*) and MnSOD (*sod-3*) and they were shown to be required for DAF-16-induced dauer formation (23, 24). The observation that *C. elegans* organismal lifespan is solely determined by DAF-16 signalling in the brain has led to the suggestion that anti-oxidant enzymes contribute to an increased cellular lifespan of neurons that

deliver neuro-endocrine signals to the organism. Our results now start to define a remarkable conservation of function. Neurons are generally considered to be post-mitotic or quiescent cells. The finding that FKHR-L1 expression is relatively high in the brain (19) allows us to speculate that the DAF-16 like Forkheads might be involved in the regulation of neuronal cellular lifespan through the maintenance of quiescence and protection from oxidative damage. Possibly, as in *C. elegans*, this might contribute to the control of organismal lifespan. In addition, quiescent/differentiated cells have upregulated levels of anti-oxidant enzymes, especially of MnSOD protein and mRNA (25, 26, 27), a property that is lacking in various tumor cells (reviewed in (28)). Our data suggest that the observations that certain tumors have low levels of $p27^{kip1}$ and/or MnSOD (reviewed in (29)) might be a consequence of the inactivation of the DAF-16-like Forkheads by activation of the PI(3)K/PKB pathway and furthermore that this might contribute to the process of oncogenic transformation. Finally, it is important to note that Cu/ZnSOD is not regulated. Apparently, Cu/ZnSOD does not need to be induced for enhanced protection during quiescence which might relate to the fact that Cu/Zn SOD is cytosolic whereas MnSOD is mitochondrial. This points to the unique function of MnSOD and illustrates the importance of its regulation by external cues. In agreement with this, overexpression of Cu/ZnSOD in MnSOD-deficient mouse fibroblasts cannot prevent neonatal lethality nor oxidative aconitase inactivation (30). In conclusion, we propose that the mammalian DAF-16-like Forkhead transcription factors AFX, FKHR and FKHR-L1 increase cellular lifespan by coupling cell-cycle exit with enhanced protection from oxidative damage.

Methods

Cell culture, retroviral infections and stable cell-lines. The DL23 cell-line was created as follows: linearized pcDNA3-HA-FKHR-L1.A3-ER (14) was transfected into DLD-1 human colon carcinoma cells by electroporation. Transfectants were selected for two weeks on 500 µg/ml geneticin. Subsequently, clones were isolated and analyzed for expression of the fusion protein. The DL23 subclone was chosen for further study. The DLD-1 and DL23 cell-lines were maintained in RPMI-1640 with standard supplements and the appropriate selection antibiotic. Retroviral infections and maintenance of immortalized wildtype and $p27^{kip1}$ -/- as well as primary wildtype and *Sod2*-/- MEFs has been described (1, 22).

Cloning and plasmids. pBabe-FKHR-L1.A3 was created by ligating a Klenow-blunted HindIII/BamHI fragment of pcDNA3-HA-FKHR-L1.A3 into Klenow-blunted BamHI-cut pBabe-puro. pcDNA3-HA-FKHR-L1.A3-ER has been described (14). pSODLUC-3340 and pSODLUC-1 were a gift of M. Yim (18). pSODLUC-3340.DBE1mut and pSODLUC-3340.DBE2mut were created by site-directed mutagenesis using the primers 5'- CTGACGTCTGTAAAGAAGCCCAG CCCTTC-3' and 5'- CATTCAGGATTGTTCTTTAACTGTT GAG-3', respectively. p1205-luc was a gift of D. Powell (31). p27GL2 has been described (1). pBabe-puro used for retroviral infections of MEFs has been described (1). pGEMZ-MnSOD was a gift of J. Wispe (32).

Western blotting, antibodies and standard immunofluorescence. Western blotting of total lysates was performed as described (4). Antibodies to MnSOD and Cu/ZnSOD were from StressGen. The antibodies to ER (C-20) and actin (I-19) were from SantaCruz. The $p27^{kip1}$ antibody was from Transduction Labs. Anti-catalase has been described (33). Standard immunofluorescence using the $p27^{kip1}$ antibody was performed as follows: cells were fixed in 4% paraformaldehyde, permeabilized and blocked with PBS containing 0.1% TX-100, 0.5% BSA and stained with anti-$p27^{kip1}$ followed by Cy3-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Labs).

Northern blotting. 2 µg of $polyA^+$-RNA (polyA-Tract, Promega) purified from 1 mg total RNA (RNAZol, TEL-TEST, Inc.) was run on a formaldehyde denaturing gel and blotted onto GeneScreen-Plus nylon membrane (NEN). The blot was hybridized using radiolabeled MnSOD (EcoRI fragment of pGEM3Z-MnSOD) and GAPDH (NotI-linearized pUC19-GAPDH) probes.

Luciferase assays. Luciferase assays were performed as described (4).

BrdU incorporation. BrdU incorporation was performed as described (1).

Microscopy and fluorescence ratio-imaging. Generally,

microscopy and fluorescence ratio-imaging were performed as described (16). Shortly, cells cultured on coverslips were placed in a temperature (37°C)-controlled coverslip holder in the microscope and incubated for 20 min with C11-BODIPY$^{581/591}$ (Molecular Probes). To this end, C11- BODIPY$^{581/591}$ was dissolved in fetal calf serum to a final concentration of 0.1 mg ml^{-1}. This stock was diluted 1000 times in PBS supplemented with 5 mM glucose, 0.5 mM $MgCl_2$ and 0.9 mM $MgCl_2$ (PBS+). After washing with fresh PBS+, images were taken with a Leica TCSNT confocal laser scanning system on an inverted microscope DMIRBE (Leica Microsystems) with an argon-krypton laser as excitation source. The green and red fluorescence of C11-BODIPY$^{581/591}$ was acquired simultaneously using double wavelength excitation (laserlines 488 and 568 nm) and detection (emission bandpass filter 530/30 nm for green and longpass 560 nm followed by a bandpass 600/30 nm for red). Oxidized and non-oxidized C11-BODIPY$^{581/591}$ are spectrally well separated and this property was used to quantify the fraction of oxidized and non-oxidized C11-BODIPY$^{581/591}$ simultaneously at any time point. Images were processed and calculated with Scion Image 1.62 on a Macintosh PowerPC. Image calculus of figure 2a was performed as follows: the red and green fluorescence of the probe was assesed at several timepoints. The fraction oxidized probe in every timepoint was calculated as the ratio of I^{green} to $I^{green}+I^{red}$, where I^{green} and I^{red} are the intensities of the green and the red fluorescent signal respectively. Images were smoothed before the algorythm was applied. The signal of the probe was filtered from the rest of the images by tresholding. The calculated fractions were averaged using the smooth filter. A false colour look-up table was applied to visualize low and high oxidation of the probe. All data are normalized, arbitrary units.

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References

1. R. H. Medema, G. J. Kops, J. L. Bos, B. M. Burgering, AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782-7 (2000).
2. S. R. Datta, A. Brunet, M. E. Greenberg, Cellular survival: a play in three Akts. *Genes Dev* **13**, 2905-27 (1999).
3. A. Brunet, et al., Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-68 (1999).
4. G. J. Kops, et al., Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**, 630-4 (1999).
5. G. Rena, S. Guo, S. C. Cichy, T. G. Unterman, P. Cohen, Phosphorylation of the Transcription Factor Forkhead Family Member FKHR by Protein Kinase B. *J Biol Chem* **274**, 17179-17183 (1999).
6. P. F. Dijkers, R. H. Medemadagger, J. J. Lammers, L. Koenderman, P. J. Coffer, Expression of the pro-apoptotic bcl-2 family member bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* **10**, 1201-4 (2000).
7. D. M. Li, H. Sun, PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. *Proc Natl Acad Sci U S A* **95**, 15406-11 (1998).
8. N. Nakamura, et al., Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. *Mol Cell Biol* **20**, 8969-82 (2000).
9. K. Lin, J. B. Dorman, A. Rodan, C. Kenyon, daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. *Science* **278**, 1319-22 (1997).
10. S. Ogg, et al., The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. *Nature* **389**, 994-9 (1997).
11. S. Paradis, G. Ruvkun, 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 (1998).
12. C. A. Wolkow, K. D. Kimura, M. S. Lee, G. Ruvkun, Regulation of C. elegans life-span by insulinlike signaling in the nervous system. *Science* **290**, 147-50 (2000).
13. K. B. Beckman, B. N. Ames, The free radical theory of aging matures. *Physiol Rev* **78**, 547-81 (1998).
14. P. F. Dijkers, et al., Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol Cell Biol* **20**, 9138-48 (2000).
15. T. D. Littlewood, D. C. Hancock, P. S. Danielian, M. G. Parker, G. I. Evan, A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. *Nucleic Acids Res* **23**, 1686-90 (1995).
16. E. H. Pap, et al., Ratio-fluorescence microscopy of lipid oxidation in living cells using C11-BODIPY(581/591). *FEBS Lett* **453**, 278-82 (1999).
17. P. Huang, L. Feng, E. A. Oldham, M. J. Keating, W. Plunkett, Superoxide dismutase as a target for the selective killing of cancer cells. *Nature* **407**, 390-5 (2000).
18. H. P. Kim, J. H. Roe, P. B. Chock, M. B. Yim, Transcriptional activation of the human manganese

superoxide dismutase gene mediated by tetradecanoylphorbol acetate. *J Biol Chem* **274**, 37455-60 (1999).

