CHAPTER 3

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

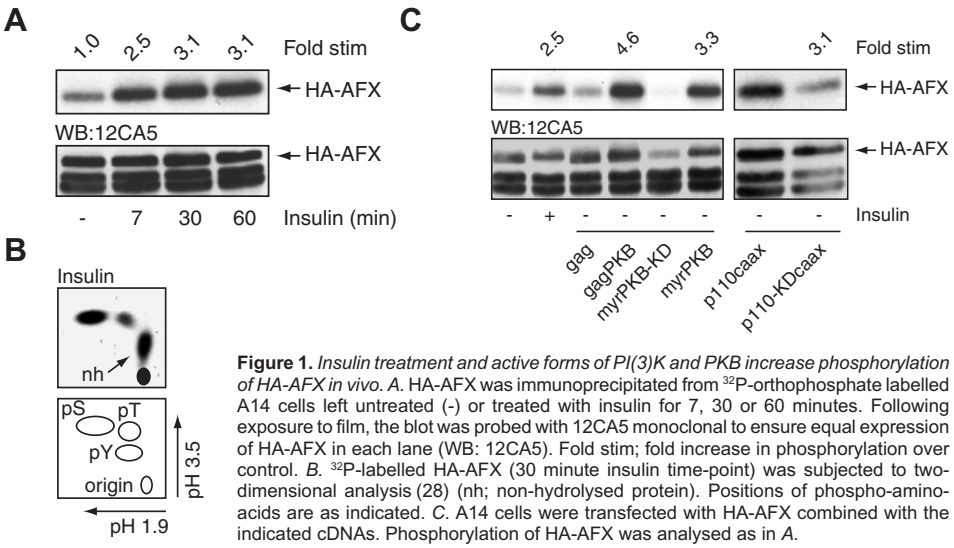

Figure 1. *Insulin treatment and active forms of PI(3)K and PKB increase phosphorylation of HA-AFX in vivo. A.* HA-AFX was immunoprecipitated from ^{32}P-orthophosphate labelled A14 cells left untreated (-) or treated with insulin for 7, 30 or 60 minutes. Following exposure to film, the blot was probed 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

