Insulin action in the brain: intracellular signaling and FoxO transcription factors

Insuline actie in het brein: intracellulaire communicatie en FoxO transcriptie factoren

(met een samenvatting in het Nederlands)

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abbreviations

ACSF	artificial cerebrospinal fluid
AMPA	alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
CK1	casein kinase 1
CNS	central nervous system
DAF	dauer formation
DYRK	dual specificity tyrosine phosphorylated and regulated kinase
EPSP	excitatory post synaptic potential
ERK1/2	extracellular regulated kinase 1 and 2
FCS	fetal calf serum
FHRE	forkhead responsive element
FKHR	forkhead in rhabdomyosarcoma
FoxO	forkhead box transcription factor of the O class
G-6-Pase	glucose-6-phosphatase
GDP	guanosine diphosphate
GFP	green fluorescent protein
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
IGF	insulin like growth factor
IRE	insulin responsive element
IRS-1	insulin receptor substrate 1
IRU	insulin response unit
LMB	leptomycin B
LTD	long term depression
LTP	long term potentiation
NES	nuclear export sequence
NLS	nuclear localization sequence
NMDA	N-methyl-D-aspartate
PBS	phosphate buffered saline
PDK-1	3'-phosphoinositide dependent kinase 1
PI3K	phosphoinositide-3-kinase
PKB	protein kinase B
PKC	protein kinase C
SGK	serum and glucocorticoid regulated kinase

Chapter 1

General Introduction

Introduction: Insulin signaling and function in the central nervous system.

Insulin is a peptide hormone secreted from the pancreas in response to increasing levels of metabolic fuels in the blood which is best known for its endocrine role in peripheral glucose homeostasis and its importance in Diabetes Mellitus. Apart from its role in the periphery insulin has a role, unrelated to glucose homeostasis, in the central nervous system (Schubert M., 2004). Insulin has been implicated with learning and memory and may have links with the pathogenesis of Alzheimer's disease and dementia. In this thesis, we address how insulin modulates intracellular signaling pathways and how it influences synaptic plasticity. In addition, we describe the identification and characterization of a novel forkhead transcription factor of the "O" class (FoxO), functioning as a transcriptional end-point of intracellular insulin signaling. This chapter describes insulin action in the nervous system and its role in neuronal plasticity and pathology.

Presence of insulin in the central nervous system

Each brain region has been attributed specific functions. Thus, knowing where insulin and its receptor are expressed in the brain can provide important information about the physiological role of insulin. Insulin was long considered to be incapable of crossing the blood–brain barrier, but it is clear now that insulin and its receptor are present in the brain (Havrankova J., 1978; Unger J.W., 1991; Adamo M., 1989; Marks J.L., 1990; Gerozissis K., 2003). Although widely distributed, the insulin receptor is enriched in specific brain regions including the olfactory bulbs, limbic system, hypothalamus and hippocampus, whereas insulin itself is particularly abundant in the hypothalamus and olfactory bulb (Schulingkamp R.J., 2000). It is, however, unclear if all the brain insulin originates from the periphery or whether it is locally synthesized in the brain. Insulin mRNA has been detected in hippocampal CA1 and CA3 regions (Devaskar S.U., 1994), suggesting the hippocampus has its own source of insulin.

Insulin and synaptic plasticity

The expression of the insulin receptor and insulin itself in the hippocampus implicates a role for brain insulin in hippocampal processes such as learning and memory. Insulin has been shown to induce changes in synaptic strength (Malenka R.C., 2003), which form the basis of learning and memory. The molecular basis of synaptic plasticity and how insulin influences these processes is described here.

Two opposite forms of activity-dependent synaptic modifications have been identified, long-term potentiation (LTP) and long-term depression (LTD). In many brain areas including the hippocampus and neocortex, the direction and the degree of the synaptic changes are a function of conditioning frequency, the level of postsynaptic depolarization and the change in postsynaptic Ca^{2+} level. LTD is obtained following low levels of postsynaptic depolarization whereas LTP is produced by a stronger depolarization (Dunwiddie T. & Lynch G., 1978; Artola A et al., 1990; Dudek S.M. & Bear M.F., 1992, 1993; Ngezahayo A., 2000). The best characterized forms of LTD and LTP require Ca^{2+} influx through the N-Methyl-D-Aspartate (NMDA) receptor (Malenka R.C., 2003). Low frequency stimulation or small Ca^{2+} influx results in LTD, whereas high frequency stimulation or high Ca^{2+} influx results in LTP (Stanton P.K., 1996). Ca^{2+} influx functions as a second messenger modulating downstream signaling cascades involved in synaptic strength,

these molecular cascades include calmodulin, calmodulin-dependent kinase II, calcineurin, protein phophatase 1 (PP1), PP2A, PP2B, protein kinase C (PKC), extracellular regulated kinase 1 and 2 (ERK1/2) and phosphatidyl-inositol-3-kinase (PI3K) (Soderling T.R., 2000; Sweatt J.D., 2004).

Insulin signaling has been implicated in both LTP and LTD, by influencing the levels of plasma membrane associated gamma-aminobutyric acid receptor receptors, by influencing NMDA receptor conductance and through regulation of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor cycling (Kneussel M., 2002; Wang Y.T., 1994; Malenka R.C., 2003). AMPA receptors mediate most of the excitatory neurotransmission in the mammalian central nervous system and also participate in forms of synaptic plasticity thought to underlie memory and learning, and the formation of neural networks during development. The AMPA receptor family is composed of four different subunits named GluR1-4 and native AMPA receptors are most likely tetramers generated by the assembly of one or more of these subunits, yielding homomeric or heteromeric receptors (O'Neill M.J., 2004). Interestingly, AMPA receptors are constitutively endocytosed from, and returned to, the plasma-membrane. This AMPA receptor cycling is facilitated by synaptic activity. AMPA or NMDA application and LTD induction all decrease the level of membrane associated AMPA receptors. Interestingly, insulin facilitates the internalization of AMPA receptors in a GluR2 dependent manner and results in a depression of excitatory transmission. Insulin induced LTD is NMDA independent, but does require a rise in intracellular Ca2+. Insulin mediated endocytosed AMPA receptors do not co-localize with intracellular compartments encompassing the recycling endocytotic machinery, suggesting that insulin utilizes additional pathways.

Insulin mediated endocytosis of AMPA receptors depends on the C-terminal cytoplasmic tail of the GluR2 subunit. The first 10 amino acids of the C-terminal tail regulate constitutive AMPA receptor endocytosis, whereas the last 15 amino acids are necessary for insulin stimulated endocytosis. Possibly phosphorylation of a tyrosine residue located in the last 15 amino acids is required for insulin induced endocytosis (Ahmadian, G., 2004). The insulin induced kinase responsible for phosphorylating the AMPA receptor C-terminal tyrosine residue has not been identified. Pharmacological intervention studies identified PP1/2 A, PP2B, calcineurin, and Ca2+ in the regulation of AMPA receptor endocytosis, the precise signaling mechanism is, however, unclear.

Opposite to LTD, LTP is associated with an increase in the expression of functional AMPA receptors on the plasma membrane. Strikingly, exocytosis of AMPA receptors is also facilitated by insulin application, but the subunits involved, differ from those involved in endocytosis. Insertion of GluR1 subunits into the plasma-membrane occurs slowly under baseline conditions and is greatly enhanced by insulin. GluR2 exocytosis is constitutively rapid and not affected by insulin (Passafaro M., 2001).

In summary, insulin influences excitatory post synaptic currents by regulating AMPA receptor levels localized on the plasma-membrane. However, the intracellular signaling mechanisms underlying the actions of insulin on AMPA receptor trafficking are at present unclear.

Neuronal Insulin Signaling
The key question arising is how biological functions of insulin are mediated in neuronal systems at the cellular and molecular level. Here, five major players (Insulin receptor, PI3K, PKB and Erk1/2 and FoxOs) of the insulin signaling-cascade are described (Fig. 1).

The insulin receptor
The insulin receptor is a tetramer composed of two extracellular α-subunits and two intracellar β-subunits (White M.F., 1997, 1998; Taha C. & Klip A., 1999). The β-subunits contains tyrosine residues that are auto-phosphorylated upon extracellular insulin binding. Tyrosine phosphorylation of β-subunits, induces specific recruitment of docking proteins. Such proteins, as SHC and the insulin receptor substrate (IRS) family (Fig1) contain SH2 and PTB domains (domains that recognize phosphorylated tyrosines). Docking protein interactions regulate many signal transduction components such as extracellular regulated kinase 1 and 2 (ERK1/2) and protein kinase B (PKB). ERK1/2 is a serine/threonine kinase that is activated through the highly conserved Ras-Raf-MEK cascade, and has been implicated in survival and learning and memory (Sweatt J.D., 2001). PKB/Akt is a PI3K activated serine/threonine kinase that influences a wide spectrum of cellular signaling events including anti-apoptosis, regulation of glycogen metabolism and protein synthesis by regulating substrates such as Bad, caspase 9, PKCs, GSK3 and forkhead transcription factors (Vanhaesebroeck B., 1999; Brunet A., 2001) Together, the ERK1/2- and the PI3K-PKB cascade appear to form the two major branches of the insulin signaling route (White M.F., 1997) (Fig 1).

PI3K
PI3K are heterodimers of a catalytic subunit (110kDa) and a regulatory or adapter subunit (Vanhaesebroeck B., 1999, 2000) and are expressed in the developing and adult nervous system. During development from stage E15-E18 in rat, expression of PI3K p110alpha is found throughout the nervous system, it then gradually restricts to the adult expression pattern (Rodgers E.E., 2002). In the adult brain, PI3K p110alpha is expressed at relatively high levels in the olfactory bulb, hippocampus and cerebellum. In all other regions, p110alpha is expressed at low levels in neurons. PI3Ks are activated by several proteins, including G-proteins and tyrosine kinases (Vanhaesebroeck B., 2000). PI3K-signaling is implicated in survival, cell cycle regulation, cell differentiation and intra-cellular trafficking (Vanhaesebroeck B., 1999, 2000; Foster F.M., 2003). Recruitment of PI3K to membrane receptors relocates PI3K to its lipid substrates where it phosphorylates the 3'-OH group of the inositol ring of phosphatidylinositol to generate the phosphoinositide phosphates PIP2 and PIP3 (Vanhaesebroeck B., 2000). These two signaling molecules trigger downstream serine/threonine kinases including 3'-phosphoinositide-dependent kinase-1 (PDK-1) and PKB (Vanhaesebroeck B., 2000). Signaling by PI3K is counter balanced by the tumor suppressor protein phosphatase: tensin homologue deleted on chromosome ten (PTEN). This protein de-phosphorylates PIP2 and PIP3 and prevents the subsequent activation of serine/threonine protein kinases (Vanhaesebroeck B., 1999, 2000). PI3K activation is sufficient to promote neuronal survival in primary cerebellar, sympathetic, sensory, motor, cortical, hippocampal, and retinal neurons and neuroblastoma cell lines. Recently, glutamatergic activity was linked to PI3K signaling.

Activated group I metabotrobic glutamate receptors recruit the scaffolding protein Homer and PI3K enhancer-large (PIKE-L). This complex activates PI3K and prevents neuronal apoptosis in response to stress (Rong R., 2003). In addition, PI3K has been linked to AMPA receptor cycling and synaptic plasticity (see above).

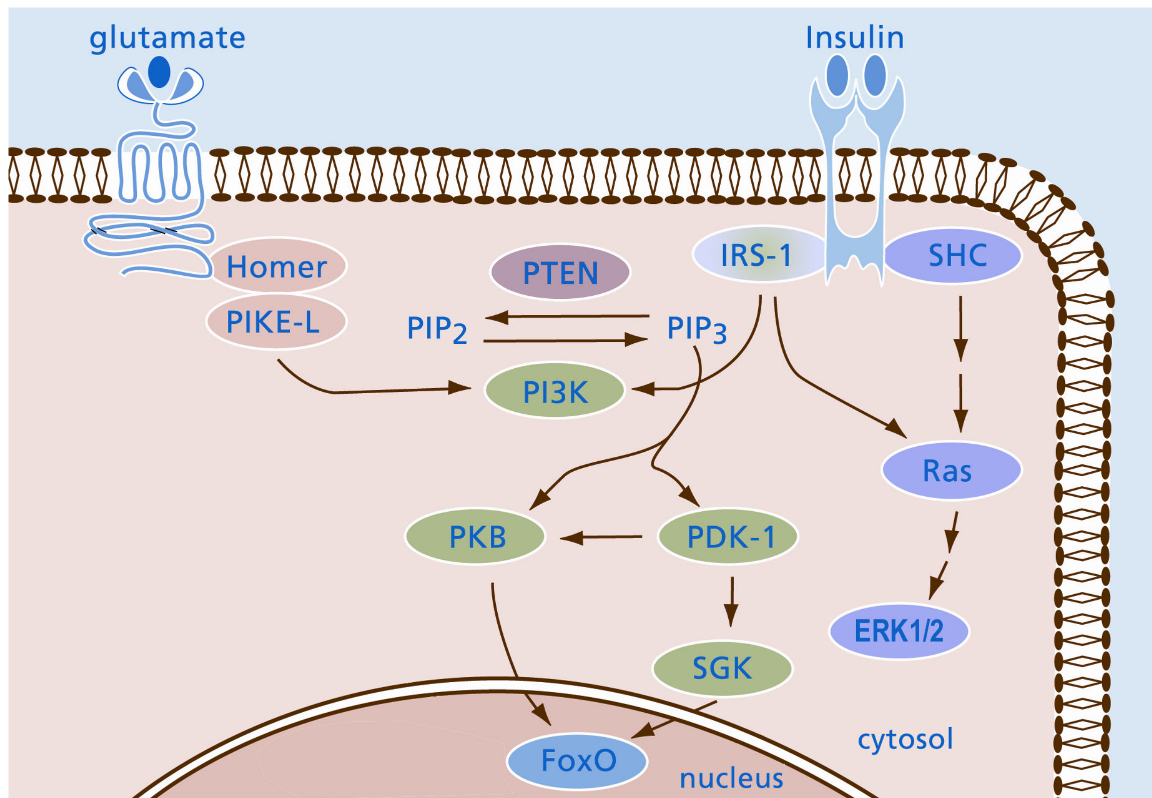

Figure1 Insulin activates the PI3K pathway and the Ras pathway by recruiting IRS-1 and SHC to the activated insulin receptor. PI3K signaling is counterbalanced by PTEN. Type 1 mGlur receptors were shown to activate the PI3K pathway. PI3K signaling activates PKB regulating nuclear FoxO transcription factors. Ras is activated through recruitment to the plasma membrane, and triggers activation of ERK1/2