19. T. Furuyama, T. Nakazawa, I. Nakano, N. Mori, Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* **349**, 629-634 (2000).
20. J. M. Mates, F. Sanchez-Jimenez, Antioxidant enzymes and their implications in pathophysiologic processes. *Front Biosci* **4**, D339-45 (1999).
21. Y. Li, et al., Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet* **11**, 376-81 (1995).
22. T. T. Huang, et al., Superoxide-mediated cytotoxicity in superoxide dismutase-deficient fetal fibroblasts. *Arch Biochem Biophys* **344**, 424-32 (1997).
23. Y. Honda, S. Honda, The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. *Faseb J* **13**, 1385-93 (1999).
24. J. Taub, et al., A cytosolic catalase is needed to extend adult lifespan in C. elegans daf-C and clk-1 mutants. *Nature* **399**, 162-6 (1999).
25. L. W. Oberley, L. A. Ridnour, E. Sierra-Rivera, T. D. Oberley, D. L. Guernsey, Superoxide dismutase activities of differentiating clones from an immortal cell line. *J Cell Physiol* **138**, 50-60 (1989).
26. S. L. Church, D. R. Farmer, D. M. Nelson, Induction of manganese superoxide dismutase in cultured human trophoblast during in vitro differentiation. *Dev Biol* **149**, 177-84 (1992).
27. A. Bravard, et al., Modifications of the anti-oxidant metabolism during proliferation and differentiation of colon tumor cell lines. *Int J Cancer* **59**, 843-7 (1994).
28. L. W. Oberley, T. D. Oberley, Role of antioxidant enzymes in cell immortalization and transformation. *Mol Cell Biochem* **84**, 147-53 (1988).
29. R. V. Lloyd, et al., p27kip1: a multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. *Am J Pathol* **154**, 313-23 (1999).
30. J. C. Copin, Y. Gasche, P. H. Chan, Overexpression of copper/zinc superoxide dismutase does not prevent neonatal lethality in mutant mice that lack manganese superoxide dismutase. *Free Radic Biol Med* **28**, 1571-6 (2000).
31. D. R. Powell, et al., Insulin inhibits transcription of the human gene for insulin-like growth factor-binding protein-1. *J Biol Chem* **266**, 18868-76 (1991).
32. J. R. Wispe, et al., Synthesis and processing of the precursor for human mangano-superoxide dismutase. *Biochim Biophys Acta* **994**, 30-6 (1989).
33. E. A. Wiemer, et al., Production and characterisation of monoclonal antibodies against native and dissassembled human catalase. *J Immunol Methods* **151**, 165-75 (1992).

CHAPTER 8

Discussion

In the G1 phase of the cell-cycle growth factors provide a cell with a license to divide. It has previously been shown that the PI(3)K/PKB and Ras signal transduction pathways can play an important role in the process of cell-cycle progression through G1 into S phase. In this thesis we have described the identification of a component shared by the PI(3)K/PKB and Ras pathways that can be involved in their effects on G1/S transition. The Forkhead transcription factors AFX, FKHR and FKHR-L1 inhibit cellular proliferation at the G1 phase and induce cellular quiescence via increased transciption of the genes encoding the $p27^{kip1}$ cell-cycle inhibitor and the p130 retinoblastoma-like pocket protein ((1) and chapter 6), with a possible involvement of superoxide radicals (chapter 7). Activation of the PI(3)K/PKB cascade directly inactivates AFX, FKHR and FKHR-L1 (2, 3, 4) leading to a release of this block on proliferation imposed by the Forkheads (1). For AFX, we show that in addition to the PI(3)K/PKB pathway, the Ras/Ral cascade is involved in inhibition of the Forkhead, possibly also through phosphorylation (3).

Regulating Forkhead activity

The AFX, FKHR and FKHR-L1 Forkhead transcription factors are inactivated by phosphorylation on multiple sites. Inactivation may involve several mechanisms. The most striking one found to date is the PKB-mediated relocalization from the nucleus to the cytoplasm ((2, 5, 6) and chapter 4). This kind of regulation of the activity of a transcription factor through phosphorylation-dependent relocalization is reminiscent of several factors in yeast. In phosphate-starved *Saccharomyces cerevisiae*, for example, the Pho4 transcription factor is unphosphorylated and nuclear, and transcribes phosphate-responsive genes (7). Addition of phosphate activates the cyclin-cdk complex Pho80/Pho85 that phosphorylates Pho4 on five serine residues (7). These multiple phosphorylations all contribute to inactivation of Pho4, by either disturbing an interaction with its transcriptional partner Pho2 or by relocalizing Pho4 to the cytoplasm. Two of the serine residues interact with the Msn5 nuclear export factor in a phosphorylation-dependent manner (8) and one serine is located within a NLS (figure 1). Phosphorylation of Pho4 therefore not only interrupts an interaction with a co-activator, it also enhances nuclear export and inhibits nuclear import, all functions carried out separately by distinct phosphorylated residues (9). Another yeast regulatory cascade that controls transcription factor activity via phosphorylation-dependent relocalization is the Tor/Msn2/Msn4 pathway. Here, the PI(3)K-like kinase Tor phosphorylates the Msn2 and Msn4 transcription factors causing them to complex with the 14-3-3-like protein Bmh2, which results in the cytoplasmic localization of Msn2 and Msn4 (10).

As with Pho4, full inhibition of AFX requires that all sites are phosphorylated. Growth factor-induced activation of either PKB or Ral only partially inactivates AFX (3) and mutation of either of the PKB sites only partially attenuates the inhibition induced by insulin (unpublished observations). This implies that the phosphorylations of the Ral-dependent site(s) and of the PKB-dependent serines 193 and 258 are all functionally relevant, but the contribution of all sites to growth factor-induced inactivation of the transcription factors is not completely understood. Interestingly, we observe similarities between Pho4/Msn2/Msn4 and Forkhead regulation. Serum starvation of mammalian cells causes unphosphorylated AFX to be nuclear and active. Subsequent addition of insulin causes phosphorylation on multiple sites (two PKB-mediated and at least one Ral-mediated, see (3)). A function for one of the residues in the inactivation of AFX has been determined and shown to be the inhibition of nuclear import

(chapter 4). PKB-mediated phosphorylation of AFX on serine 193 functionally inactivates a NLS located around that residue, causing the continuously shuttling transcription factor to be detained in the cytoplasm (chapter 4), much like phosphorylation of serine 152 (SP4) in Pho4 (figure 1) (9).

What role might the other phosphorylated residues play in the inhibition of AFX, with respect to nuclear exclusion or otherwise? First, one or more phosphorylated residues might serve as a specific binding sequence for cytoplasmic anchors, such as the Bmh2-binding to the Msn2 and Msn4 transcription factors in budding yeast (10). Two of the three phosphorylated PKB sites in FKHR-L1 are responsible for binding the 14-3-3ζ protein (2), which, as suggested by the authors, might fulfill the function of cytoplasmic anchoring (figure 1). However, it remains to be demonstrated that Forkheads complex with 14-3-3 proteins upon growth factor treatment of cells and it is unknown whether this complex is formed in the cytoplasm or in the nucleus. Besides the possibility that 14-3-3 proteins bind the Forkheads in the cytoplasm where they function as a cytoplasmic anchor, 14-3-3-Forkhead complex formation might occur in the nucleus. In this way, 14-3-3 could provide a NES, as was shown for the phosphorylation-dependent nuclear export of the cdc25 protein by the 14-3-3 family member Rad24 (11). Although the Forkheads have a classical NES themselves, it does not appear to function as a NES, since deletion, despite having an effect on nuclear export of AFX, does not disturb binding to the Forkhead exportin Crm1 (see discussion of chapter 4). This leaves the possibility that an additional protein in the Forkhead/Crm1 complex might provide the required NES. Conversely, 14-3-3 binding might have nothing to do with regulating nuclear-cytoplasmic localization, but rather may affect