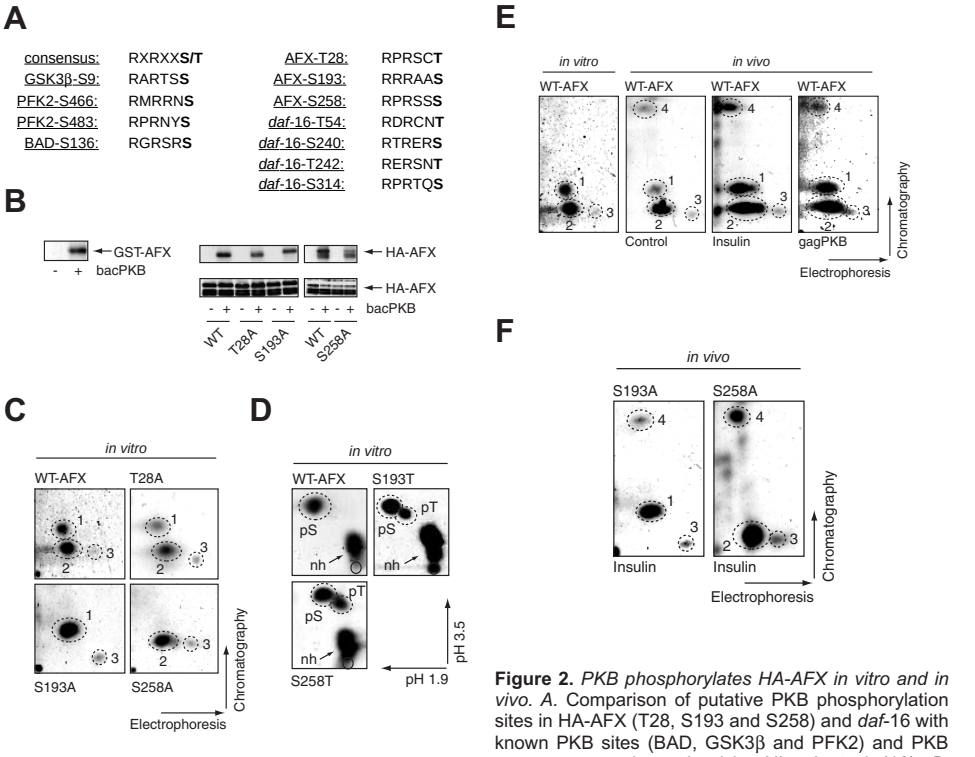

Figure 2. *PKB phosphorylates HA-AFX in vitro and in vivo. A.* Comparison of putative PKB phosphorylation sites in HA-AFX (T28, S193 and S258) and *daf*-16 with known PKB sites (BAD, GSK3β and PFK2) and PKB consensus as determined by Allessi *et al*. (10). *B.* Immunoprecipitated wild-type HA-AFX and 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).

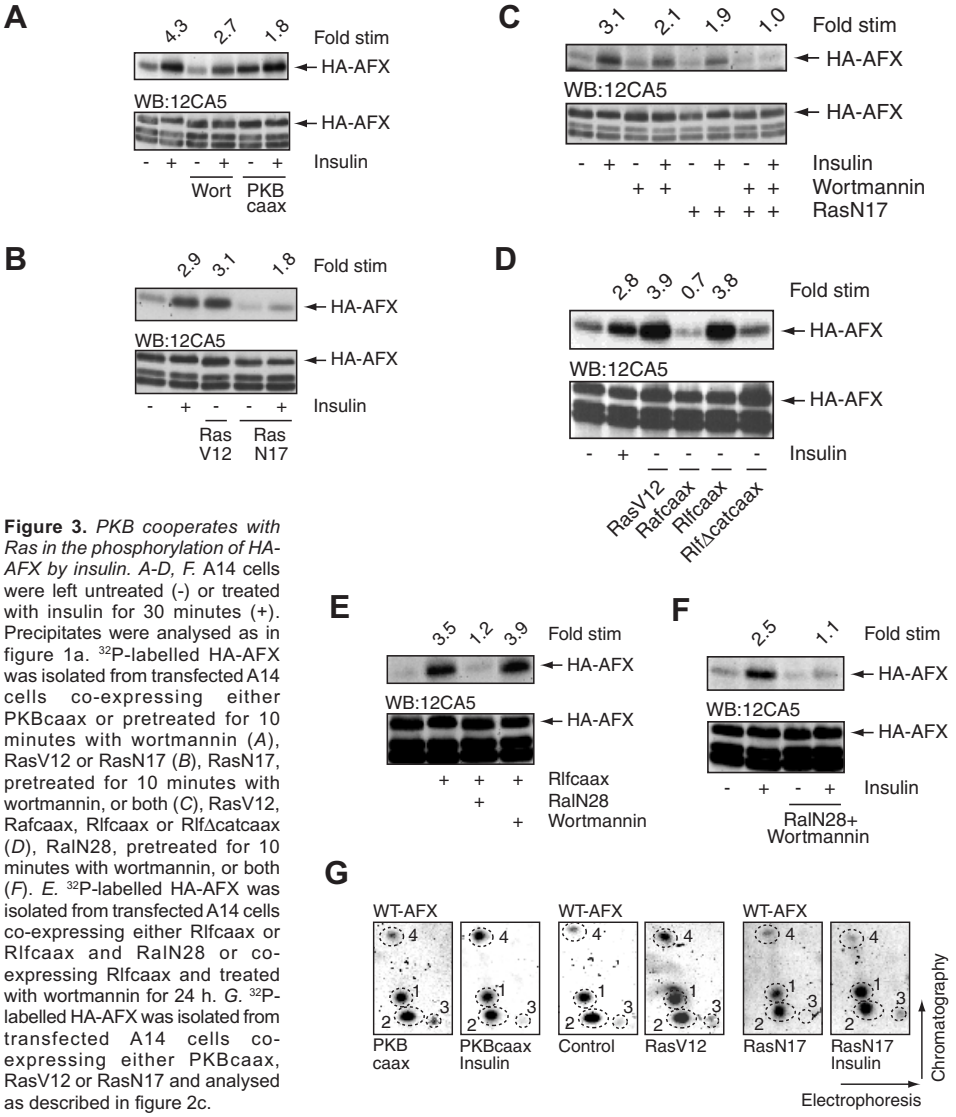

Figure 3. *PKB cooperates with Ras in the phosphorylation of HA-AFX by insulin. A-D, F.* A14 cells were left untreated (-) or treated with insulin for 30 minutes (+). Precipitates were analysed as in figure 1a. ^{32}P-labelled HA-AFX was isolated from transfected A14 cells co-expressing either PKBcaax 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).