PKB

One of the main downstream mediators of PI3K signaling is PKB (Akt), a serine/threonine kinase structurally related to protein kinase A (PKA) and protein kinase C (PKC). It belongs to the AGC family of protein kinases. They share similarity in the catalytic domain and in the mechanism of activation (Scheid M.P., 2003). PKB is extensively studied, partly because it was shown that PKB inactivates proteins of the apoptotic machinery in response to many ligands such as the Bcl-2 family member Bad (Datta S.R., 1999; Brunet A., 2001). PKB mediates and is not exclusively activated by insulin, because its activation is coupled to PI3K.There are three widely expressed isoforms of PKB: PKBα, PKBβ and PKBγ. The proteins consist of three functionally distinct regions: an N-terminal pleckstrin homology domain, a catalytic domain and a C-terminal hydrophobic motif (Scheid M.P., 2003). PKB isoforms are recruited to the plasma membrane by PIP2 and PIP3 where PKB is phosphorylated on two specific sites required for full catalytic activity (Lawlor M.A., 2001; Scheid M.P., 2003). At least one

of these sites is phosphorylated by PDK-1, a major regulator of AGC kinases, also recruited by phosphoinositides (Alessi D., 2001). Activated-PKB detaches from the plasma membrane and translocates to the cytosol and nucleus where it phosphorylates serine or threonine residues. Activated PKB in the hippocampus was associated with neuronal protection against hypoxic stress and nitric oxide toxicity (Yamaguchi A., 2001; Matsuzaki H., 1999). In addition, activated PKB prevents neuronal apoptosis by preventing transcriptional activity of p53 (Yamaguchi A., 2001).

ERK1/2

ERK1/2 forms a parallel branch, next to PI3K signaling, downstream of the insulin receptor (Fig 1) and is implicated in multiple forms of synaptic plasticity and neuronal survival (Sweatt J.D., 2001). ERK1/2 is activated by at least two multi-step routes downstream of the insulin receptor (see above). The adapter protein SHC binds to the tyrosine phosphorylated C-terminal tail of the insulin receptor. Grb2, another adapter protein, binds to SHC via its SH2 domains. Besides an SH2 domain, Grb2 contains two SH3 domains which mediate interactions with proline-rich regions in Sos, a Ras guanine nucleotide exchange factor. Loading of membrane bound Ras with GTP results in its activation and additional recruitment of the serine/threonine kinase Raf, which in turn activates the dual specificity kinase MEK. MEK phosphorylates ERK1/2 on threonine and tyrosine residues which trigger its activation. The activation of ERK1/2 can induce activation of several transcription factors, including CREB, Elk-1 and c-Myc (Weeber E.J., 2002). ERK1/2 activates CREB indirectly via phosphorylation and activation of a member of the pp90RSK family of S6 kinases, RSK2 (Sweatt J.D., 2004). Interestingly, the RSK2 gene, when mutated in humans, causes a form of mental retardation: Coffin-Lowry Syndrome (Trivier E., 1996). ERK1/2 activity is counterbalanced by specific ERK1/2 phosphatases, which can be induced by several stimulants which include glucocorticoids, nitric oxide, serum and insulin (Pervin S., 2003; Begum N. & Ragolia L., 2000). In the hippocampus ERK1/2 activation is regulated by a wide variety of neurotransmitter receptors such as NMDA receptors, adrenergic receptors, dopamine receptors, muscarinic acetylcholine receptors and metabotropic glutamate receptors. These findings indicate diverse ERK1/2 regulation in the hippocampus, and suggest a broad role for ERK1/2 in both short-term and long-term forms of hippocampal synaptic plasticity (Sweat J.D., 2001).

The Forkhead box O (FoxO) family of transcriptional activators

The finding that FoxO transcription factors are regulated by PKB (Kops G., 1999) has linked this group of transcription factors to neuronal insulin signaling. Transcription factors of the forkhead family have an important role in development and function of an organism (Kaufmann E., 1995). Since the discovery of the winged helix structure (forkhead domain) in *Drosophila*, more than 90 genes containing the forkhead domain have been identified, in species ranging from yeast to humans (Kaufmann E., 1997). Daf-16, a forkhead transcription factor in *Caenorhabditis elegans,* has been extensively studied for its role in controlling longevity and dauer formation (Ogg S., 1997). In mice Daf-16 has four described orthologues, FoxO1, FoxO3, FoxO4 and FoxO6. These proteins form the FoxO-class of forkhead transcription factors. FoxO1, FoxO3, and FoxO6 are expressed in the central nervous system whereas FoxO4 is not. Over recent

years it has become evident that FoxO factors are insulin sensitive transcription factors with an array of downstream targets and interacting partners (van der Heide L.P., 2004). Central to insulin mediated inhibition of FoxO factors is a shuttling mechanism that drives nuclear FoxO to the cytosol, thereby terminating its transcriptional function. Several intra-molecular domains, including phosphorylation sites, are necessary for FoxO factors to shuttle efficiently from nucleus to cytosol. Interestingly, FoxO6, a recently described FoxO family member, displays unique shuttling dynamics adding information about the mechanism underlying translocation. Overexpression of wild-type FOXO3a and its non-phosphorylated mutant induced apoptosis in cultured hippocampal neurons (Zheng W.H., 2002) in a process that may involve the induction of the pro-apoptotic gene Bim (Gilley J., 2003). IGF-1 rapidly induces the phosphorylation of endogenous FoxO3 in hippocampal neurons in a PI3K/PKB dependent manner, blocks nuclear translocation of FoxO3 and promotes survival in parallel to the phosphorylation of PKB and FoxO3 (Zheng W.H., 2002). In chapter 4 FoxOs will be discussed in more detail.

Insulin in CNS pathology

As described above, insulin affects processes that underlie learning and memory and induces signal transduction pathways involved in neuronal survival and synaptic plasticity. Moreover, insulin is also implicated in several diseases. Impairments in the insulin signalling pathway in the brain have been implicated in aging, Alzheimer's disease and cognitive defects in diabetes (Gispen W.H. & Biessels G.J., 2000; Hoyer S., 1998; Frolich L., 1998). Aging is associated with reductions in the level of both insulin and its receptor in the brain (Frolich L., 1998). In Alzheimer's disease this age-related reduction in cerebral insulin levels appears to be accompanied by functional changes of the insulin receptor (Frolich L., 1998), leading to the qualification of Alzheimer's disease as "an insulin-resistant brain state" (Hoyer S., 1998). In elderly non-demented, non-diabetic individuals chronic hyperinsulinemia is associated with cognitive decline, even after adjustment for possible confounding factors like cardiovascular disease and glucose levels (Kalmijn S., 1995; Stolk R.P., 1997). In contrast to the apparent negative association between chronic hyperinsulinemia and cognitive functioning, acute insulin administration, while keeping glucose at fasting levels, actually improves memory in individuals with Alzheimer's disease, as well as in healthy controls (Craft S., 1999).

Mechanisms of insulin mediated pathology in the CNS

Which mechanisms underlie the potential adverse effects of defective cerebral insulin signalling? Some suggest that simply disturbances in cerebral glucose metabolism are involved (Hoyer S., 1998), but others suggest links with the formation of advanced glycation end products and oxidative stress (Facchini F.S., 2000).
Interestingly, insulin favours the accumulation of oxidized molecules through two different pathways. The first pathway involves insulin induced inhibition of the proteasome, a large intracellular multienzymatic proteolytic complex (Pacifici R.E., 1989) that is responsible for the degradation of almost all oxidised molecules. Insulin decreases both the peptide-degrading and ubiquitin-dependent proteolytic activities of the proteosome (Hamel F.G., 1997; Duckworth W.C., 1998; Bennett R.G., 2000). The second pathway through which hyperinsulinaemia may favour the accumulation of oxidised-proteins was recently demonstrated in *Caenorhabditis Elegans*. In this species

DAF-16, a FoxO transcription factor, is a target of insulin-mediated signalling (Ogg S., 1997). DAF-16 increases the defence against oxidative stress (Johnson T.E. 2000; Honda Y. & Honda S., 1999) and increase life-span (Lin K., 1997). Insulin inactivates these transcription factors, eliminating these effects (Kops GJ. & Burgering B.M., 1999).

The insulin receptor knock-out
Additional information about the role of insulin signaling in the brain and its involvement in pathology is provided by studies that analyzed the effect of knocking out the insulin receptor specifically in neurons. The brains of neuronal insulin receptor knockout (NIRKO) mice do not display alterations in development and structure, nor show increased apoptosis in vivo (Schubert M., 2004). However, they do display a large increase in the phosphorylation of tau. Tau is a microtubule–associated protein that promotes tubulin polymerization and stabilizes microtubles. Hyperphosphorylated tau is a major component of neurofibrillary lesions characteristic of Alzheimer's disease and other brain pathologies. NIRKO mice however, do not display deficits in spatial learning, long term memory and brain glucose metabolism. Possibly, the absence of insulin receptor signalling is compensated through other signaling routes.

Clearly insulin in the central nervous system affects cognition in some way and may underlie the cognitive deficits observed in aging, Alzheimer's disease and diabetes. Knowledge concerning the mechanism underlying the actions of insulin, may provide more insight into how insulin affects these processes in the central nervous system and possibly lead to treatment. The apparent pathological effects of hyperinsulinemia form a dilemma when treating diabetes patients as its treatment with insulin is likely to affect cerebral insulin levels and insulin signaling. It is difficult, however, to separate these "direct" effects of alterations in insulin homeostasis on the brain from the consequences of the accompanying alterations in peripheral and central glucose homeostasis, which in themselves can affect the brain. For example, in type 2 diabetics, the individuals who are treated with insulin have the highest risk to develop dementia (Ott A., 1999). It is, however, unknown, if this observation just reflects the severity of diabetes in this subgroup of patients, or whether it is related to insulin treatment itself.

Outline of this thesis

Insulin in the central nervous system affects processes that involve learning and memory, synaptic plasticity and neuronal survival. However, the mechanism by which insulin affects these processes is unclear. In addition, the mechanism underlying the negative influence of hyperinsulinemia in the pathology of disease is also not known. The aim of this thesis was to explore how insulin modulates intracellular signaling routes and how these routes relate to long lasting changes in synaptic plasticity. In chapter 2, the intracellular routes activated in response to insulin in cells of neuronal origin were investigated. In addition to this, the effect of insulin on intracellular crosstalk between kinases was studied. Since insulin has been described to have both beneficial and negative effects related to the period of insulin administration (see above), short- and

long-term insulin treatment and its effects on the activation of signaling routes were investigated.
Since insulin has been shown to have an effect on synaptic plasticity, the effect of insulin on synaptic plasticity in the hippocampus was investigated (chapter 3). Additionally, a signal transduction route which functions as a mediator of insulin signaling we described in chapter 2 was studied. In chapter 4, a review of the literature on FoxO proteins and their regulation by insulin and insulin like factors is provided. The upstream signaling components which regulate FoxO proteins and the transcriptional mechanism regulating FoxO target genes and function are described. Finally, the in vivo relevance of FoxO factors in regulating metabolism and homeostasis is discussed. In chapter 5, the identification of FoxO6 from brain tissue, a novel member of the FoxO subgroup of forkhead transcription factors which serves as a transcriptional end-point of insulin signaling is described. In chapter 6, the intra-molecular mechanisms underlying FoxO6 function are described to gain more insight into how insulin or other growth factors influence the activity of FoxO6. In chapter 7, the results obtained are summarized and their relevance for insulin signaling, neuronal metabolism and survival is discussed.

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Chapter 2

Insulin inhibits extracellular regulated kinase 1/2 phosphorylation in a phosphatidylinositol 3-kinase-dependent manner

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J Neurochem. 2003 Jul;86(1):86-91

Insulin inhibits ERK1/2 phosphorylation in a PI3-Kinase dependent manner in Neuro2a cells

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Insulin signaling is well studied in peripheral tissue, but remains relatively understudied in neuronal tissue. To gather more insight about neuronal insulin signaling we looked at protein kinase B (PKB) and extracellular regulated kinase 1 and 2 (ERK1/2) regulation in serum deprived neuro2a cells. Insulin dose-dependently phosphorylated PKB but reduced phosphorylation of ERK1/2. Both processes were PI3 Kinase (PI3K) dependent. Interestingly blockade of PI3K in combination with insulin induced phosphorylation of ERK1/2. The phosphorylation of ERK1/2 was mediated through the highly conserved Ras-Raf-MEK-ERK1/2 pathway as its phosphorylation could be blocked with a specific inhibitor of mitogen-activated protein/ERK kinase (MEK). Prolonged exposure to high concentrations of insulin resulted in a desensitised PI3K-PKB route. The insulin iduced inhibition of ERK1/2 phosphorylation was also diminished, when the PI3K-PKB route was desensitised. Blockade of PI3K in combination with insulin, however, still resulted in an unaltered MEK dependent phosphorylation of ERK1/2. We conlude that PI3K is an important integrator of insulin signaling in neuro2a cells as it regulates activation of PKB and inhibition of ERK1/2 and is sensitive to the duration of the insulin stimulus.