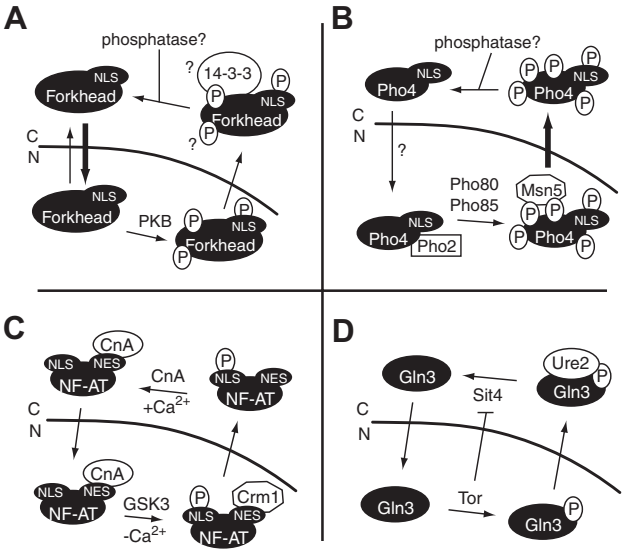


Figure 1. *Models for the regulation of transcription factors activity by phosphorylation-dependent relocalization.* The Forkheads AFX, FKHR and FKHR-L1 are inhibited by phosphorylation on multiple sites. PKB-dependent phosphorylation of one site is responsible for masking a NLS, leading to cytoplasmic localization (panel A; see also chapters 2 and 4). The role of the other phosphorylation sites is unclear. Possibly, they interrupt binding to a co-factor, as was shown for the yeast Pho4/Pho2 complex (panel B). Alternatively, the phosphorylated residues could create a binding site for a cytoplasmic anchor, such as 14-3-3 proteins, as was shown for the yeast Msn2 and Msn4 transcription factors that bind Bmh2 (see text). How the Forkheads are relocalized to the nucleus is unknown. Possibly, a phosphatase dephosphorylates the residues leading to unmasking of the NLS, as was shown for calcineurin (CnA)-dependent dephosphorylation of NF-AT (panel C). Additionally, dephosphorylation could lead to release from the putative cytoplasmic anchor, similar to Sit4-mediated re-activation of the yeast Gln3 transcription factor through release of the cytoplasmic Ure2 protein (panel D). See text for further details. N, nucleus; C, cytoplasm. Panel C was adapted from (17). Panel D was adapted from (10).

transcriptional activity of the Forkheads more directly. The association of the *C. elegans* DAF-16 Forkhead with 14-3-3 proteins, for instance, was recently suggested to inhibit the DNA-binding capacity of DAF-16 (12).

Second, phosphorylation might disturb an interaction with a co-factor, like phosphorylation of serine 223 (SP6) of Pho4 disrupts binding to the co-activator Pho2 (9). Recently, the p300/CREB-binding protein (CBP) was reported to be a co-activator for the *C. elegans* Forkhead transcription factor DAF-16 as well as for FKHR, but no evidence was presented as to whether phosphorylation of a specific site could inhibit the interaction between the Forkhead and p300/CBP (13). Third, phosphorylation of a specific residue might affect the rate of nuclear export, although we suggest in chapter 4 that for AFX, Crm1 binding is not affected by phosphorylation of the Forkhead. Nevertheless, a LMB-sensitive, Crm1-independent export pathway may exist, and phosphorylation of one of the sites could mediate the association of an exportin with the Forkheads in a similar manner as the yeast Msn5 exportin associates with Pho4 via phosphorylated serines 114 and 128 of Pho4 (9). Fourth, phosphorylation might affect the stability of the protein, as was shown for phosphorylation-dependent ubiquitin-mediated targetting of $p27^{kip1}$ to the proteasome (14, 15). However, no change in the amount of AFX protein was detected even after prolonged treatment of A14 cells with insulin (unpublished observations), arguing against this mode of regulation. Finally, phosphorylation in the C-terminus (serine 258 in AFX) might directly affect the affinity of the transactivating domain for the basal transcription machinery, and phosphorylation in the DNA-binding domain (serine 193 in AFX) could alter its affinity for Forkhead-specific promoter sequences. Elucidating the way in which the phosphorylation on all sites affects AFX function will be important for understanding the exact mechanism by which a growth factor can regulate these Forkheads and thereby affect cellular proliferation.

What causes nuclear relocalization of AFX when growth factor signalling is terminated? Depriving cycling HeLa cells of serum for ninety minutes causes a relocalization of AFX from the cytoplasm to the nucleus (chapter 4). This is unlikely to be due to high turnover of AFX and the nuclear accumulation of newly synthesized AFX protein, since preliminary experiments suggest that AFX is highly stable ($t_{1/2} > 10$ hours, unpublished observations). Hence, it seems more likely that in the absence of PKB activity AFX relocalization to the nucleus is caused by its dephosphorylation. Several studies in yeast and mammalian cells on other transcription factors have already reported the existence of such a mechanism of activation. When phosphorylated, the yeast GATA-like Gln3 transcription factor is bound to the cytoplasmic Ure2 protein. Nitrogen starvation, however, activates the type 2A-like phosphatase Sit4 that dephosphorylates Gln3, freeing it from Ure2 thereby causing Gln3 to be imported into the nucleus (10). Addition of nutrients activates Tor which in turn inactivates Sit4, leading to cytoplasmic localization and thus inactivation of Gln3 (figure 1). In mammalian systems, the localization of the T-cell transcription factor NF-AT is regulated by the phosphatase calcineurin. In the absence of calcium signalling, NF-AT is phosphorylated by GSK3 which causes masking of a NLS but not of a NES, resulting in Crm1-mediated export and cytoplasmic localization (16). In the presence of calcium, however, calcineurin is activated and binds to the region in NF-AT containing the NES but in addition dephosphorylates the NLS-masking domain, causing nuclear accumulation of NF-AT (figure 1) (17). All these studies provide elegant mechanisms for the phosphorylation-dependent activation of transcription factors through relocalization.

Whether or not such mechanisms apply to AFX re-activation remains to be investigated and this will await the identification of the functional role of all growth factor-induced phosphorylation events on AFX.

Apoptosis versus cell-cycle arrest

As determined thus far, overexpression of AFX, FKHR and FKHR-L1 can have two short-term effects: apoptosis and cell-cycle arrest. Which of these events occurs upon Forkhead expression seems to largely depend on the cellular context. All non-transformed haematopoietic cells (and some transformed ones) tested to date die upon Forkhead activation, either by Forkhead overexpression or withdrawal of a required survival factor that normally inactivates the transcription factors. In this way, programmed cell death of human CTLL2 T lymphocytes (R. H. Medema, personal communication), human UT-7/EPO leukemia cells (18), primary human erythropoid progenitor cells (19) and mouse Ba/F3 pre-B cells (20) has been linked to Forkhead activity. Furthermore, a non-phosphorylatable form of FKHR-L1 (FKHR-L1.A3) can cause apoptosis in human Jurkat lymphoma cells (1, 2). In cell-types that are not derived from the haematopoietic compartment, however, overexpression of Forkheads causes cell-cycle arrest/quiescence. Mouse A14 fibroblasts (1), MEFs (1), human U2OS osteosarcoma cells (1), human U87MG glioblastoma cells (1), human DLD-1 colon carcinoma cells (chapter 6 and 7), human 786-0 renal carcinoma cells (21) and human CCL39 fibroblasts (22) arrest in the G1 phase of the cell cycle in response to Forkhead activation. Importantly, no non-transformed haematopoietic cells have yet been reported to arrest and survive upon Forkhead expression and conversely, few non-haematopoietic cells have been reported to die when Forkheads are activated. With respect to the latter, overexpression of a non-phosphorylatable constitutively active version of the Forkheads can cause some apoptosis in chinese hamster ovary CHO-K1 cells (6), human LNCaP prostate carcinoma cells (21), human CCL39 and 293T fibroblasts (2, 23) and post-mitotic neurons (2), albeit to a much lower extent than in haematopoietic cells. Altogether, it seems that in cells that continuously require an anti-apoptotic signal to survive, such as cells from the haematopoietic compartment, Forkhead overexpression results in apoptosis. The anti-apoptotic signals (for instance IL-3 for the Ba/F3 cells) will thus likely induce survival at least in part via the inactivation of the Forkheads, and subsequent removal of the signal will activate the Forkheads hence causing apoptosis. On the other hand, in cells like fibroblasts that do not need a continuous survival signal and that upon growth factor withdrawal exit the cell-cycle rather than undergo apoptosis, Forkhead expression results in a cell-cycle block. We therefore propose that in haematopoietic cells PKB activation causes survival at least in part via Forkhead inactivation in addition to BAD and caspase-9 inactivation, but that in non-haematopoietic cells PKB-mediated Forkhead inactivation contributes to cell-cycle progression rather than survival (see figure 5c of chapter 7).

Why do cells derived from the haematopoietic compartment die upon Forkhead activation whereas other cells enter quiescence? Several answers might apply to this question. The target genes of Forkheads in haematopoietic cells could be different from the ones in other cell-types. Two Forkhead gene targets associated with apoptosis are *Bim* and *FasL*. Although no FasL protein levels after Forkhead activation have been measured in any of the two cell-types, Bim expression is seemingly restricted to cells from the haematopoietic lineage ((24, 25) and chapter 6). This is in agreement with observations made in chapter 6 where DLD-1 cells that exit the cell-cycle when a conditionally active FKHR-L1.A3

is turned on do not express detectable levels of Bim, neither before nor after Forkhead activation.