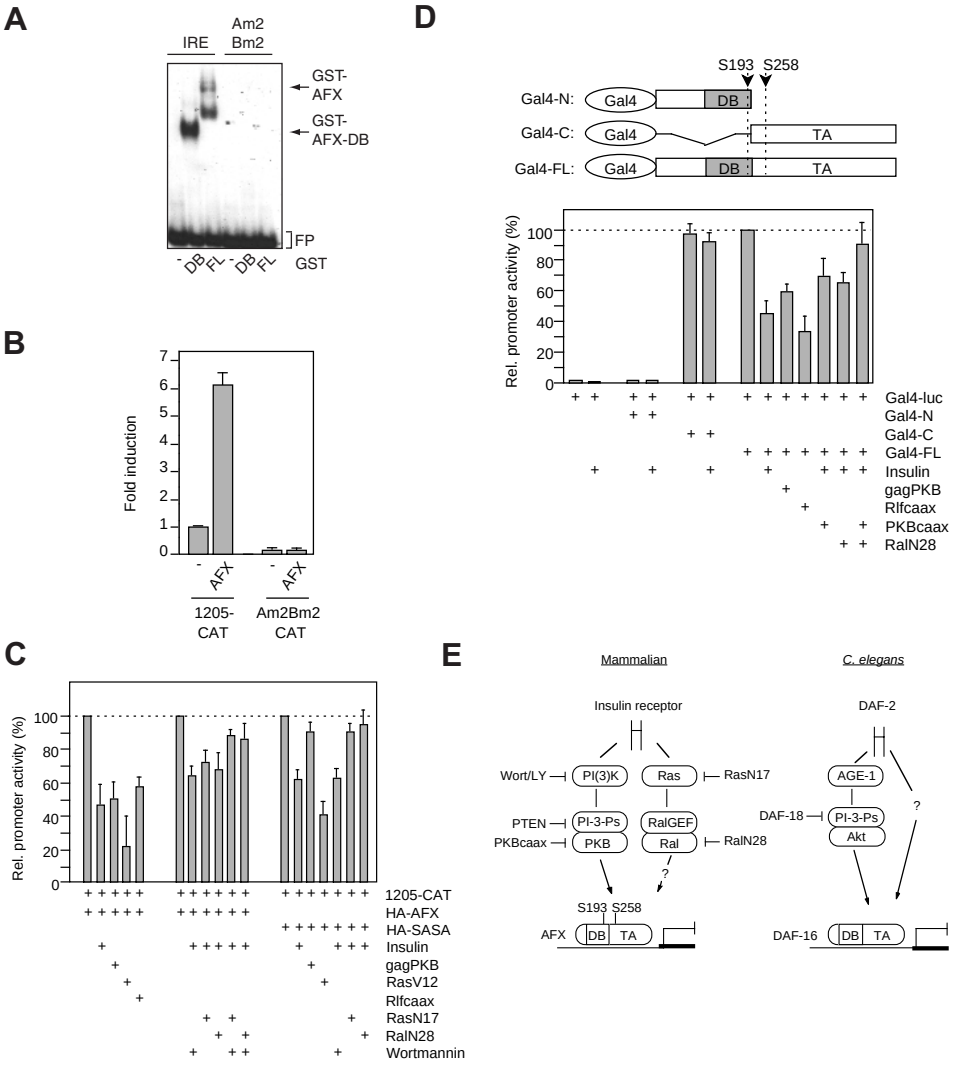

Figure 4. *Phosphorylation of HA-AFX by PKB regulates transcriptional activity. A.* GST protein alone (-), GST-AFX-DB (DB) or GST-AFX (FL) were incubated with ^{32}P-labelled oligonucleotides encompassing the IRE or a mutated IRE (Am2Bm2) of the IGFBP-1 promoter. Faster migrating complexes in the GST-AFX 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).

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