Insulin and Insulin like growth factors (IGFs) have been studied in peripheral tissue for their involvement in glucose metabolism. Besides explicit functions in the periphery IGFs have also been implicated in regulation of brain metabolism and its development. In contrary to IGFs Insulin's role in the central nervous system remains relatively understudied. Insulin was long considered to be incapable of crossing the blood brain barrier but insulin and its receptor are now known to be present in the brain. Although glucose uptake by the brain is considered to be mainly insulin-insensitive (1) insulin does affect cerebral glucose utilisation to some extent (2;3) analogous to its role in the periphery. In addition, brain insulin does seem to play a role in the regulation of food intake and body weight (4) and it may act as a "neuromodulator", influencing the release and re-uptake of neurotransmitters (5) and probably also learning and memory (2;6).

The intracellular protein kinase cascades involved in peripheral and neuronal insulin signaling appear to share several basic signal transduction components. In muscle, adipose tissue and neurons insulin associated responses appear to be mediated by

intracellular kinases such as protein kinase B (PKB) also known as Akt and extracellular regulated kinase 1 and 2 (ERK1/2) (7)
Activation of the insulin receptor leads to tyrosine phosphorylation of its intracellular β-subunits, resulting in the specific recruitment of docking proteins such as Src homology 2 containing protein (SHC) and the insulin receptor substrate (IRS) family (8) Interactions between these docking proteins result in the regulation of many signal transduction components. Insulin receptor SHC signaling results in activation of ERK1/2 through the subsequent activation of Ras-Raf and MEK. ERK1/2 is serine/threonine kinase, that in neurons, has been associated with survival and learning and memory (9). The other branch of the insulin signalling cascade, the insulin receptor-IRS, regulates activation of the PI3K-PKB route. PKB is a serine/threonine kinase that influences a wide spectrum of cellular signaling events including survival, regulation of glycogen metabolism and protein synthesis (10). Together the Insulin-SHC-Ras-Raf-MEK-ERK1/2- and the IRS--PI3K-PKB route form the two major branches of intracellular insulin signaling (8).
We focused on the regulation of both PKB and ERK1/2, to gain more insight in neuronal insulin signal transduction. We studied the dynamics between these two routes with the use of specifc inhibitors. Using serum deprived Neuro2a cells we show that insulin results in a PI3K dependent phosphorylation of PKB whereas insulin decreases basal levels of ERK1/2 phosphorylation. In addition to the regulation of PKB and ERK1/2 we show that the PI3K-PKB route is sensitive to the duration of the insulin stimulus whereas the Ras-Raf-MEK-ERK1/2 route is not. These findings implicate PI3K as a main regulator of neuronal signal transduction through its ability to regulate both the PKB and ERK1/2 route.

Materials and Methods

Cells and cell-culture. Neuroblastoma Neuro2a cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 units/ml penicillin, 100 units units/ml streptomycin plus 2mM L-glutamine in a humidified atmosphere and 5% CO_2. For each experiment cells were seeded at high confluence in FCS containing growth medium and grown for an additional 24h. After 24h FCS containing growth medium was removed and replaced with serum-free medium and grown for an additional 24h. 1h before experiments medium was removed to remove dead cells and replaced with fresh serum-free medium. Specific inhibitors of MEK: PD98059 (50μM) and PI3K: LY294002 (50μM) were pre-incubated 1h before experiments to ensure complete saturation of the cells with the inhibitor. Subsequently cell medium was replaced with serum-free growth medium containing freshly prepared insulin and in experiments using inhibitors with specific inhibitors to ensure inhibitors are not washed out. Cells were incubated for the indicated amount of time before further processing. In the experiments concerning an overnight incubation of insulin cells were incubated for 24h with 100nM of insulin in serum-free growth medium. Before secondary insulin stimulus, insulin containing growth medium was replaced with serum-free medium and incubated for an additional hour. During this hour inhibitors were also added. After 1h cell medium was removed and replaced with medium containing insulin and/or specific inhibitors.

Western blotting. After incubation of the cells with insulin and/or specific inhibitors medium was removed and cells were rinsed twice with ice-cold phosphate buffered saline (PBS). Cells were then lysed with ice-cold lysis buffer containing 50mM TRIS, 1mM EDTA, 1mM EGTA, 0.5% Triton X-100, 1mM phenylmethylsulfonyl fluoride, 100mM sodium fluoride and 1mM sodium vanadate on ice. Insoluble material was removed from the sample by centrifugation at 12,000 g for 1min. Total protein in the supernatant was determined by the BCA method (Pierce). Concentrated SDS sample buffer containing 66mM Tris/HCL pH 6.8, 3% (w/v) SDS, 5% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 2% (v/v) β-mercapthoethanol was added to the samples before samples were heated for 5min at 100°C. Protein samples were corrected for protein concentration using diluted SDS sample buffer. Equal amounts of lysate protein (15-20μg) were separated on 11% SDS gel electrophoresis. After electrophoresis protein was transferred to polyvinylidene difluoride membranes (Amersham) using a semi-dry Biorad Blotting apparatus, according to manufacturer's instructions. Protein transfer and blotting efficiency was checked with coomassie-stain (50% methanol, 10% acetic acid and 0.1% Coomassie brilliant blue R 250). After staining with coomassie blots were de-stained and washed with PBS. Blots were either used directly for detection or stored at 4°C.

Protein detection

Blots were blocked overnight with 5% (w/v) BSA, 0.1% Tween-20 in TBS at 4°C for ERK1/2 or phospho ERK1/2 detection. Blots were blocked in 4% non-fat milk powder, 0.05% Tween-20 in PBS for 1h for PKB, phospho PKB (both Thr308 and Ser473)and phospho GSK3beta (Ser9) detection. After blocking blots were incubated with primary antibodies overnight at 4°C in a heat-sealable plastic bag. For ERK1/2 or phospho ERK1/2 detection polyclonal antibodies (New England Biolabs) were diluted 1/1000 in 5% (w/v) BSA, 0.1 % (v/v) Tween-20 in TBS. For PKB, phospho PKB (Ser 473), phospho PKB (Thr 308) and phospho GSK3beta detection, polyclonal antibodies (New England Biolabs) were diluted in PBS containing 3% (w/v) non-fat milk-powder and 0.05% (v/v) Tween-20. After incubation with the primary antibodies blots were washed extensively with wash-buffer for 40min (TBS containing 0.1% (v/v) Tween-20 for ERK1/2 and phospho ERK1/2 or PBS containing 0.5% (w/v) non-fat milkpowder and 0.05% (v/v) Tween-20. After washing blots were incubated with horseradish peroxidase coupled goat-anti-rabbit GARPO 1/2000 (Sigma) diluted in the antibody specific wash-buffer for 1h at RT. Blots were again extensively washed with wash-buffer for 40min before detection. Immunoreactivity was visualised using the enhanced chemiluminescence detection kit (ECL, Boehringer Mannheim) and hyperfilm (Amersham). Each experiment was at least performed in triplicate. Results shown are representative examples of experiments performed. Statistical analysis was performed on densitometric values with a student's two-tailed *t* test.

Results

To investigate the signal transduction routes activated by insulin we treated neuro2a cells with insulin and assayed the phosphorylation of PKB and ERK1/2 using phospho-specific antibodies.

Immuno-detection with a phospho-specific antibody shows that baseline levels of phosphorylated PKB were undetectable whereas insulin treatment resulted in a dose-dependent increase in the phosphorylation of PKB at both Thr308 and Ser473 (Fig1). No time dependent effects could be observed as 15,30 and 60 min of insulin treatment all resulted in comparable PKB phosphorylation. in the following experiments we detected PKB using the phospho PKB Ser473 antibody since both residues on PKB were dose-dependently phosphorylated by insulin. Phosphorylation on both Thr308 and Ser473 leads to PKB kinase activity (11) which is confirmed by the insulin induced increase in GSK3beta phosphorylation, a PKB target (11) (Fig1).

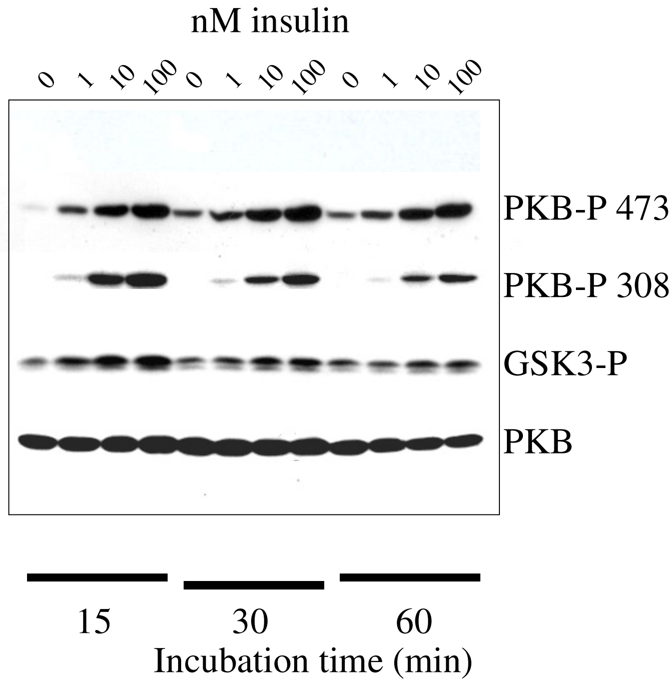

Fig1 **Insulin induces phosphorylation of PKB** Western blot showing dose dependent effects of insulin on the phosphorylation of PKB, on both its phosphorylation sites, and phosphorylation of GSK3, a PKB target. Total PKB levels are shown as a loading control. Cells were incubated with 0, 1, 10, and 100nM of insulin for 15min, 30min or 60min.

Blockade of PI3K with LY294002 completely inhibited the increase in insulin induced phosphorylation of PKB (Fig2). Blockade of MEK with PD98059 had no effect on insulin induced PKB activation. To test the insulin response of the IRS-Raf-Ras-MEK-ERK1/2 route we took the same approach as for PKB. Detection with a phospho specific ERK1/2 antibody shows a high basal phosphorylation of ERK1/2 (Fig3). Insulin stimulation resulted in an unexpected decrease in phosphorylation of ERK1/2 (Fig3).This decrease was, as it was for PKB, dose- and not time dependent. Because the increase in PKB phosphorylation matched the decrease in ERK1/2 phosphorylation and to examine a possible interaction we tested the effect of PI3K inhibition on this route. Stimulation with 100nM of insulin in combination with PI3K blockade resulted in an increase in ERK1/2 phosphorylation (Fig4). Both basal and stimulated phosphorylation of ERK1/2 could be blocked by inhibiting the upstream kinase of ERK1/2; MEK (Fig4). Detection with antibodies directed at total PKB and ERK1/2 revealed that the observed insulin induced phosphorylation effects were phosphorylation specific and not due to alterations in protein content.

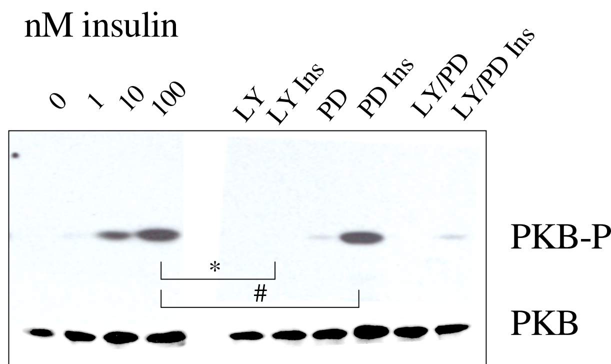

Fig2 **PKB activation is PI3K dependent**. PI3K inhibition but not MEK inhibition (#) blocks insulin induced PKB phosphorylation (* $p < 0.05$). Cells were serum deprived for 24h before treatment with insulin for 30min. Left: Western blot showing dose-response effects of 0, 1, 10 and 100nM insulin on PKB phosphorylation. Right: Western blot showing effects of specific inhibitors on insulin (100nM) induced PKB phosphorylation. Total PKB levels are shown as a loading control.
Insulin = Ins. LY = LY294002, PD = PD98059

Because short stimulations with high insulin concentrations had such a dramatic effect on PI3K-PKB and Ras-Raf-MEK-ERK signaling, we examined the effect of a prolonged exposure to high insulin. Pre-treatment of 100nM of insulin led to a decrease in the PKB response to a novel insulin stimulus at every concentration tested (Fig5). The decrease in PKB phosphorylation was observed as early as 4h after incubation with high insulin (data not shown). Maximal reductions in PKB phosphorylation were obtained when high insulin was incubated for 12h to 24h.