On the other hand, Forkheads might regulate anti-apoptotic genes in non-haematopoietic cells but not in haematopoietic cells. In this respect, the gene for MnSOD is an interesting candidate target, since preliminary experiments suggest that upon Forkhead activation MnSOD is not upregulated in Ba/F3 cells (unpublished observations). MnSOD overexpression can elicit a survival signal in many cell types (26, 27, 28, 29, 30, 31), presumably by reducing the accumulation of superoxide radicals in the membrane of the mitochondria thereby preventing leakage of cytochrome *c* from that membrane (32). Possibly, selective upregulation of MnSOD by Forkhead transcription factors contributes to the distinct responses of different cell-types to Forkhead activity.

Finally, different cell-types might respond differently to Forkhead-mediated expression of the same geneproduct. $p27^{kip1}$, for instance, potently arrests the cell-cycle upon AFX or FKHR-L1 expression in A14 cells, MEFs, DLD-1 and 786-0 cells (1, 21), whereas a similar $p27^{kip1}$ regulation by FKHR-L1 in Ba/F3 cells causes apoptosis (20). Elucidating the differences between cell types in their response to Forkhead activity will be helpful in understanding specificity of PKB signalling in particular and possibly of signal transduction events in general.

Forkheads and cellular transformation

Several studies have provided some compelling data that Forkhead inactivation is one of the events that contributes to cellular transformation upon loss of PTEN expression. Forkhead overexpression in PTEN-negative tumor cells has the same effect on cellular proliferation as overexpression of PTEN in these cells: cell-cycle arrest in the G1 phase of the cell-cycle and/or apoptosis (1, 21). In addition, ectopically expressed FKHR is cytosolic in PTEN-negative renal and prostate carcinoma cells, and expression of a FKHR mutant that cannot be phosphorylated by PKB causes apoptosis in the prostate carcinoma cells and a G1 arrest with a concomitant $p27^{kip1}$ upregulation in the renal carcinoma cells (21). All this suggests that PTEN overexpression might mediate its effects by activating the Forkheads through the inactivation of PKB. Following from this, one may also suggest that PTEN-negative cells may contain inactive Forkhead protein, which may play a role in the emergence of the transformed phenotype of those cells.

Might *PTEN* deletions or mutations alone be enough to fully inhibit the Forkheads, in particular AFX? Since we have shown that both Ras and PI(3)K signalling are required for full growth factor-induced inhibition of AFX, one might predict that it is not (3). This would be in agreement with recent findings that both oncogenic Ras (RasD12) and constitutively active PKB (myristoylated PKB) need to be expressed in astrocytes and neural progenitor cells in mice to induce glioblastoma formation (33), indicating that either Ras and PKB have separate but complementary effectors and/or that both need to be activated to fully inhibit a shared target, for instance the Forkheads. On the other hand, both hyperactive forms of Ras (RasV12) or PKB (gagPKB) alone can fully inhibit AFX in a reporter assay (3) and the RasV12 protein in addition can cause constitutive activation of PKB in various cell-types (34, 35). Investigating whether cells that contain genomic *PTEN* deletions or mutations have additional mutations of the *Ras* gene and *vice-versa* and whether Forkheads are fully inactivated in these cells will contribute to the clarification of the role of Forkheads in cellular transformation and of the role of the two pathways in controlling Forkheads in tumor cells (figure 2).

How would Forkhead inactivation

promote cellular transformation? In tumor cells from haematopoietic origin, Forkhead inactivation might be part of one of the survival signals that contribute to transformation. On the other hand, in cells from tumors that arise from quiescent tissues, for example brain, Forkheads might play a role in enhanced and unlicensed proliferation. Such cells have had to re-enter the cell-cycle at one point during their evolution into a cancer cell. Active FKHR-L1 and AFX induce and maintain a state of cellular quiescence, at least in non-haematopoietic cells and subsequent inactivation of these Forkheads reverses this (chapter 6). Interestingly, the *PTEN* gene is often found deleted in glioblastoma tumor cells derived from originally quiescent neuronal tissue, and Forkhead inactivation due to loss of PTEN function in such cells might be an important step for re-entry into the cell-cycle (36, 37). It will be of interest to see whether or not in general Forkheads are inactive in such tumor cells but are active in the normal quiescent counterparts. In accordance with a role for inactive Forkheads in transformation via cell-cycle re-entry are the observations that the Forkhead target genes encoding $p27^{kip1}$, p130 and MnSOD are generally low in tumor cells (38, 39, 40, 41).

How might Forkhead-mediated regulation of MnSOD expression participate in cellular transformation? Of all anti-oxidant enzymes, MnSOD in particular has been associated with cell-cycle progression. MnSOD is low during S-phase and high in G0 in several cell-lines including fibroblasts (42) and overexpression of MnSOD in NIH3T3 cells can block cellular proliferation (43). Concordant with a role for MnSOD in cell-cycle control is an observation made twelve years ago that superoxide radicals enhance proliferation of fibroblasts (44) although to date no data has been provided to suggest a mechanism by which this may occur. Importantly, overexpression of MnSOD can revert the transformed phenotype of a variety of tumor cell-lines, including those transformed by the Ras oncogene ((45, 46, 47) and reviewed in (42)) and it was suggested that superoxide radicals are required for cellular transformation, possibly by acting as second messengers (48). Maintaining high levels of MnSOD might therefore be one of the ways in which Forkheads inhibit proliferation. Interestingly, in a recent study the MnSOD promoter was found to be mutated in

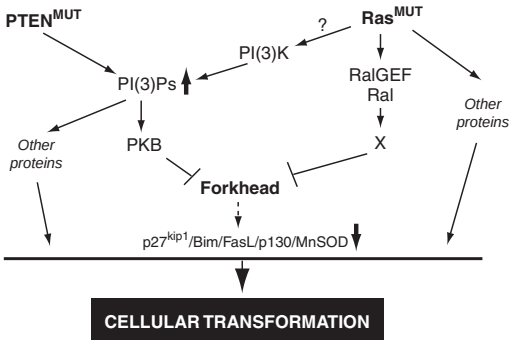


Figure 2. *Model for contributions of PI(3)K and Ras signalling pathways to cellular transformation via regulation of the Forkheads.* Under normal circumstances, activation of both PKB and Ral is required for full inhibition of AFX and possibly also of the other two Forkheads. Oncogenic mutations in PTEN ($PTEN^{MUT}$) and Ras (Ras^{MUT}) might contribute to cellular transformation by a combined effort to reduce expression of the genes encoding $p27^{kip1}$, Bim, FasL, p130 and MnSOD, thus inducing survival or cell-cycle re-entry and cell-cycle progression, depending on the cellular context. Conversely, $PTEN^{MUT}$ or Ras^{MUT} alone might be sufficient to accomplish the same effect. Hyperactive PKB (via $PTEN^{MUT}$) or Ras (Ras^{MUT}) alone can fully inhibit the Forkheads and Ras^{MUT} may accomplish this by activating the PI(3)K pathway in addition to Ral, again leading to the activation of the two pathways that are required for Forkhead inactivation in the normal situation.

five out of fourteen human cancers examined (49), suggesting that transcriptional inactivation of the gene encoding MnSOD may be an important component of cellular transformation. However, analyzing cellular proliferation of *Sod2*-/- cells upon infection with Forkheads did not reveal any function of MnSOD in cell-cycle control by the Forkheads (unpublished observations). Control of MnSOD expression by Forkhead transcription factors might thus merely be required to protect quiescent cells from oxidative stress (chapter 7), and low levels of MnSOD protein in tumor cells might therefore just be a consequence rather than a cause of Forkhead inactivation and cell-cycle re-entry.

AFX and FKHR were originally identified as part of t(2;13) and t(X;11) chromosomal translocations that are associated with ALL and rhabdomyosarcomas, respectively (50, 51). In these translocations, the transactivation domains of AFX and FKHR fuse with the DNA-binding domains of the MLL and PAX3 transcription factors. Although this excludes Forkhead target genes as direct mediators of the transforming capacity of the oncogenic MLL-AFX and PAX3-FKHR fusion products, those genes might still be indirectly regulated by the fusions. Cells that contain PAX3-FKHR, for example, have increased levels of the RTKs PDGF receptor and MET, although the gene for neither of these proteins is a target for FKHR or PAX3 separately (52, 53). Both of these RTKs are very potent activators of PI(3)K, which in turn is able to inactivate FKHR. This process would thereby have effects on cellular survival and proliferation. This might suggest that the t(2;13) translocation that already results in the loss of one functional allele of FKHR can inactivate the second functional allele by upregulating PI(3)K-activating RTKs. The transforming capability of the PAX3-FKHR fusion product could therefore at least in part be due to its ability to inactivate FKHR or even any of the other two Forkheads (figure 3). In a similar manner, the way that the MLL-AFX fusion protein contributes to the onset of leukemia can be viewed in a new light. The *AFX* gene lies on the X chromosome, meaning that males have only one allele (51). A translocation of the *AFX* gene to chromosome 11 would therefore not only result in the potent transcriptional activator MLL-AFX, but also in the functional knockout of AFX itself in males. This latter effect could then conceivably contribute to the leukemia.

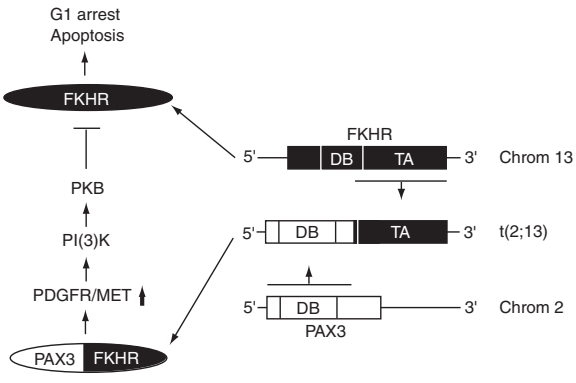


Figure 3. *Model for transforming capacity of the oncogenic PAX3-FKHR fusion.* In some rhabdomyosarcomas, 3' sequences of one *FKHR* allele on chromosome 13, containing sequences encoding its transactivation domain (TA), have fused with 5' chromosome 2 sequences. This creates a fusion between PAX3, with its DNA-binding domain (DB), and FKHR-TA. This hybrid transcription factor increases gene expression of the RTKs PDGF receptor (PDGFR) and MET, that both are potent activators of PI(3)K. In this way, the fusion protein might activate PKB which would lead to inhibition of FKHR protein encoded by the remaining functional *FKHR* alelle, ultimately affecting survival and cell-cycle progression. This signalling cascade might contribute to the oncogenic properties of the t(2, 13) translocation.

In conclusion, we have identified the AFX Forkhead transcription factor as a protein that is regulated by pathways that are controlled by the proto-oncogene Ras and the tumor suppressor PTEN. AFX and its closest relatives FKHR and FKHR-L1 can arrest the ongoing cell-cycle, causing cycling cells to enter a state of quiescence. Importantly, these Forkheads, when overexpressed, have the ability to inhibit proliferation of a variety of tumor cell-lines. This suggests that, in order to proliferate and survive, tumor cells have somehow inactivated endogenous AFX, FKHR and FKHR-L1. Indeed, FKHR is cytoplasmic in PTEN-negative renal and prostate tumor cells, and protein levels of the gene targets of the Forkheads ($p27^{kip1}$, p130 and MnSOD) are generally low in tumor samples. Finding ways to re-activate the Forkheads, possibly through relocalizing them to the nucleus, might therefore provide a valuable tool to inhibit the proliferation of such cells not only in culture but also in a living organism.

References

1. R. H. Medema, G. J. Kops, J. L. Bos, B. M. Burgering, AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782-7 (2000).
2. A. Brunet, et al., Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-68 (1999).
3. G. J. Kops, et al., Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**, 630-4 (1999).
4. G. Rena, S. Guo, S. C. Cichy, T. G. Unterman, P. Cohen, Phosphorylation of the Transcription Factor Forkhead Family Member FKHR by Protein Kinase B. *J Biol Chem* **274**, 17179-17183 (1999).
5. W. H. Biggs, 3rd, J. Meisenhelder, T. Hunter, W. K. Cavenee, K. C. Arden, Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A* **96**, 7421-7426 (1999).
6. H. Takaishi, et al., Regulation of nuclear translocation of forkhead transcription factor AFX by protein kinase B. *Proc Natl Acad Sci U S A* **96**, 11836-41 (1999).
7. E. M. O'Neill, A. Kaffman, E. R. Jolly, E. K. O'Shea, Regulation of PHO4 nuclear localization by the PHO80-PHO85 cyclin-CDK complex. *Science* **271**, 209-12 (1996).
8. A. Kaffman, N. M. Rank, E. M. O'Neill, L. S. Huang, E. K. O'Shea, The receptor Msn5 exports the phosphorylated transcription factor Pho4 out of the nucleus. *Nature* **396**, 482-6 (1998).
9. A. Komeili, E. K. O'Shea, Roles of phosphorylation sites in regulating activity of the transcription factor Pho4. *Science* **284**, 977-80 (1999).
10. T. Beck, M. N. Hall, The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature* **402**, 689-92 (1999).
11. A. Lopez-Girona, B. Furnari, O. Mondesert, P. Russell, Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature* **397**, 172-5 (1999).
12. C. M. Cahill, et al., PI-3 kinase signalling inhibits DAF-16 DNA binding and function via 14-3-3 dependent and 14-3-3 independent pathways. *J Biol Chem* **402**, (epub) (2000).
13. N. Nasrin, et al., 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 (2000).
14. A. C. Carrano, E. Eytan, A. Hershko, M. Pagano, SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat Cell Biol* **1**, 193-9. (1999).
15. H. Sutterluty, et al., p45SKP2 promotes p27Kip1 degradation and induces S phase in quiescent cells. *Nat Cell Biol* **1**, 207-14. (1999).
16. C. R. Beals, C. M. Sheridan, C. W. Turck, P. Gardner, G. R. Crabtree, Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* **275**, 1930-4. (1997).
17. J. Zhu, F. McKeon, NF-AT activation requires suppression of Crm1-dependent export by calcineurin. *Nature* **398**, 256-60 (1999).
18. Y. Kashii, et al., A member of Forkhead family transcription factor, FKHR-L1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. *Blood* **96**, 941-9 carry this site. (2000).
19. S. Uddin, S. Kottegoda, D. Stigger, L. C. Platanias, A. Wickrema, Activation of the Akt/FKHR-L1 pathway mediates the antiapoptotic effects of erythropoietin in primary human erythroid progenitors. *Biochem Biophys Res Commun* **275**, 16-9 (2000).
20. P. F. Dijkers, et al., Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol Cell Biol* **20**, 9138-48 (2000).
21. N. Nakamura, et al., Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. *Mol Cell Biol* **20**, 8969-82 (2000).
22. A. Brunet, et al., Protein Kinase SGK Mediates

Survival Signals by Phosphorylating the Forkhead Transcription Factor FKHR-L1 (FOXO3a). *Mol Cell Biol* **21**, 952-965 (2001).

23. E. D. Tang, G. Nuñez, F. G. Barr, K. L. Guan, Negative Regulation of the Forkhead Transcription Factor FKHR by Akt. *J Biol Chem* **274**, 16741-16746 (1999).
24. L. O'Connor, et al., Bim: a novel member of the Bcl-2 family that promotes apoptosis. *Embo J* **17**, 384-95 (1998).
25. P. F. Dijkers, R. H. Medemadagger, J. J. Lammers, L. Koenderman, P. J. Coffer, Expression of the pro-apoptotic bcl-2 family member bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* **10**, 1201-4 (2000).
26. J. N. Keller, et al., Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J Neurosci* **18**, 687-97. (1998).
27. S. K. Manna, H. J. Zhang, T. Yan, L. W. Oberley, B. B. Aggarwal, Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor-kappaB and activated protein-1. *J Biol Chem* **273**, 13245-54. (1998).
28. M. W. Epperly, et al., Overexpression of the human manganese superoxide dismutase (MnSOD) transgene in subclones of murine hematopoietic progenitor cell line 32D cl 3 decreases irradiation-induced apoptosis but does not alter G2/M or G1/S phase cell cycle arrest. *Radiat Oncol Investig* **7**, 331-42. (1999).
29. M. Fujimura, et al., Manganese superoxide dismutase mediates the early release of mitochondrial cytochrome C and subsequent DNA fragmentation after permanent focal cerebral ischemia in mice. *J Neurosci* **19**, 3414-22. (1999).
30. K. K. Kiningham, T. D. Oberley, S. Lin, C. A. Mattingly, D. K. St Clair, Overexpression of manganese superoxide dismutase protects against mitochondrial-initiated poly(ADP-ribose) polymerase-mediated cell death. *Faseb J* **13**, 1601-10. (1999).
31. M. Kuroda, et al., Overexpression of manganese superoxide dismutase gene suppresses spontaneous apoptosis without a resultant alteration in in vivo growth of the mouse fibrosarcoma, FSa-II. *Anticancer Res* **20**, 7-10. (2000).
32. P. Huang, L. Feng, E. A. Oldham, M. J. Keating, W. Plunkett, Superoxide dismutase as a target for the selective killing of cancer cells. *Nature* **407**, 390-5. (2000).
33. E. C. Holland, et al., Combined activation of Ras and Akt in neural progenitors induces glioblastoma formation in mice. *Nat Genet* **25**, 55-7. (2000).
34. A. Kauffmann-Zeh, et al., Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385**, 544-8. (1997).
35. A. Khwaja, P. Rodriguez-Viciana, S. Wennstrom, P. H. Warne, J. Downward, Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *Embo J* **16**, 2783-93. (1997).
36. P. Kleihues, H. Ohgaki, Phenotype vs genotype in the evolution of astrocytic brain tumors. *Toxicol Pathol* **28**, 164-70 (2000).
37. H. K. Ng, P. Y. Lam, The molecular genetics of central nervous system tumors. *Pathology* **30**, 196-202 (1998).
38. L. W. Oberley, T. D. Oberley, Role of antioxidant enzymes in cell immortalization and transformation. *Mol Cell Biochem* **84**, 147-53 (1988).
39. A. Baldi, et al., Differential expression of the retinoblastoma gene family members pRb/p105, p107, and pRb2/p130 in lung cancer. *Clin Cancer Res* **2**, 1239-45 (1996).
40. T. Susini, et al., Expression of the retinoblastoma-related gene Rb2/p130 correlates with clinical outcome in endometrial cancer. *J Clin Oncol* **16**, 1085-93 (1998).
41. R. V. Lloyd, et al., p27kip1: a multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers. *Am J Pathol* **154**, 313-23 (1999).
42. T. D. Oberley, J. L. Schultz, N. Li, L. W. Oberley, Antioxidant enzyme levels as a function of growth state in cell culture. *Free Radic Biol Med* **19**, 53-65. (1995).
43. N. Li, T. D. Oberley, L. W. Oberley, W. Zhong, Inhibition of cell growth in NIH/3T3 fibroblasts by overexpression of manganese superoxide dismutase: mechanistic studies. *J Cell Physiol* **175**, 359-69. (1998).
44. G. A. Murrell, M. J. Francis, L. Bromley, Modulation of fibroblast proliferation by oxygen free radicals. *Biochem J* **265**, 659-65. (1990).
45. J. J. Li, L. W. Oberley, D. K. St Clair, L. A. Ridnour, T. D. Oberley, Phenotypic changes induced in human breast cancer cells by overexpression of manganese-containing superoxide dismutase. *Oncogene* **10**, 1989-2000. (1995).
46. T. Yan, L. W. Oberley, W. Zhong, D. K. St Clair, Manganese-containing superoxide dismutase overexpression causes phenotypic reversion in SV40-transformed human lung fibroblasts. *Cancer Res* **56**, 2864-71. (1996).
47. E. W. Lam, et al., Immunolocalization and adenoviral vector-mediated manganese superoxide dismutase gene transfer to experimental oral tumors. *J Dent Res* **79**, 1410-7. (2000).
48. J. Q. Yang, S. Li, F. E. Domann, G. R. Buettner, L. W. Oberley, Superoxide generation in v-Ha-ras-transduced human keratinocyte HaCaT cells. *Mol Carcinog* **26**, 180-8. (1999).
49. Y. Xu, et al., Mutations in the promoter reveal a cause for the reduced expression of the human manganese superoxide dismutase gene in cancer cells. *Oncogene*