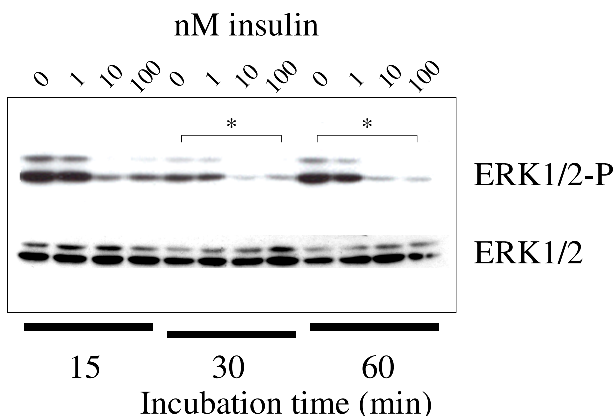

Fig3 **Insulin inhibits ERK1/2 phosphorylation**. Western blot showing dose dependent effects of insulin on the phosphorylation of ERK1/2. Cells were incubated with 0, 1, 10, and 100nM of insulin for 15min, 30min or 60min. ERK1/2 phosphorylation was detected with a phospho-specific anti ERK1/2 antibody. The phospho specific ERK1/2 antibody recognises both ERK isoforms ERK1(upper band) and ERK2 (lower band). Total levels of ERK1/2 are shown as a loading control. 100nM insulin significantly inhibited ERK1/2 phosphorylation at timepoints 30 and 60 (* $p < 0.05$).

Discussion

Insulin has the ability to affect many intracellular signal-transduction components (7). The two major signaling branches activated by insulin in peripheral cells are the PI3K-PKB (7) and Ras-Raf-MEK-ERK1/2 (7) route. Both are of major importance for general cell metabolism. We investigated neuronal insulin signal transduction by focusing on the regulation of PKB and ERK1/2.

We find that serum deprived Neuro2a cells had no basal PKB phosphorylation whereas insulin treatment resulted in a dose- and not time dependent increase in the phosphorylation of PKB on both its phosphorylation sites (Fig1). PKB phosphorylation

matched the phosphorylation of GSK3beta, a PKB substrate. The observed PKB phosphorylation and insulin induced GSK3beta phosphorylation make it highly likely that PKB itself is indeed activated under these conditions as is described by others (11).

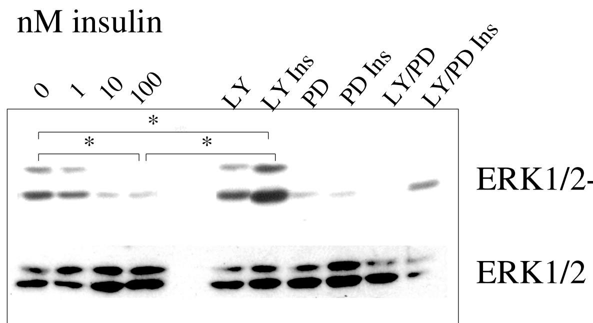

Fig4 **ERK1/2 inhibition is PI3K dependent**. Cells were serum deprived for 24h before treatment with insulin for 30min. Left: Western blot showing dose-response effects of 0, 1, 10 and 100nM insulin on ERK1/2 phosphorylation. Right: Western blot showing effects of specific inhibitors on insulin (100nM) induced ERK1/2 phosphorylation. LY294002 leads to an increase in ERK1/2 phosphorylation, compared to controls, when treated in combination with insulin (* $p < 0.05$). Total ERK1/2 levels are shown as a loading control.
Insulin = Ins. LY = LY294002, PD = PD98059.

The increase in PKB phosphorylation could be blocked with LY294002 (Fig2) implicating PI3K as the PKB upstream kinase. PI3K-PKB activation is in compliance with studies performed by others (11;12). Surprisingly serum deprived neuro2a cells show high levels of basal ERK1/2 phosphorylation (Fig3). ERK1/2 phosphorylation is clearly mediated by the Ras-Raf-MEK route as basal levels could be reduced by MEK inhibition (Fig4).

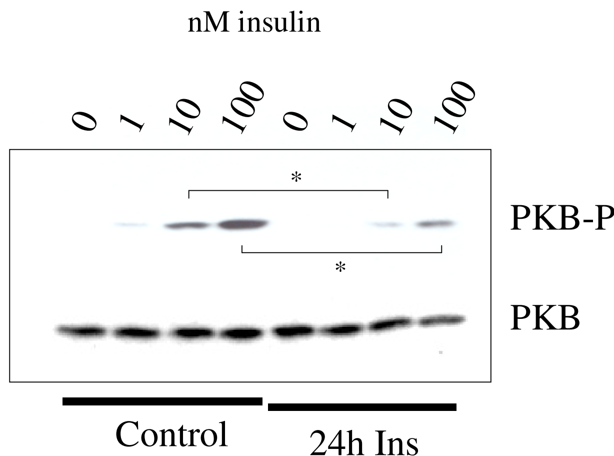

Fig5 **Prolonged insulin treatment leads to insulin resistance**. Western blot showing effects of 100nM 24h pre-treatment of insulin to a novel insulin stimulus. Figure shows treatment of control cells (left 4 lanes) with 0, 1, 10, 100nM of insulin and insulin pre-treated cells (right 4 lanes) with 0, 1, 10, 100nM of insulin for 30min. 24h of insulin treatment results in insulin resistance (* $p < 0.05$). Total PKB levels are shown as a loading control

The high levels of ERK1/2 phosphorylation in unstimulated neuro2a cells may reflect a neuro specific characteristic as usually ERK1/2 is not phosphorylated after long periods of serum deprivation as shown in cells of muscular and adipose origin (13;14). Interestingly insulin stimulation resulted in a dose-dependent reduction of ERK1/2 phosphorylation (Fig3), which is the exact opposite as usually described in literature (7;8;12). We could effectively block the insulin induced reduction of ERK1/2 phosphorylation with an inhibitor of PI3K (Fig4). In addition to this insulin application in combination with PI3K inhibition even resulted in a clear increase in ERK1/2

phosphorylation which suggests that the Ras-Raf-MEK-ERK pathway is normally activated by insulin as is the PI3K-PKB route. It is clear that these presumed separate routes must actually interact.

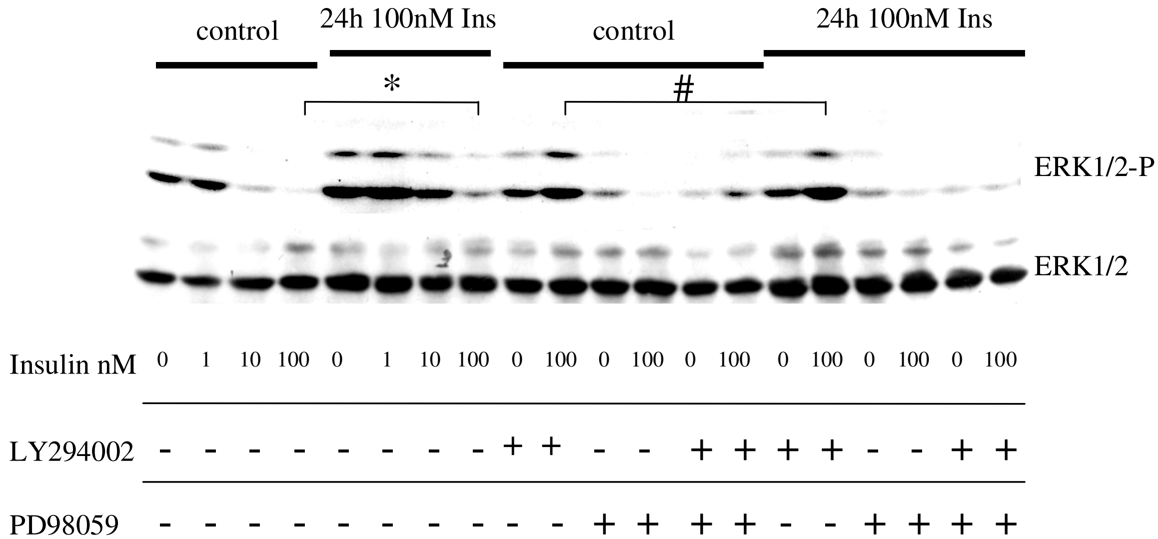

Fig6 **Insulin resistance relieves insulin induced ERK1/2 inhibition**. 24h of insulin treatment results in an impaired insulin induced inhibition of ERK1/2 phosphorylation (* $p < 0.05$). Insulin treatment in combination with PI3K inhibition still induces normal ERK1/2 phosphorylation after overnight high insulin treatment (#).
Insulin = Ins. LY = LY294002, PD = PD98059.

Two possibilities exist for the insulin induced inhibition of ERK1/2, the first being the inhibition of a ERK1/2 kinase the second being induction of a ERK1/2 phosphatase. The latter has been studied in smooth muscle cells (13). In vascular smooth muscle cells, insulin stimulation results in the rapid induction of mitogen-activated protein kinase phosphatase-1 (MKP-1) within the same timeframe as in our system. MKP-1 is a phosphatase responsible for the inactivation of ERK1/2. Inhibition of PI3K signaling by wortmannin (a PI3K inhibitor) abolishes the MKP-1 induction by insulin. Although MKP-1 is described as an deactivator of ERK1/2 insulin still activates ERK1/2 above baseline in these studies (13), which is a major discrepancy to our study.
The option that an ERK1/2 kinase is inhibited has been examined by Rommel et al. It has been shown that the Raf-MEK-ERK pathway can be inhibited by PKB in differentiated myotubes but not in their undifferentiated myoblast precursors (15). The authors suggest that regulation of a Raf-Akt interaction (15;16), underlying the ERK1/2 inhibition, might be mediated by stage-specific modification of these proteins or by stage-specific accessory proteins (Fig7). This regulation might be intact in cells of neuronal origin also.

Because short stimulations of high insulin concentration had such dramatic effects on PI3K-PKB and Ras-Raf-MEK-ERK1/2 signaling we tested the effect of high-prolonged concentrations of insulin. When stimulated with a secondary insulin stimulus PKB phosphorylation is less pronounced in cells treated with high overnight insulin as

compared to controls (Fig5). The reduction in ERK1/2 phosphorylation in response to insulin is also diminished (Fig6), which is in compliance with a model whereby the PI3K-PKB route actively inhibits the Ras-Raf-MEK-ERK1/2 route (Fig7). Inhibition of the PI3K route after overnight high insulin treatment still results in a normal insulin induced increase in ERK1/2 phosphorylation (Fig6) showing that this route is unaffected by the treatment. The reduction in insulin induced PKB phosphorylation and diminished reduction in ERK1/2 phosphorylation seem to underlie a desensitisation effect in response to high concentrations of insulin. Since the desensitisation did not affect the insulin induced phosphorylation of ERK1/2 when PI3K is inhibited, it appears that the observed desensitisation is PI3K-PKB route specific. Many examples exist for insulin induced desensitisation ranging from increased proteolysis of the IRS (17;18) to negative feedback by downstream insulin-IRS induced kinases (19-21) Although it is unclear why our model desensitises to high concentration of insulin it is clear that only the PI3K route is affected and not the Ras-Raf-MEK-ERK1/2 pathway.

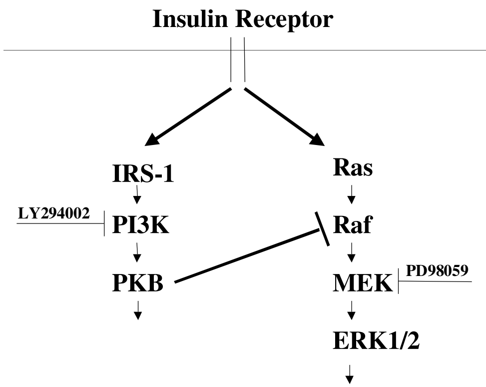

Fig7 **Hypothetical scheme of PKB ERK1/2 crosstalk**. Possible insulin signaling cascade based on experimental results and literature showing PKB mediated inhibition of Raf. Insulin receptor activates both PI3K and Ras routes but PKB inhibits Raf and thereby causes inhibition of ERK1/2 phosphorylation. Blockade of PI3K with LY294002 relieves inhibition of Raf by PKB and restores insulin induced phosphorylation of ERK1/2.

In summary, our study shows a regulation of neuronal insulin signaling that differs from other tissues . We show that the concentration and duration of the insulin stimulus regulates interactions of intracellular signaling routes. We show that the Ras-Raf-MEK-ERK1/2 is sensitive to and is inhibited by the PI3K-PKB route (Fig7). Further we have shown that high concentrations of insulin lead to a desensitisation of the PI3K-PKB route and not the Ras-Raf-MEK-ERK1/2 route, which could potentially have profound effects on downstream ERK1/2 transcription factors.
In vivo studies will have to clarify whether neuronal in-vivo insulin signaling has the characteristics we observed in our in-vitro neuronal model. It would be interesting to see if PI3K has a comparable role as insulin sensor and integrator in the brain. In this scenario the regulation of ERK1/2 by insulin and PI3K would of course be a very important aspect of insulin signaling.

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Chapter
3

Insulin modulates hippocampal activity-dependent synaptic plasticity in an N-methyl-D-aspartate (NMDA) receptor and phosphatidyl-inositol-3-kinase (PI3K) dependent manner

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submitted

Insulin modulates hippocampal activity-dependent synaptic plasticity in an N-methyl-D-aspartate (NMDA) receptor and phosphatidyl-inositol-3-kinase (PI3K) dependent manner

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Insulin and its receptor are both present in the central nervous system and have been implicated in neuronal survival and multiple forms of synaptic plasticity. Here we show that insulin application to hippocampal slices results in activation of PI3K and PKB, and results in an induction of long-term depression (LTD) in hippocampal CA1 neurons if combined with low frequency synaptic stimulation (0.033 Hz) and blockade of GABAergic transmission. The LTD induced in the presence of insulin was inhibited by APV, a specific NMDA receptor antagonist, or facilitated by lowering the extracellular Mg^{2+} concentration to relieve the NMDA receptor of its Mg^{2+} block. Inhibition of PI3K signaling or discontinuing synaptic stimulation prevented this LTD. Evaluation of the frequency-response curve of synaptic plasticity, by determining the changes induced by 0.033-, 1-, 10-, 50-, and 100 Hz revealed that insulin shifts the frequency response curve of synaptic plasticity to the left. In the presence of insulin LTD was induced at 0.033 Hz and LTP at 10 Hz, whereas in the absence of insulin 1 Hz induced LTD and 100 Hz induced LTP.

These results show that that insulin modulates the frequency-response curve of activity-dependent synaptic plasticity, which requires activation of NMDA receptors and the PI3K pathway. This effect of insulin on hippocampal synaptic plasticity suggests that insulin functions as a neuromodulator of synaptic plasticity.

Long-lasting changes in synaptic strength underlie experience-dependent plasticity, which has been suggested to form the basis of learning and memory (1). Two opposite

forms of activity-dependent synaptic modifications have been identified: long-term potentiation (LTP) and depression (LTD). In many brain areas including the hippocampus and neocortex, the direction and the degree of the synaptic change are a function of conditioning frequency, the level of postsynaptic depolarization and the change in postsynaptic Ca^{2+} level. LTD is obtained following low frequency stimulation (1 Hz), low levels of postsynaptic depolarization and a small rise in the intracellular Ca^{2+} level, whereas LTP is produced by high frequency stimulation (100 Hz), a stronger depolarization and a large rise in the intracellular Ca^{2+} level (2)(3)(4)(5). The best characterized forms of LTD and LTP require Ca^{2+} influx through the NMDA receptor (1). This Ca^{2+} influx functions as a second messenger that modulates downstream signaling cascades involved in the regulation of synaptic strength. These cascades include Calmodulin, CAMKII, Calcineurin, PP1, PP2A, PP2B, PKC, ERK1/2 and PI3K (6)(7).

Insulin has long been considered to be a peripheral hormone incapable of crossing the blood–brain barrier. However, the presence of insulin and its receptor have now been established in the brain (8)(9)(10)(11)(12). Although widely distributed, the insulin receptor is concentrated in specific brain regions including the dendritic fields of hippocampal neurons and the olfactory bulbs and the adrenergic terminals in the hypothalamus (13), whereas insulin itself is particularly abundant in the hypothalamus and olfactory bulb (14). Brain insulin plays a role in the regulation of food intake and body weight (15)(16), and it may act as a neuromodulator, influencing the release and reuptake of neurotransmitters (17), neuronal survival (18)(19) and probably also learning and memory (13,20). Insulin binding to the insulin receptor leads to intracellular recruitment of insulin receptor substrates (IRS) and activation of phosphoinositide-3-kinase (PI3K) signaling (21)(22)(23). PI3K has many downstream targets, which include survival promoting kinases such as protein kinase B (PKB) and serum and glucocorticoid-regulated kinase (SGK) (24). Besides PI3K signaling direct recruitment of the adapter protein SHC to the insulin receptor leads to activation of the extracellular regulated kinase pathway (ERK1/2) (21). Insulin induced activation of ERK1/2 has been suggested to underlie changes in synaptic plasticity and hippocampal dependent learning and memory (20)(25)(26)(27). A role for PI3K in synaptic plasticity is also emerging as PI3K signaling has been implicated in the induction and maintenance of LTP (28)(29) and specific forms of LTD. DHPG application, which selectively activates type I mGluR receptors, results in a phosphorylation of phosphoinositide dependent kinase 1 (PDK-1), PKB, and mammalian target of rapamycin (mTOR), and induces mGluR dependent LTD. Specific kinase inhibitors of PI3K or mTOR prevent this DHPG-induced LTD (30). mTOR is suggested to regulate immediate protein synthesis that is required for DHPG-induced LTD. Since mTOR has also been implicated in the regulation of LTP (31), this downstream PI3K substrate is very likely to be involved in multiple forms of synaptic plasticity.

Interestingly, insulin application to hippocampal neurons results in a depression of excitatory synaptic transmission, due to increased AMPA receptor endocytosis (32), however the intracellular signaling cascades involved in this insulin-induced LTD remain unclear. We addressed how insulin mediates LTD induction in the CA1 area of the hippocampus and investigated the underlying mechanism by studying the involvement of

the NMDA receptor activation and PI3K signaling. Our results show that LTD in the presence of insulin depends on low frequency synaptic activity (0.033 Hz) and NMDA receptor activation. In addition, we show that this form of LTD is indeed PI3K-dependent. Stimulation at different frequencies revealed that insulin shifts the frequency response curve of synaptic plasticity in an NMDA-dependent manner. This suggests that insulin or insulin-like compounds function as neuromodulators of activity-dependent synaptic plasticity.

Materials and methods

Preparation of Hippocampal slices. Hippocampal slices (450 µm) were prepared from 2-week-old male Wistar rats (70-80 g) after Isoflurane anesthesia and decapitation, as described previously (33). The slices were allowed to recover for at least 1 h at room temperature in artificial CSF saturated with 95% O_2 and 5% CO_2. The composition of the artificial CSF was (in mM) NaCl, 124; KCl, 3.3; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 2.5; $NaHCO_3$, 20; and glucose, 10.

Immunoblotting. Hippocampal slices were prepared and treated as described above. 6-7 slices were incubated with or without 500 nM insulin in ACSF saturated with 95% O_2/ 5% CO_2 at 30°C. After 15 min slices were homogenized with ice cold lysis buffer containing: 50mM TRIS, 1mM EDTA, 1mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100mM sodium fluoride and 1mM sodium vanadate on ice. After homogenisation triton X-100 (0.5% (v/v)) was added to the lysates and incubated for 10min on ice. Total protein in the supernatant was determined by the BCA method (Pierce). Concentrated SDS sample buffer containing 66mM Tris/HCL pH 6.8, 3% (w/v) SDS, 5% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 2% (v/v) β-mercapthoethanol was added to the samples before they were heated for 10min at 100°C. Equal amounts of sample protein (15-20µg) were separated by eletrophoresis on 11% SDS polyacrylamide gels. After electrophoresis, protein was transferred to polyvinylidene difluoride membranes (Amersham) using a semi-dry Biorad Blotting apparatus, according to manufacturer’s instructions. Protein transfer and blotting efficiency was checked with coomassie-stain (50% methanol, 10% acetic acid and 0.1% Coomassie brilliant blue R 250). After staining with coomassie blots were de-stained and washed with PBS.

Protein detection. For PKB and phospho-PKB detection blots were blocked in 4% non-fat milk powder, 0.05% Tween-20 in PBS for 1h. Subsequently blots were incubated with primary antibodies overnight at 4°C in a heat-sealable plastic bag. Total PKB and phospho PKB (Ser 473) polyclonal antibodies (New England Biolabs) were diluted in PBS containing 3% (w/v) non-fat milk-powder and 0.05% (v/v) Tween-20. After incubation with primary antibodies blots were washed extensively with wash-buffer for 40min (PBS containing 0.5% (w/v) non-fat milk powder and 0.05% (v/v) Tween-20). Blots were incubated with horseradish peroxidase coupled goat-anti-rabbit (Sigma) diluted in wash-buffer for 1h at RT. After extensive washing immunoreactivity was visualised using the enhanced chemiluminescence detection kit (ECL, Boehringer

Mannheim) and hyperfilm (Amersham). Each experiment was performed in duplo and repeated several times. Results shown are representative examples of experiments performed.

Electrophysiological recordings. Slices were transferred to a submerged recording chamber were they were perfused at a rate of 1-2 ml/min with artificial ACSF saturated with O_2/CO_2 at 30°C. To prevent epileptiform activity during blockade of GABAergic inhibition, a surgical cut was made between CA3 and CA1. For intracellular recording of excitatory postsynaptic potentials (EPSPs), cells were impaled with sharp glass microelectrodes of 60-80 MΩ filled with KAcetate (2 M, pH 7.5) and potentials were recorded using an Axoclamp-2B amplifier (Axon Instruments, Foster City, CA, USA) in bridge mode. The resting membrane potential (RMP) was determined when the membrane potential had stabilized after impalement of the cell (within 3 min). Schaffer collateral-commissural fibers were stimulated using a bipolar stainless steel stimulating electrode (0.1 mm diameter) placed in the stratum radiatum. At the beginning of each recording, a stimulus-response curve was obtained by using five stimulus intensities (I1-I5) ranging from stimulus intensities evoking threshold responses (I1) to intensities eliciting maximal responses (I5). The stimulus intensity that evoked half-maximum EPSPs was used throughout the experiment (test frequency 0.033 Hz). Only cells with stable synaptic responses, bridge-balance, RMP and input resistance were used for analysis. The data were normalized to the averaged value of the initial slope of the EPSP, obtained during the 15-min period prior to the application of the conditioning stimulus.
In all experiments the conditioning stimulation was applied in the presence of 10 μM bicuculline (Tocris Cookson (Bristol, UK) and 500 nM insulin except when stated otherwise. DL-2-amino-5-phosphono-valeric acid (APV) Sigma (St Louis, USA) or LY294002 (New England Biolabs, USA) were added 15 min before the conditioning. To test whether a conditioning stimulation had a significant effect on the slope of the EPSP, a Wilcoxon's matched-pair test was used in which the baseline responses were compared with responses at 30 min after conditioning. To compare the effect of different experimental protocols, we first performed an overall analysis of variance and subsequently a Student's *t*-test for independent samples; all averages are listed as mean ± SEM.

Results

Insulin activates the PI3K-PKB pathway in hippocampal slices. To investigate insulin signaling in the regulation of synaptic plasticity we first assessed whether insulin activated the PI3K pathway. Treatment of coronal hippocampal slices with insulin for 15min resulted in PKB phosphorylation on Thr473. PKB phosphorylation increased more than 2.5 fold as compared to controls (Figure 1). Total PKB levels remained unaltered. Thr473 is phosphorylated by an upstream kinase dependent on PI3K activity. Therefore insulin induced PKB phosphorylation on Thr473 can be considered an indirect measure of PI3K activity. For PKB to achieve full catalytic activity phosphorylation of Thr473 and Ser308 is required. We have previously shown that phosphorylation of PKB at Thr473 by insulin parallels phosphorylation of Ser308 and results in an increase in

catalytic activity (23). Remarkable is the high level of PKB phosphorylation under non-stimulated conditions, which could reflect the role this kinase has in survival and maintenance of cellular integrity.

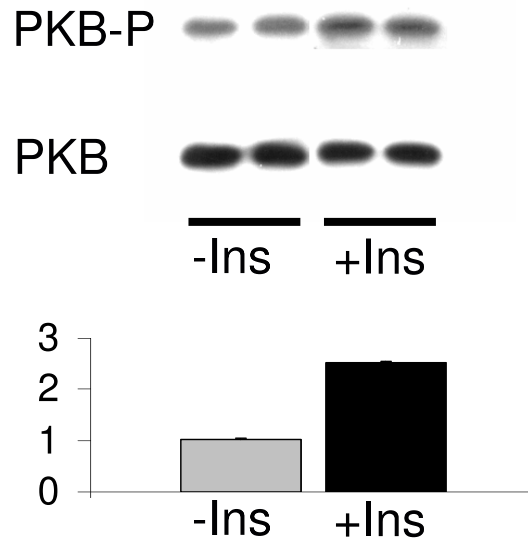

Figure 1. Insulin activates PKB in hippocampal slices. Hippocampal slices were treated for 15min with 500 nM insulin in ACSF and cell lysates were analyzed for PKB phosphorylation using a phospo-specific antibody.
Upper panel: Insulin increased phosphorylation of PKB (PKB-P) compared to controls. Blots were stripped and reprobed with an antibody that recognizes PKB independently of its phosphorylation state as a loading control (lower 4 lanes). The experiment was performed in duplicate.
Lower panel: densitometric quantification of results. Controls (- Ins) were set at 1 and compared relative to insulin-treated conditions (+ Ins). Insulin treatment caused a 2.7 fold increase in PKB phosphorylation.

Insulin application induces LTD in CA1 neurons. In the presence of bicuculline, insulin application resulted in a rapid and robust decrease in excitatory post synaptic potential (EPSP) to 47.6 ± 8.8 % ($p<0.05$), which was maximal after 10 min of insulin application and remained stable for the remainder of the experiment (Figures 2A and 2E). The results obtained are similar to what others have shown (32), and demonstrate the insulin responsiveness of our experimental preparation.
Subsequently, we investigated the requirement of the NMDA receptor in insulin-mediated LTD induction. In the presence of APV, a specific antagonist of the NMDA receptor, insulin did not affect EPSPs: after 30 min of insulin application the slope of the EPSPs were 103.4 ± 8.8 % ($p>0.05$, Figures 2B and 2E). This shows that NMDA receptor blockade blocks the induction of insulin-LTD and suggests that insulin-LTD is NMDA receptor-dependent.
With intact GABAergic inhibition (in the absence of bicuculline), application of 500 nM insulin did not affect the EPSPs (Figures 2A and 2E; 108.7 ± 8.8 %, $p>0.05$, 30 min after insulin was added). Bicuculline facilitates NMDA receptor activation by reducing GABAergic inhibition. Therefore, we tested whether insulin could induce LTD when the extracellular Mg^{2+} concentration was lowered from 1.3 mM to 0.2 mM, which also results in an increased activation of NMDA receptors. Under low extracellular Mg^{2+} concentrations, insulin significantly reduced the EPSP to 76.1 ± 5.1 % ($p<0.05$, Figures

2C and 2E), although the magnitude of the LTD observed under low Mg^{2+} concentration was not as high as with bicuculline.