18, 93-102. (1999).
50. J. E. Sublett, I. S. Jeon, D. N. Shapiro, The alveolar rhabdomyosarcoma PAX3/FKHR fusion protein is a transcriptional activator. *Oncogene* **11**, 545-52 (1995).
51. A. Borkhardt, et al., 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 (1997).
52. J. A. Epstein, B. Song, M. Lakkis, C. Wang, Tumor-specific PAX3-FKHR transcription factor, but not PAX3, activates the platelet-derived growth factor alpha receptor. *Mol Cell Biol* **18**, 4118-30 (1998).
53. J. P. Ginsberg, R. J. Davis, J. L. Bennicelli, L. E. Nauta, F. G. Barr, Up-regulation of MET but not neural cell adhesion molecule expression by the PAX3-FKHR fusion protein in alveolar rhabdomyosarcoma. *Cancer Res* **58**, 3542-6 (1998).

Summary

A large number of cells in the human body is quiescent, but can be induced to enter the cell-division cycle (cell-cycle) by small peptide growth factors excreted by themselves or surrounding cells. These growth factors bind to a cell-surface receptor that subsequently transduces the growth signal across the plasma membrane to the cell's interior. Upon receptor activation many cascades of protein modifications take place, which ultimately result in the licencing of DNA duplication and subsequent cell division. Human cancer cells have, through genetic alterations, aquired the ability to proliferate in the absence of extra-cellular growth factor. This lead to the hypothesis that constitutive activation of signalling pathways normally controlled by growth factors may contribute to the phenotype of uncontrolled proliferaton often associated with human cancer cells. One cascade that is activated by growth factors, and dysregulated in certain types of human tumors, is the PI(3)K pathway. PI(3)K is a lipid-kinase that, under normal circumstances, is recruited to and activated by ligand-bound growth factor receptors. When activated, PI(3)K produces 3' phosphorylated phosphoinositides that act as second messengers to recruit a variety of proteins to the plasma membrane, one of which is PKB. Once properly localized, PKB is activated by upstream kinases and released from the plasma membrane to phosphorylate its substrates that participate in a variety of cellular processes including glycolysis, protein synthesis, survival, transcription, and cellular proliferation. Importantly, PI(3)K signalling and thus PKB activity is negatively controlled by the PTEN tumor suppressor, a protein often found mutated in human tumors.

At the time of the start of the study described in this thesis a number of PKB substrates had been identified that could explain at least certain aspects of the regulation of glycolysis, protein synthesis and survival by PKB, but no effectors had been found that could mediate its control over transcription and proliferation. We set out to identify transcription factors regulated by PKB, and, inspired by a study in the nematode *C.elegans*, we identified AFX, a Forkhead transcription factor with two close relatives named FKHR and FKHR-L1. **Chapter3** describes this identification, and shows in detail that the PI(3)K/PKB pathway participates in growth factor-induced inhibition of AFX transcriptional activity. PKB does so by direct phosphorylation of AFX on two serine residues. In addition to and independent of the PI(3)K/PKB pathway, the Ras/RalGEF/Ral route is also involved in growth factor-induced inhibition of AFX. In **chapter 4** the research to elucidate the mechanism by which PKB-dependent phosphorylation of AFX inhibits its activity is described. In this chapter, we show that activation of PKB results in a relocalization of AFX from the nucleus to the cytoplasm. In quiescent, serum-starved cells, AFX is active and nuclear at steady-state, although it constitutively shuttles in and out of the nucleus. Once a cell has been given a growth factor, PKB is activated after which it translocates to the nucleus. There, it phosphorylates AFX on the two serine residues, one of which is located at the heart of a non-classical NLS. Subsequently, since the phosphorylation of the serine residue in the NLS by PKB has inactivated that NLS, PKB-phosphorylated AFX is no longer able to re-enter the nucleus after it has been exported. **Chapter 5** describes the functional consequence of the inhibition of AFX, FKHR and FKHR-L1 by PKB. We show that AFX can arrest the ongoing cell-cycle at the G0/G1 phase. AFX, and

presumably also its two family members, does so by actively transcribing the gene encoding the $p27^{kip1}$ cdk-inhibitor. This leads to an increase in the amount of $p27^{kip1}$ protein to a level sufficient to fully inhibit the activity of cyclin E/cdk2 complexes that are essential for proper G1/S transition. Activation of PKB, in turn, inhibits AFX and thereby stimulates cell-cycle progression. In **chapter 6**, we refine the function for the three Forkheads transcription factors as described in chapter 5. We show that FKHR-L1 activation not only arrests cell proliferation, but that it actually induces a state of cellular quiescence, even in human tumor cells. For this study, we created a conditionally active FKHR-L1 protein that is specifically activated by a synthetic ligand named 4OHT. Prolonged addition of 4OHT to cells expressing this conditionally active Forkhead resulted in quiescent cells and allowed the identification of genes specifically induced by Forkhead activity. In this way, we were able to identify the p130 pocket protein, a regulator of quiescence, as a candidate target gene. In **chapter 7** we use the same inducible system to investigate the contribution of the Forkheads to the cellular protection from oxidative damage, a function described for the *C. elegans* othologue DAF-16. We show that conditional activation of FKHR-L1 indeed protects cells from the damaging effects of reactive oxygen species. A mammalian cell contains several anti-oxidant enzymes that can scavenge such reactive oxygen species. We show that activation of Forkheads results in the upregulation of at least two of such enzymes, MnSOD and catalase. For the Forkhead-induced increase in MnSOD, we provide evidence that FKHR-L1 directly regulates the activity of the promoter for the MnSOD-encoding gene. Interestingly, in *C. elegans* the DAF-16 pathway regulates organismal longevity through similar enzymes, presumably by affecting the lifespan of longevity-regulating neurons. Since some of the Forkheads are markedly expressed in brain tissue of mice, this may indicate by analogy that the Forkhead pathway in mammals can likewise regulate organismal lifespan.