To address if the LTD in the presence of insulin required evoked release of glutamate, insulin was applied in the absence of synaptic stimulation. Insulin application for 15 min in the absence of electrical stimulation revealed that directly after electrical stimulation resumed the slope of the EPSPs was still at baseline levels (99.7 ± 14.5 %, $p>0.05$, Figures 2D and 2E). Thereafter, during normal synaptic stimulation at 0.033 Hz the EPSPs decreased gradually (26.9 ± 7.5 % after 30 min of insulin, $p<0.05$, Figure 2D). These results show that synaptic stimulation is required for insulin-mediated LTD induction.

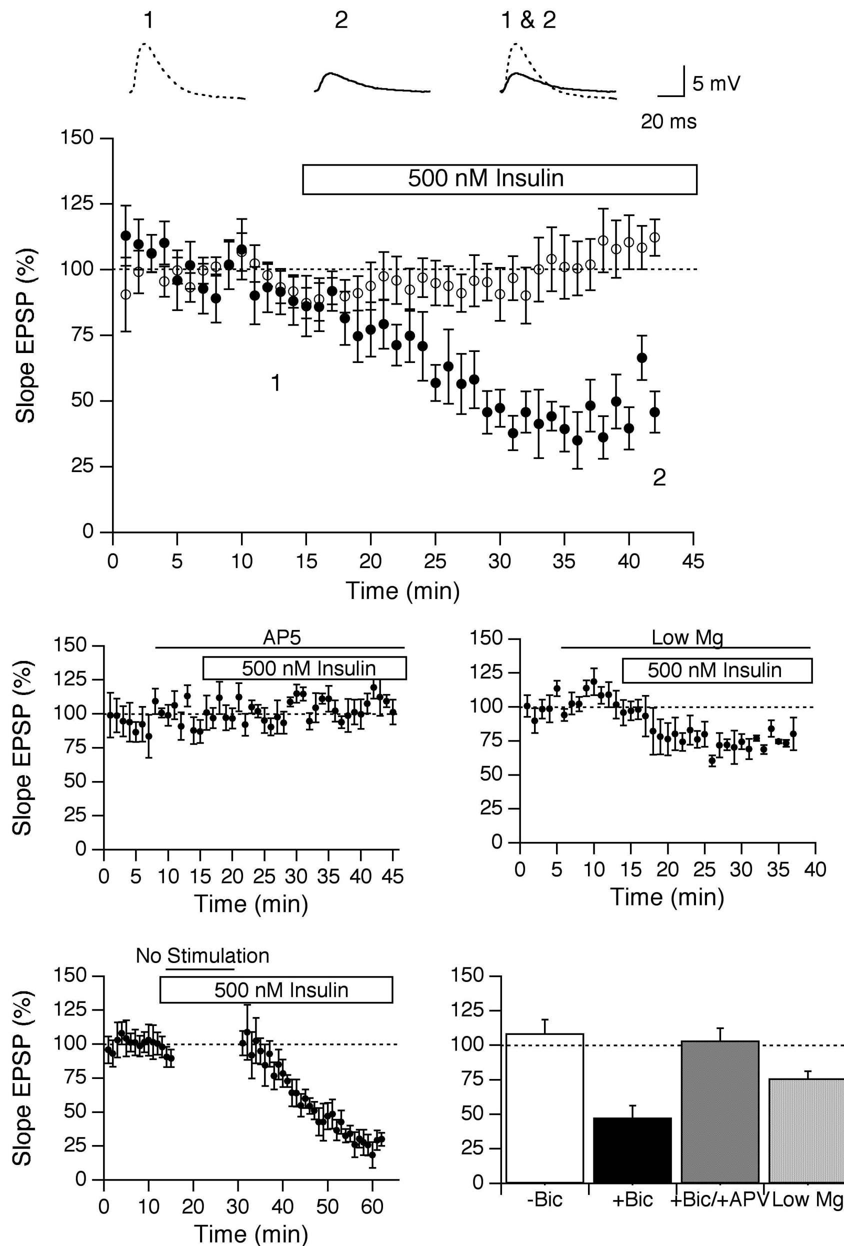

Figure 2 The LTD induced in the presence of insulin is NMDA receptor-dependent

A. EPSPs were recorded in CA1 pyramidal cells after stimulation (0.033 Hz) of Schaffer collateral-commisural fibres. GABAergic inhibition was blocked by the addition of 10 μM bicuculline. Insulin application in the presence of bicuculline (filled circles) resulted in LTD within 15 min and persisted for at least 30 min. When insulin was applied with intact inhibition (no bicuculline present; open circles) no change in the EPSPs was induced. Typical examples of EPSPs, recorded at the indicated time point are shown as insets.
B. To test whether the LTD in the presence of insulin requires activation of NMDA receptor, 500 nM of insulin was applied in the presence of 100 μM APV. In the presence of APV, insulin did not induce any change in the EPSPs.
C. The involvement of NMDA receptors in the LTD induction in the presence of insulin was further investigated by lowering the extracellular Mg^{2+} concentration from 1.3 mM to 0.2 mM. In 0.2 mM Mg^{2+} and 500 nM insulin the slope of the EPSPs reduced significantly.
D. To address whether the LTD induced in the presence of insulin depended on evoked synaptic activation of NMDA receptors, 500 nM insulin was applied in the absence of afferent fibre stimulation. After stimulation was resumed the slope of the EPSPs was not changed, only after stimulation at 0.033 Hz was continued, LTD developed.
E. Bar graph summarizing the results of the experiments shown in panels A through C. The bar graph represents the values of the last 5 minutes in the presence of 500 nM insulin in the absence of bicuculline (open bar), presence of bicuculline (black bar), the combined presence of bicuculline and APV (dark grey bar) and low extracellular Mg^{2+} (light grey bar). * indicates $p<0.05$, compared to baseline.

Insulin mediated LTD is PI3K-dependent. Since PI3K activation has been implicated in insulin signaling and synaptic plasticity, we investigated whether PI3K activation is required for insulin-mediated LTD induction. LY294002 is a cell permeable specific inhibitor of PI3K activity, and has been shown to inhibit insulin-induced PKB activation (23). Pre-treatment of hippocampal slices with LY294002 did not affect baseline EPSPs. In the presence of LY294002 the EPSPs were reduced to 77.4 ± 9.9% ($p<0.05$; Figure 3), which is a significantly smaller reduction than in the absence of LY294002 (47.6 ± 8.8%, $p<0.05$, Figure 3B). The reduced magnitude of the insulin-induced LTD in the presence of the PI3K inhibitor LY294002 provides evidence that PI3K is required for insulin-induced LTD .

Insulin shifts the frequency response curve of synaptic plasticity. To test whether application of 500 nM insulin has an effect on activity-dependent synaptic plasticity, we compared the frequency-response functions for the induction of LTD and LTP, in the absence of GABAergic inhibition, in control conditions and in the presence of insulin. Stimulation at test frequency (0.033 Hz) did not induce any change in control conditions, but it induced LTD in the presence of insulin (see Figure 2A). In control conditions, slices receiving 1 Hz stimulation showed substantial LTD (65.3 ± 3.1% of baseline, n=5, $p\leq0.05$). Stimulation at 10 Hz did not induce any significant change (105.1 ± 6.2% of baseline, n=5, $p>0.05$), whereas 50 and 100 Hz stimulations resulted in LTP (156.4 ± 17.8% and 162.4 ± 21.4% of baseline for 50 and 100 Hz stimulation respectively, n=4, $p<0.05$; Figure 4). The LTD-LTP crossover point of the frequency-response function for induction of LTD and LTP was between 1 and 10 Hz. In the presence of 500 nM insulin, stimulation at 0.033 Hz resulted in LTD (see Figures 2 and 4) and stimulation at 1 Hz did not induce any change (104.4 ± 3.1% of baseline, n=4, $p<0.05$: Figure 4). All the higher frequencies yielded LTP (184.1 ± 6.2%, 174.3 ± 12.6%, 171.3 ± 18.4% of baseline for 10, 50 and 100 Hz stimulation respectively, n=4, $p<0.05$; Figure 4). Therefore, bath

application of 500 nM insulin resulted in a leftward shift of the frequency-response function for the induction of LTD and LTP (Figure 4), the LTD-LTP cross-over point now being around 1 Hz.

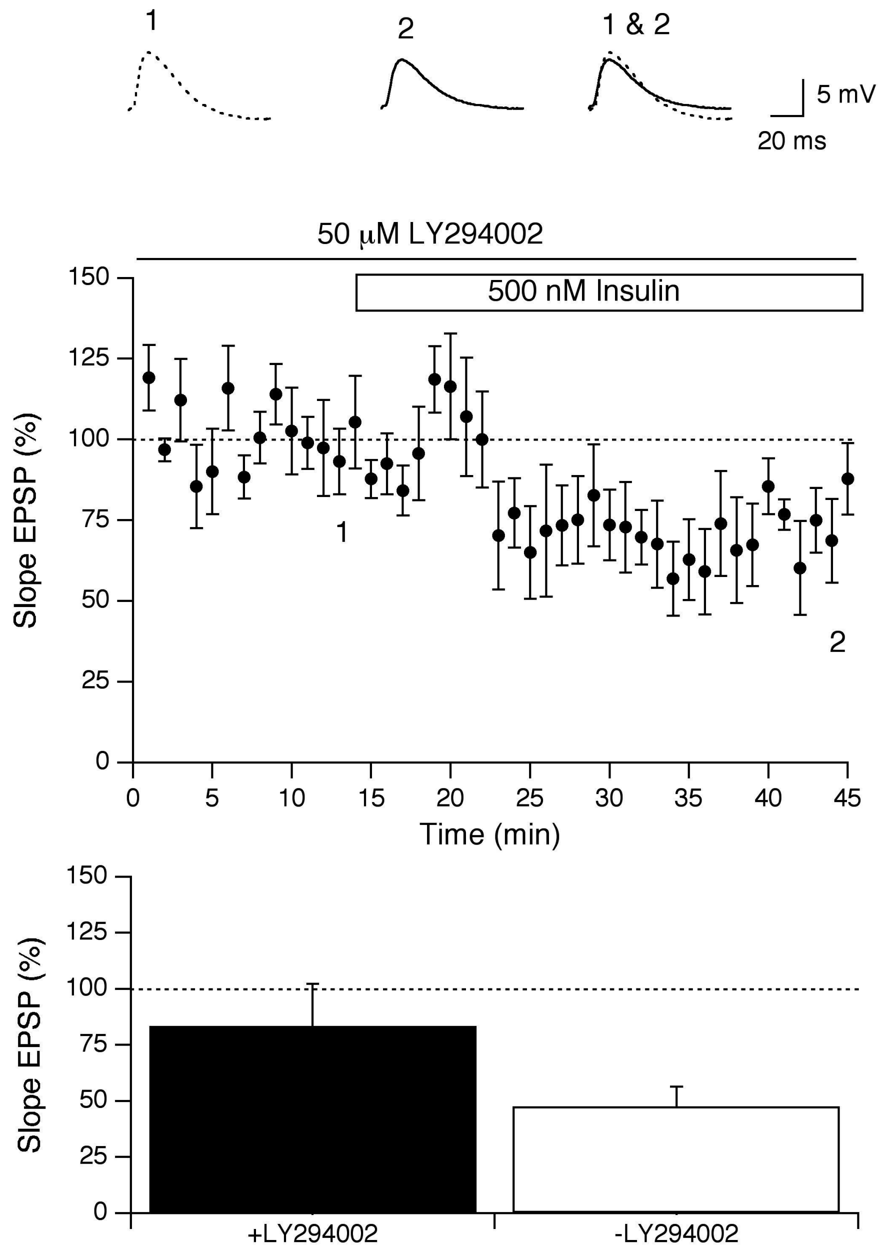

Figure 3 The LTD in the presence of insulin is PI3K-dependent

A. The involvement of the PI3K pathway was assessed by determining the effect of the specific PI3K inhibitor LY294002 (50 μM) on the LTD induced in the presence of 500 nM insulin. In the presence of LY294002 the magnitude of the LTD was strongly reduced. Typical examples of EPSPs, recorded at the indicated time point are shown as insets.
B. Summary bar graph representing the values of the last 5 minutes in the presence of 500 nM insulin. In the combined presence of LY294002 and insulin (filled bar) the slope of the EPSP at

the end of the insulin application is not significantly different anymore from baseline, whereas in the absence of LY294002 (open bar, see Figure 2) LTD was induced. * indicates $p<0.05$, compared to baseline.
Lower panel Comparison of EPSP slopes with and without LY294002 in combination with insulin.LY294002 decreases the insulin induced LTD significantly (black bar) as compared to control (open bar)

To address whether both the LTD and the LTP in the presence of insulin were NMDA receptor-dependent, we determined if APV application prevented the LTD induction as well as the LTP induction. In the presence of APV, stimulation at 0.033 Hz did not induce any change (103.4 ± 8.8, n=4, $p>0.05$, Figures 2 and 4B), whereas the LTP induced at 10 Hz in the presence of insulin was also completely blocked (184.1 ± 6.2% in the absence of APV and 98.8 ± 7.5% in the presence of APV, n=4). The block of LTD and LTP induction in the presence of insulin by APV indicate that both are NMDA receptor-dependent.

Discussion

Insulin application to hippocampal slices results in the induction of LTD when synapses are activated at 0.033 Hz and GABAergic inhibition is blocked ((32)(34) and Figure 2). We investigated the mechanism underlying this form of LTD. Our results show that insulin potently activates PI3K signaling (Figure 1) and mediates LTD induction at the CA1 Schaffer collateral synapses in an activity-, frequency-, NMDA receptor- and PI3K dependent manner (Figures 3 and 4). Furthermore, insulin-mediated LTD induction was part of a NMDA receptor dependent shift in the frequency response curve of activity-dependent synaptic plasticity (Figure 4). Insulin shifts this curve to the left, thereby enabling both LTD and LTP induction at lower frequencies. Therefore our results suggest that insulin or insulin-like compounds may function as neuromodulators that set the threshold for NMDA receptor-dependent LTD and LTP induction in the hippocampus.

The shift in the frequency-response curve is frequency- and NMDA receptor-dependent (Figure 4) and may therefore share its underlying mechanism with insulin mediated-LTD induction. For this reason insight on insulin mediated-LTD induction is relevant for understanding the effect of insulin on synaptic plasticity. Previous studies have shown that an insulin-induced depression of excitatory synaptic transmission is dependent on intracellular Ca^{2+} (32). The mechanism underlying the release or the source of the intracellular Ca^{2+} required for insulin LTD is, however, not known. Here we show that that the NMDA receptor is required for insulin-mediated LTD induction (Figure 2). Possibly the NMDA receptor provides the rise in intracellular Ca^{2+} that is required for insulin-mediated LTD induction. APV, a specific antagonist of the NMDA receptor effectively blocked insulin-mediated LTD induction as did the absence of synaptic stimulation at 0.033Hz. Insulin-mediated LTD induction required blockade of GABAergic transmission with bicuculline or picrotoxin and lowering of the extracellular Mg^{2+} concentration, to unblock the NMDA receptor, abolished the requirement to block GABAergic inhibition. These findings underline the NMDA receptor-dependence of this

form of LTD. In vivo GABAergic inhibition may set the spatio-temporal conditions for synaptic plasticity and hippocampal-dependent learning and memory (35). Others have reported that insulin-mediated LTD induction is not NMDA receptor-dependent and instead depends on L-type voltage-activated Ca2+ channels (VACCs) (34). If indeed only Ca^{2+} influx through VACCs were required for insulin-mediated LTD induction, APV treatment should not block the induction of insulin-mediated LTD. Our finding that APV treatment blocked the insulin mediated shift in the frequency-response curve indicates an involvement of the NMDA receptor in this effect. A combination of NMDA receptor activation and VACCs is unfeasible, since APV treatment fully prevented both the LTD and LTP induction the presence of insulin. Thus, in our hands, the effects of insulin on the frequency-response curve appear to be fully dependent on the NMDA receptor.

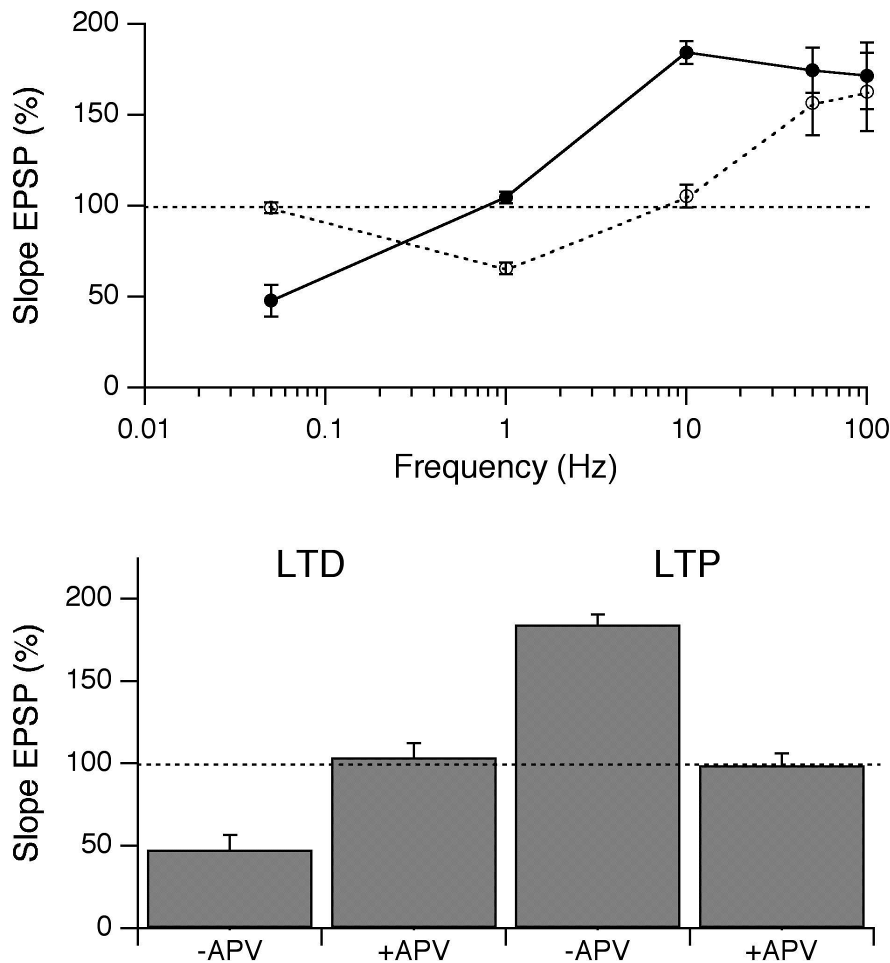

Figure 4 The frequency response curve for synaptic plasticity is shifted to the left in the presence of Insulin

A. The change in the slope of the EPSP at the end of the insulin application is plotted against the frequency (0.033, 1, 10, 50 and 100 Hz). The baseline level (100%) is indicated by the dotted line. In control conditions (open circles), synaptic stimulation at 0.033 Hz had no effect on the slope of the EPSP, at 1 Hz LTD is induced, at 10 Hz the EPSPs are not changed and at 50 or 100 Hz LTP

is induced. In the presence of 500 nM insulin (filled circles), synaptic stimulation at 0.033 induced LTD, stimulation at 1 Hz did not affect the EPSPs and stimulation at 10, 50 and 100 Hz induced LTP. All experiments were performed in the presence of 10 μM bicuculline to block GABAergic inhibition.

B. To address the question whether both the LTD at 0.033 Hz and the LTP at 1 Hz in the presence of insulin are dependent on NMDA receptor activation, experiments were performed in the presence of the NMDA receptor antagonist APV. Both the LTD (see also Figure 2B) and the LTP were prevented in the presence of this NMDA receptor antagonist.

Previous studies have implicated PI3K activity in synaptic plasticity. Here we show that LTD induced by 0.033 Hz stimulation in the presence of insulin is PI3K-dependent as the induction of insulin LTD could be prevented by LY294002, a specific PI3K inhibitor. Interestingly, LTP induction in the dentate gyrus of the rat is inhibited by wortmannin, an irreversible PI3K inhibitor (28) and exerts an inhibitory effect on KCl-stimulated glutamate release and calcium influx (28). Later it was reported that PI3K activity is not needed for the induction of LTP itself but is rather required for LTP maintenance in the hippocampal CA1 region (29). Therefore PI3K activity may be required for the insulin-induced shift in the frequency response curve of synaptic plasticity since it appears to be an important mediator of regular LTD and LTP.
A possible explanation how insulin and PI3K shift the frequency response curve lays in the potentiation of NMDA receptor currents by protein tyrosine kinases (PTKs) (Wang Y.T., 1994). Interestingly pp60c-src, a PTK, is activated by insulin in a PI3K dependent manner (36), potentiates NMDA receptor currents through tyrosine phosphorylation (37) and induces LTP in the CA1 area of the hippocampus (38). Consequently the observed insulin-induced shift in the frequency-response curve of synaptic plasticity (Figure 4) might result from a PI3K-pp60c-src-dependent enhancement of NMDA receptor currents. Remarkably a similar mechanism has been proposed for the effect leptin has on synaptic transmission in the CA1 field of the hippocampus (39).
Another interesting candidate to interact with PI3K signaling is PKC which has been implicated in the DHPG-induced leftward shift in the frequency response curve of synaptic plasticity (40). The PI3K pathway and the PKC pathway have been shown to interact at multiple levels and can influence each others activity. For instance, insulin-induced PI3K activity can induce certain PKC isoforms that provide negative feedback on IRS-1, which decreases subsequent PI3K activity (41). Activated PKC-zeta can directly bind to and inactivate PKB activity (42). Interestingly, a calpain cleaved form of PKC-zeta named PKM has been implicated in both LTP and LTD (43), but whether PKM interacts with PKB is unknown. Possibly insulin and PI3K recruit PKC to mediate changes in synaptic plasticity.

Our results demonstrate that insulin shifts the frequency response curve of synaptic plasticity to the left, thereby facilitating LTD and LTP induction at lower frequencies. NMDA receptor activation and PI3K signaling are presumably central to these effects, acting as important regulators of synaptic plasticity. Many growth factors in the brain in addition to insulin act on the PI3K pathway. These include IGF-I (44), BDNF (45) and NGF (46). Interestingly, these factors have all been implicated in survival and the modulation of synaptic plasticity. IGF-I, insulin's closest relative has also been implicated in hippocampal neurogenesis (47) and learning and memory (34). This

suggests insulin and insulin-like factor have a pro-survival as well as a neuromodulatory function by acting on the PI3K pathway, linking survival directly to synaptic plasticity.

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Chapter 4

The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation

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The Ins and Outs of FoxO Shuttling: Mechanisms of FoxO translocation and transcriptional regulation.

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Forkhead members of the O class (FoxO) are transcription factors that function under the control of insulin/insulin-like signaling. FoxO factors have been associated with a multitude of biological processes including cell-cycle, cell death, DNA repair, metabolism, and protection from oxidative stress. Central to the regulation of FoxO factors is a shuttling system, which confines FoxO factors to either the nucleus or the cytosol. Shuttling of FoxO requires protein phosphorylation within several domains and the association with 14-3-3 proteins and the nuclear transport machinery. Description of the FoxO shuttling mechanism contributes to the understanding of FoxO function in relation to signaling and gene regulation.

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1 Introduction

Initial studies on FoxO factors were performed on fusions of PAX3 [1] or PAX7 [2] to FKHR (forkhead in rhabdomyosarcoma) which results in a transcription factor with altered transcriptional activity [1] implicated in alveolar rhabdomyosarcomas. FKHR has now been renamed to FOXO1, according to the novel nomenclature, and is the first identified member of the FoxO family of transcription factors. Now FoxO proteins have been identified in several different organisms including C. elegans, Zebrafish, Drosophila, mouse, rat and man. In mouse 4 different FoxO members have been identified to date: FoxO1, FoxO3, FoxO4 and FoxO6 [3, 4] (Figure 1). Over recent years it has become evident that FoxO factors are insulin sensitive transcription factors with an array of downstream targets and interacting partners. Central to insulin mediated inhibition of FoxO factors is a shuttling mechanism that regulates FoxO localization to the cytosol, thereby terminating its transcriptional function.
Several intra-molecular domains, including phosphorylation sites, are necessary for FoxO factors to shuttle efficiently from nucleus to cytosol. Interestingly, FoxO6, a recently described FoxO family member, displays unique shuttling dynamics adding information to the mechanism underlying translocation [4]. We will discuss the complex mechanism of intracellular FoxO shuttling in a step by step model, by reviewing the upstream FoxO kinases and chaperones involved in FoxO shuttling. In addition, we summarise FoxO mediated transcriptional regulation and in-vivo function which are both under direct control of the shuttling system.

2 Signaling to FoxO proteins

FoxO factors are regulated by several signal transduction cascades (Figure 2). The main regulator of FoxO function is the PI3K pathway, whereas FoxO function is 'fine-tuned' by the casein kinase 1 and DYRK1A pathway. These kinases regulate the intracellular localization and function of FoxO proteins by phosphorylating FoxO factors within several different intra-molecular domains (Figure 3).

2.1 Phosphorylation of FoxO proteins through the PI3kinase-PKB pathway

Phosphoinositide 3-Kinases (PI3Ks) are heterodimers of a catalytic subunit (110kDa) and a regulatory or adapter subunit [10, 11]. PI3Ks are activated by several proteins, including G-proteins and Tyrosine kinases[11]. PI3K-signaling is implicated in survival, regulation of the cell cycle, cell differentiation and intra-cellular traffic processes [10, 11, 12]. Recruitment of PI3K to membrane receptors relocates PI3K to its lipid substrates where it phosphorylates the 3'-OH group of the inositol ring of phosphatidylinositol to generate the phosphoinositide phosphates PIP2 and PIP3 [11]. These two signaling molecules trigger downstream serine/threonine kinases including 3'-phosphoinositide-dependent kinase-1 (PDK-1) and Protein Kinase B (PKB) [11]. Signaling by PI3K is counter balanced by the tumor suppressor protein phosphatase and tensin homologue deleted on chromosome ten (PTEN). This protein de-phosphorylates PIP2 and PIP3 and prevents the subsequent activation of serine/threonine protein kinases [10, 11]. Recently glutamatergic activity was linked to PI3K signaling. Activated group I metabotrobic

glutamate receptors recruit the scaffolding protein Homer and PI3K enhancer-large (PIKE-L). This complex activates PI3K and prevents neuronal apoptosis in response to stress [13].