In conclusion, this thesis describes the identification of a novel component of the PI(3)K/PKB signalling cascade, the Forkhead transription factor AFX, that is responsible for some of the effects on cellular proliferation that have been observed for this pathway. Moreover, the data from chapter 6 imply that the PI(3)K/PKB/Forkhead pathway is particularly important in the regulation of cell-cycle exit and entry, a feature that may be significant in the context of a human cancer cell. From all the data described in this thesis, we propose the following model: In normal quiescent human cells, the Forkhead transcription factor is active and contributes to the maintenance of the quiescent state. When cells are presented with a growth factor or when certain oncogenic mutations take place (i.e. in the gene for PTEN), the PI(3)K/PKB pathway is activated, leading to inhibition of the Forkheads, down-regulation of the genes for $p27^{kip1}$, p130 and MnSOD, and subsequent entry into the proliferative state (figure 1). Possibly, this series of events might significantly contribute to the emergence of certain types of human cancers.

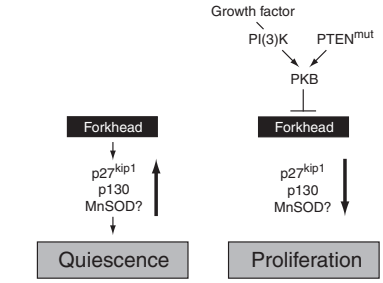


Figure 1. *Model for Forkhead-mediated control of proliferation.* In the absence of growth factor, Forkheads are active and maintain the quiescent state via the upregulation of $p27^{kip1}$, p130 and possibly MnSOD (left). Once PKB gets activated, either by growth factors or by oncogenic mutations in upstream regulators such as PTEN ($PTEN^{mut}$), Forkhead activity is inhibited, leading to cell-cycle entry and proliferation.

Samenvatting (voor de normale mens)

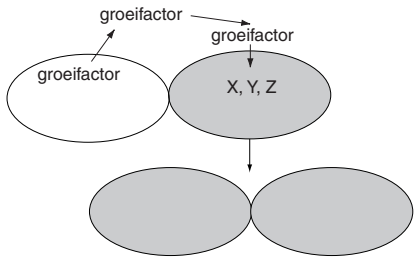
Het menselijk lichaam bestaat uit allerlei geïntegreerde onderdelen met ieder een specifieke taak. Een voorbeeld van zo'n onderdeel is een orgaan, zoals de longen, of een systeem, zoals de bloedsomloop. Al deze onderdelen zorgen ervoor dat het lichaam optimaal blijft functioneren. Elk van de onderdelen bestaat uit allerlei soorten weefsels. Zo hebben de longen weefsels die de zuurstof-opname verzorgen, maar ook weefsels die het mogelijk maken dat een long kan uitzetten. Een weefsel is op zijn beurt opgebouwd uit cellen. Deze cellen zijn de eigenlijke werkpaarden van het lichaam. Het zijn de cellen die zuurstof opnemen, transporteren, verwerken en omzetten tot energie, die zorgen dat een spier samentrekt, enzovoorts.

In een cel spelen zich een groot aantal processen af. Er moet bijvoorbeeld zuurstof worden omgezet in energie, er moet contact gehouden worden met naburige cellen, er moet snel gereageerd kunnen worden op veranderende omstandigheden, er moeten bouwstenen worden aangemaakt en afgebroken, en ga zo maar door. Al deze processen worden uitgevoerd door eiwitten. In één cel zijn duizenden van deze eiwitten werkzaam in honderden diverse processen.

Zo nu en dan zijn er cellen die vervangen moeten worden. Dit houdt in dat er een reeks processen moet worden uitgevoerd die leidt tot celdeling: het verdubbelen van één cel tot twee cellen. Cellen in de maagwand, bijvoorbeeld, zijn behoorlijk slijt-gevoelig en moeten eens in de zoveel tijd vervangen worden, wat inhoudt dat gezonde buurcellen gaan delen om het tekort aan te vullen.

De regulatie van celdeling is een delicaat proces. Als er te weinig celdeling plaatsvindt, worden versleten cellen niet aangevuld, wat de functie van een weefsel kan aantasten. Als er echter te veel celdeling plaatsvindt, kan er een tumor ontstaan. Daarom is het geheel van processen die de celdeling verzorgt zeer goed gecontroleerd, zowel door de cel zelf, door de buurcellen, als door het lichaam. Naburige cellen controleren de celdeling door het uitscheiden van een groeifactor. Zo'n groeifactor is een klein eiwit dat kan binden aan specifieke eiwitten op de buitenkant van een cel (figuur 1 en 2). Wanneer een groeifactor aan een cel bindt, veranderen allerlei eiwitten in zo'n cel van gedaante. Er wordt bijvoorbeeld ineens een bepaald soort eiwitten aangemaakt, andere worden ineens actief terwijl ze daarvoor nog in een rusttoestand verkeerden. Uiteindelijk dragen al deze veranderingen bij aan de celdeling (figuur 1).

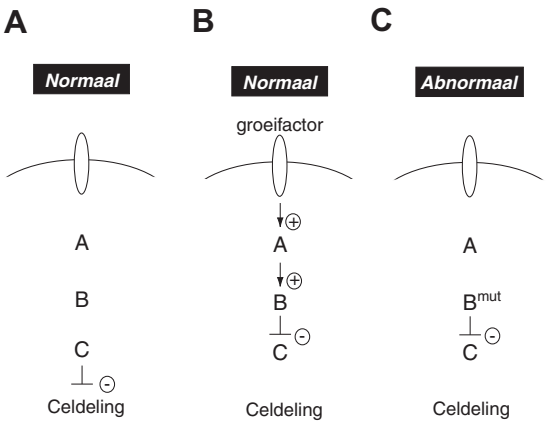
Uit het hierboven beschrevene valt af te leiden dat er een gevaarlijke situatie kan ontstaan wanneer een cel in afwezigheid van zo'n groeifactor gaat delen. Dit kan gebeuren wanneer ineens een bepaald soort eiwit wordt aangemaakt, of wanneer een bepaald eiwit, wat eigenlijk behoort te slapen, actief wordt. Het gevolg is dat een cel zou kunnen gaan delen terwijl er geen versleten cellen vervangen hoeven te worden. Dit



Figuur 1. *regulatie van celdeling door buurcellen.* De witte cel scheidt een groeifactor uit die bindt aan de grijze cel. Hierdoor worden er in de grijze cel allerlei processen (X, Y, Z) in gang gezet die uiteindelijk leiden tot celdeling.

kan leiden tot een overmaat aan cellen, en wellicht uiteindelijk tot een tumor.

Hoe kan het dat een cel zoiets gaat doen? Dit heeft alles te maken met genen. Genen zijn stukken DNA, die een code zijn voor de aanmaak van een bepaald eiwit. Je kan het vergelijken met een handleiding. Stel dat je een computer wilt maken en je koopt hiervoor een handleiding. Zo'n handleiding is niets meer dan inkt op papier, maar het is een code voor de handelingen die je moet verrichten om de computer te bouwen. Zo werkt ook een cel als hij eiwitten wil maken. Hij leest hiervoor de juiste handleiding (gen) en hij weet dan welke bouwstenen hij moet gebruiken om het juiste eiwit te bouwen. De hele verzameling van genen is dus een blauwdruk van een cel. De codes voor de aanmaak van alle eiwitten staan erin. Het is belangrijk dat alle genen worden verdubbeld op het moment dat een cel gaat delen, zodat beide cellen uiteindelijk weer een volledige set van genen en dus een volledige blauwdruk meekrijgen. Om terug te komen op de vraag aan het begin van deze alinea: hoe komt het dat een cel zonder toestemming gaat delen? Dit komt doordat er wel eens een foutje sluipt in bepaalde belangrijke genen. Deze foutjes (ook wel mutaties genoemd) kunnen allerlei oorzaken hebben: Ze kunnen ontstaan door factoren van buitenaf, zoals sigarettenrook, maar ook door fouten in het kopiëren en verdubbelen van de genen wanneer een cel deelt. Het foutje in het gen levert ook een foutief eiwit op, wat geen effect hoeft te hebben op de werking van het eiwit, maar wat soms wel het geval is. Het foutje kan ervoor zorgen dat het eiwit nooit meer actief wordt, dat het onmiddellijk wordt afgebroken, dat het constant actief wordt, etcetera. Mocht er een foutje zijn gemaakt in een gen dat codeert voor een eiwit dat een rol speelt in celdeling, dan bestaat de mogelijkheid dat het foutje ervoor zorgt dat een eiwit wordt aangemaakt dat continu het signaal blijft geven dat de cel moet delen. Een voorbeeld is gegeven in figuur 2. Stel dat er drie genen zijn die coderen voor drie eiwitten, A, B en C. In een rustende cel (figuur 2a) zijn A en B inactief, maar C actief, en deze laatste remt de celdeling. Wanneer er groeifactor bindt aan de cel wordt A geactiveerd. A activeert vervolgens B, en B remt C. De uitkomst van dit schema is dat er celdeling optreedt wanneer er groeifactor bindt (figuur 2b). Stel dat er een foutje optreedt in het gen voor eiwit B, en dat dit foutje ervoor zorgt dat B altijd actief is. B is dan niet meer afhankelijk van A (en dus niet meer afhankelijk van de groeifactor) om C uit te zetten (figuur 2c). Dit kan ertoe leiden

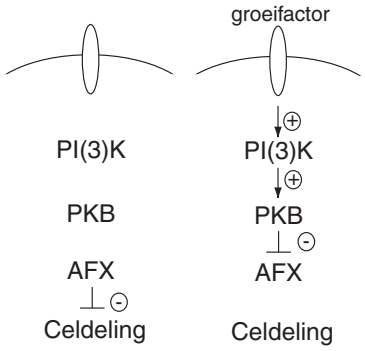


Figuur 2. *Schema's van gereguleerde en niet-gereguleerde celdeling. A*. In een cel in rusttoestand is eiwit C actief en remt (-) celdeling. *B*. In aanwezigheid van groeifactor wordt eiwit A geactiveerd (+), die op zijn beurt eiwit B activeert, die op zijn beurt eiwit C remt. De uitkomst van deze gebeurtenissen is celdeling. *C*. In een tumorcel kan het volgende zijn gebeurd: er is een mutatie geslopen in het gen dat codeert voor eiwit B. Dit levert een foutief eiwit B op (B^{mut}), dat door het foutje permanent aan staat, zelfs zonder groeifactor of activiteit van eiwit A. Zonder de juiste toestemmingen remt eiwit B^{mut} eiwit C. De uitkomst is wederom celdeling.

dat een rustende cel zonder toestemming zomaar gaat delen. De twee cellen die ontstaan uit deze deling hebben nu allebei het foutje, gaan dus weer zonder toestemming delen, enzovoorts, totdat er zoveel cellen zijn gevormd dat er sprake kan zijn van een tumor.

In dit proefschrift beschrijf ik onderzoek naar zo'n A-B-C route. In dit geval heet eiwit A PI(3)K, eiwit B heet PKB en eiwit C heet AFX. In menig kankercel staat deze route permanent aan doordat er een foutje is geslopen in een bepaald gen dat codeert voor een eiwit dat deze route normaal uitzet.

In normale cellen wordt PI(3)K (indirect) geactiveerd door groeifactoren. PI(3)K activeert PKB en PKB remt AFX (figuur 3), net zoals in het A-B-C voorbeeld van figuur 2b. **Hoofdstuk drie** gaat over de ontdekking dat AFX door PKB wordt geremd. Voordat hoofdstuk drie werd gepubliceerd, was bekend dat PKB geactiveerd kon worden door PI(3)K en ook dat er waarschijnlijk een eiwit C onder controle van PKB zou staan. Wij zijn vervolgens op zoek gegaan naar de identiteit van eiwit C en vonden AFX.



Figuur 3. *Schema's van de regulatie van celdeling door de PI(3)K-PKB route.* Net zoals in het voorbeeld in figuur 2 remt AFX in een rustende cel de celdeling (links). Activatie van PI(3)K door groeifactoren leidt tot activatie van PKB, wat leidt tot remming van AFX, wat uiteindelijk leidt tot celdeling.

Hoofdstuk vier beschrijft in detail de wijze waarop PKB de activiteit van AFX remt. Wij laten zien dat PKB ervoor zorgt dat de locatie van AFX in de cel verandert. Zonder PKB activiteit (en dus zonder groeifactor) zit AFX in de kern van de cel, daar waar de genen liggen opgeslagen. AFX zit daar omdat het bijdraagt aan het vertalen van de codes van enkele van die genen naar functionele eiwitten. Wanneer PKB actief wordt, verandert AFX op zodanige wijze dat hij niet meer in de kern aanwezig kan zijn. Met andere woorden: PKB zorgt ervoor dat AFX niet meer kan bijdragen aan het lezen van genen. PKB inactiveert AFX.

Tot dusver hebben we aangetoond dat er in de mens een bepaalde A-B-C-route bestaat en hebben we vele details van deze route opgehelderd. De belangrijke volgende vraag is: we weten dat PI(3)K en PKB betrokken zijn bij celdeling en tumorvorming, maar wat heeft AFX hiermee te maken? Zou het kunnen zijn dat de genen die door AFX worden aangestuurd en die door PKB te activeren niet meer worden vertaald, invloed uitoefenen op de celdeling? In **hoofdstuk vijf en zes** beschrijven we twee genen die mogelijk met behulp van AFX worden vertaald naar eiwitten die de celdeling verhinderen. We laten zien dat de celdeling geremd kan worden door AFX te activeren in cellen. De celdeling wordt niet alleen geremd door AFX, maar de cel wordt zelfs in rusttoestand gebracht. Tevens laten we zien dat activering van PKB dit op zijn beurt kan verhinderen (figuur 3). Een belangrijke observatie die we in deze hoofdstukken beschrijven, is dat activering van AFX in tumorcellen de celdeling van deze tumorcellen remt. Dit betekent dat AFX in staat is om de ongecontroleerde deling van tumorcellen (die vaak geen groeifactor meer van naburige cellen nodig hebben) te remmen. Dit biedt mogelijke perspectieven op nieuwe behandelingen van kanker; wellicht kan in de toekomst door AFX te activeren, de groei van tumoren geremd

worden.

Tenslotte bespreken we in **hoofdstuk zeven** een derde gen dat door AFX wordt gereguleerd en dat mogelijk ook betrokken is bij de regulatie van celdeling. Dit gen is beroemd geworden door zijn rol in de bescherming van de cel tegen radicalen. Radicalen zijn moleculen die schadelijk zijn voor de cel, aangezien ze allerlei structuren, zoals eiwitten en genen, kunnen beschadigen. Zodoende kunnen er foutjes in genen sluipen, wat een oorzaak kan zijn van kanker, maar ook van veroudering (van zowel de cel als het organisme). Het eiwit waar het derde gen voor codeert, is een eiwit dat zulke schadelijke radicalen opruimt. Het is daarom mogelijk dat AFX naast bescherming tegen kanker ook bescherming tegen veroudering biedt.

Samenvattend hebben we in vier jaar onderzoek het volgende gevonden: PKB remt AFX en AFX remt celdeling. PKB activeert dus celdeling door AFX te remmen (figuur 3). Onderzoek naar tumorcellen laat zien dat PKB daarin vaak geactiveerd is. Men denkt dat dit bijdraagt aan de ongecontroleerde celdeling van die tumorcellen. Ons onderzoek geeft een mogelijke verklaring naar de werking hiervan: wij denken dat ongecontroleerde celdeling van sommige tumorcellen komt doordat PKB actief is en AFX remt.

Curriculum vitae

Geert Kops werd geboren op 18 april 1974 aan de Leliestraat 14 te Dongen. In 1992 behaalde hij het VWO-diploma aan het Orduynen College te 's-Hertogenbosch. Vervolgens begon hij aan de studie Biologie aan de Universiteit Utrecht. Zijn propedeutisch examen werd afgelegd in 1993, waarna hij zich ging specialiseren in de moleculaire celbiologie. Onderzoeksstages werden afgelegd bij de afdeling Moleculaire Celbiologie aan de Universiteit Utrecht onder supervisie van Dr. Paul van Bergen en Henegouwen, en aan de 'Medicine Branch' van het National Cancer Institute, National Institutes of Health in Bethesda, USA, onder supervisie van Dr. Katja Wosikowski, Dr. Susan Bates en Prof. Dr. Piet Borst van het Nederlands Kanker Instituut te Amsterdam. In februari 1997 werd het doctoraal-diploma Biologie behaald. Het promotie-onderzoek, zoals beschreven in dit proefschrift, is gedaan in de periode van 1 april 1997 tot 1 april 2001 onder begeleiding van Dr. Ir. Boudewijn Burgering en Prof. Dr. Hans Bos bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum Utrecht.

Publications

Wosikowski K, Schuurhuis D, Kops GJPL, Saceda M, Bates SE. Altered gene expression in drug-resistant human breast cancer cells. *Clin Cancer Res* **12** (1997) 2405-14.

Kops GJPL, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, Burgering BMT. Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398** (1999), 630-4.

Kops GJPL, Burgering BMT. Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. *J Mol Med* **77** (1999), 656-65. Review.

Medema RH*, Kops GJPL*, Bos JL, Burgering BMT. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404** (2000), 782-7.
**These authors contributed equally*

Brownawell AM, Kops GJPL, Macara IG, Burgering BMT. 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** (2001) 3534-46.

Kops GJPL, Dansen TB, Wirtz KWA, Medema RH, Dijkers PF, Coffer PJ, Huang TT, Bos JL, Burgering BMT. Coupling of cell-cycle regulation and protection from oxidative damage by DAF-16-like Forkhead transcription factors. *To be submitted.*

Kops GJPL*, Medema RH*, Glassford J, Lam E, Essers, MAG, Dijkers PF, Coffer PJ, Burgering BMT. Control of cell-cycle exit and entry by Protein kinase B: Forkhead transcription factors regulate the p130 pocket protein. *To be submitted.*
**These authors contributed equally*

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G

..... . Dus.