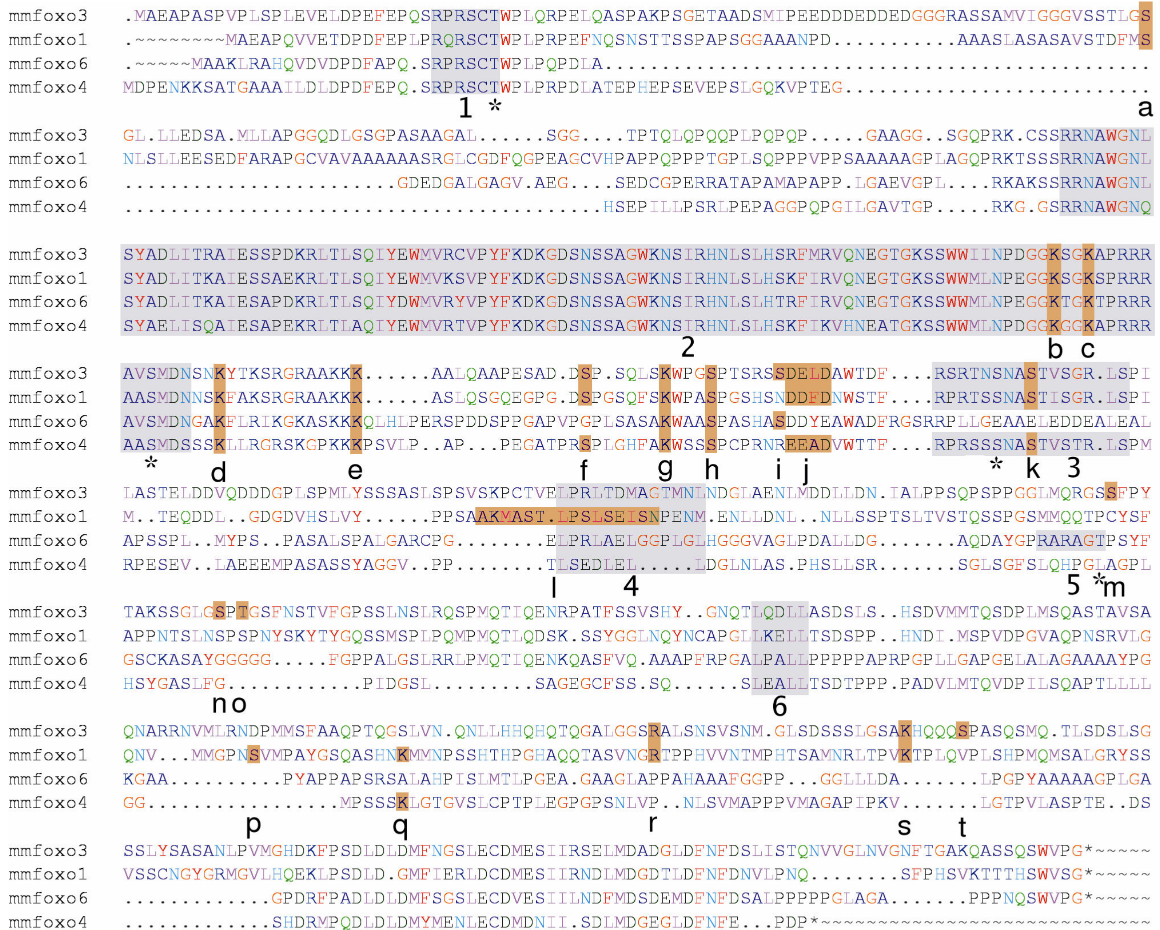

Figure 1: Alignment of all known mouse FoxO proteins. Conserved regions are indicated by boxes and are numbered accordingly. 1: N-terminal PKB motif: involved in 14-3-3 binding. 2: Forkhead domain: mediates interactions with the DNA, containing a PKB motif. Phosphorylation of this site is required for the N-and C-terminal PKB motif to be phosphorylated. This site is also involved in 14-3-3 binding and the regulation of DNA binding. 3: C-Terminal PKB motif followed by two CK1 sites and a DYRK1A site, which regulates the speed of nuclear export. This entire stretch is absent in FoxO6. 4: Nuclear export sequence. 5: Optimal PKB motif in FoxO6 with unknown function. 6: LxxLL motif, possibly involved in the recruitment of nuclear receptors. * (below sequence): PKB-Phosphorylated S/T residues. a, f, h, i, m, n and t: serine residue possibly phosporylated after stress stimuli [5]. o: threonine residue possibly phosporylated after stress stimuli [5]. b, d, e, g and s: Lysine residues possibly acetylated after stress stimuli [5]. b, c and q: Lysine residues acetylated by CBP [6]. k, p and l: Residues possibly involved in regulation of transcriptional activity [7]. j: Caspase-3-like cleavage motif [8]. r :Protease cleavage site [9].

One of the main downstream mediators of PI3K signaling is PKB, also known as Akt. It is a serine/threonine kinase structurally related to protein kinase A (PKA) and protein kinase C (PKC), and belongs to the AGC family of protein Kinases. They share similarity in the catalytic domain and in the mechanism of activation [14]. PKB is extensively studied, partly because it was shown that PKB inactivates proteins of the apoptotic

machinery such as the Bcl-2 family member Bad [15, 16]. There are three widely expressed isoforms of PKB: PKBα, PKBβ and PKBγ. The proteins consist of three functionally distinct regions: an N-terminal pleckstrin homology domain, a catalytic domain and a C-terminal hydrophobic motif [14]. PKB isoforms are recruited to the plasma membrane by PIP2 and PIP3 where PKB is phosphorylated on two specific sites required for full catalytic activity [17, 14]. At least one of these sites is phosphorylated by PDK-1, a major regulator of AGC kinases that is also recruited by phosphoinositides [18]. Activated-PKB detaches from the plasma membrane and translocates to the cytosol and nucleus where it phosphorylates serine or threonine residues within a PKB phosphorylation motif in target proteins [17].

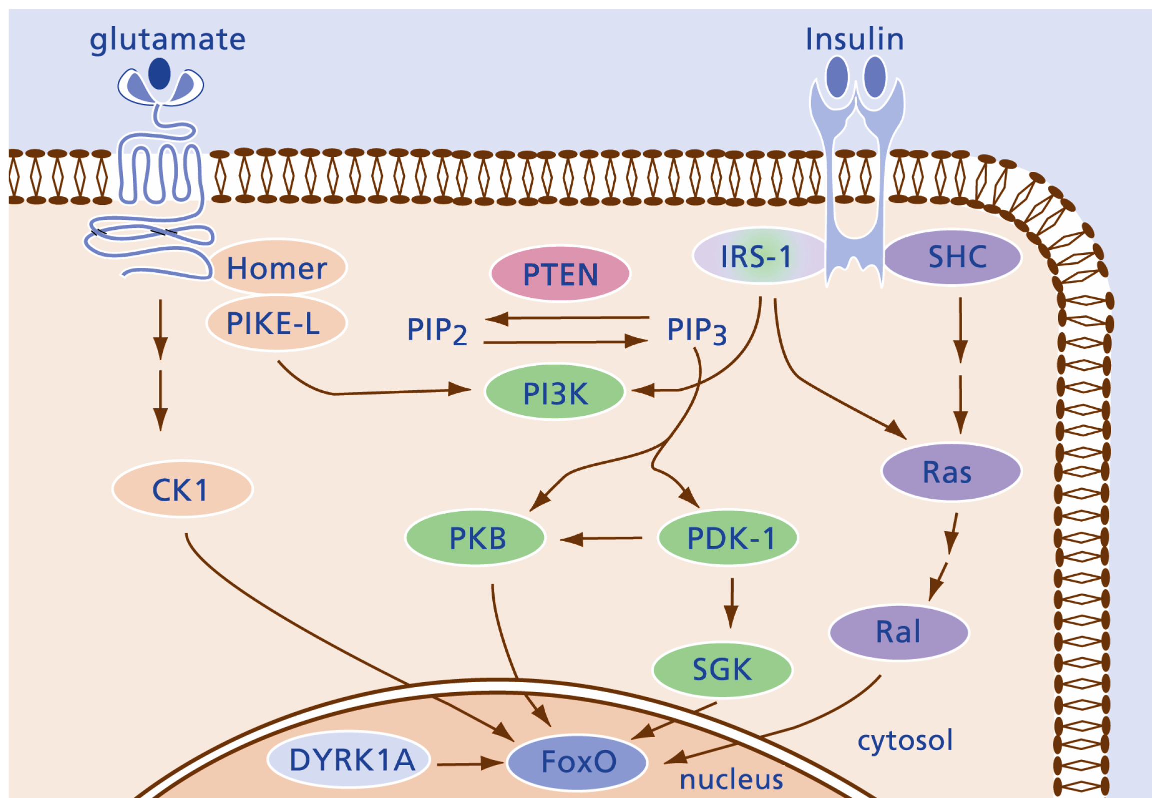

Figure 2: Schematic representation of the events related to the cascade of signaling from extracellular to intracellular, influencing the activity of FoxO proteins. The PI3K pathway forms the central component of FoxO regulation by acting on PKB and SGK. The signaling is fine-tuned by the CK1 and the Ras-Ral pathway, which converse at FoxOs. The regulation of DYRK1A is unknown but it is described to phosphorylate FoxOs, thereby influencing their function.

Murine FoxO1, FoxO3, FoxO4 and FoxO6 contain three highly conserved putative PKB recognition motifs (Figure 3)(consensus sequence: RXRXXS/T [19]) [4]. An N- and C-terminal site and one located in the forkhead domain. The C. elegans FoxO, DAF-16, contains an additional overlapping PKB motif in the forkhead domain. Interestingly, FoxO6 lacks the consensus C-terminal PKB motif. However, an optimal PKB phosphorylation motif is present in the far C-terminus, but it is questionable whether it is an in vivo substrate (data not shown). All FoxO protein have been shown to require the

consensus N-terminal PKB site and the PKB site located in the forkhead domain to translocate from nucleus to cytosol [20]. The two phosphorylated residues are essential components for translocation as they influence the nuclear localisation signal (NLS) function [20] and the association with 14-3-3 proteins (see below) [21]. Besides binding of 14-3-3 the N-terminal PKB site also regulates the association of FoxOs to p300. In the absence of growth factors p300 binds to the N-terminal part of FOXO3. Growth factor addition and phosphorylation of the N-terminal PKB motif disrupts the interaction of p300 with FOXO3 [22], thereby possibly preventing acetylation of FOXO3 and directly influencing FoxO mediated transactivation. An important role is reserved for the C-terminal PKB phosphorylation site. Phosphorylation of this motif accelerates nuclear export [23, 4, 24]. The importance of this C-terminal PKB site is underlined by FoxO6, which lacks the third conserved PKB phosphorylation site. Differences in subcellular localisation are very pronounced comparing FoxO6 to FoxO1 and FoxO3. Under most conditions FoxO6 is located in the nucleus, whereas FoxO1 and FoxO3 have a predominant cytosolic localisation after PKB activation [4]. The domain surrounding the third PKB site is important for the efficiency of translocation and is a target for additional signaling events. The lack of a third PKB site at the proper position in the protein has therefore clear functional consequences [4].

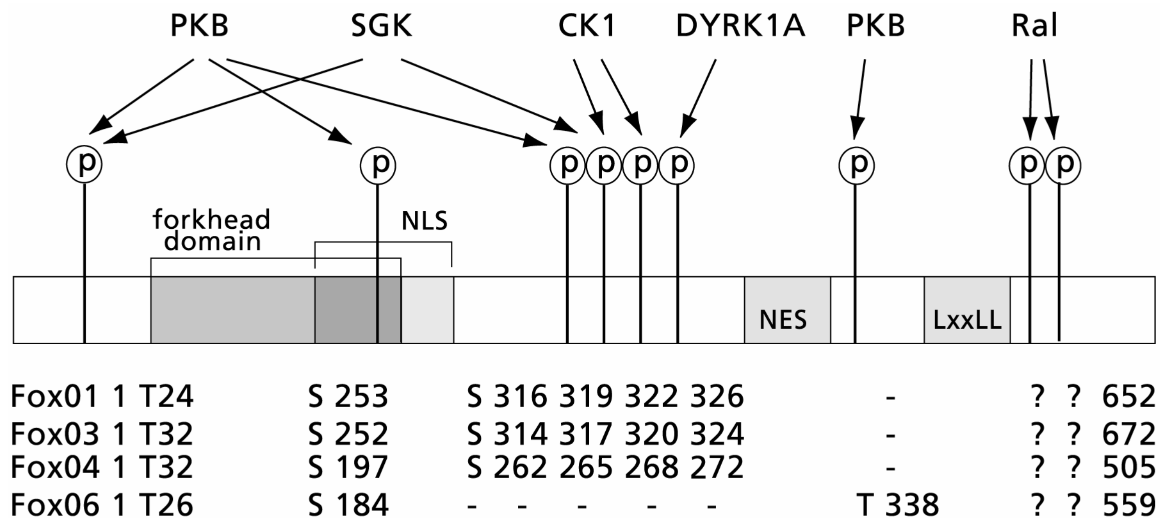

Figure 3: Schematic representation of the primary amino acid sequence of FoxO proteins. The residues that can be phosphorylated by the accompanying kinases are indicated. The exact position of the phospho-residues is indicate below. The total number of residues is indicted on the far right.

2.2 FoxO phosphorylation by SGK

A structurally related family member of PKB, which also phosphorylates FoxO factors [24], is Serum and Glucocorticoid regulated Kinase (SGK). Similar to PKB, SGK is enzymatically activated by PI3K and PDK-1 [18], although it is not recruited to the plasma membrane by PIP2 and PIP3 since it does not contain a pleckstrin-homology domain [25]. Serum, glucocorticoids and stressors stimulate the SGK promoter, which results in rapid transcription of the SGK gene and an induction of the protein [26, 27]. Since PKB and SGK are able to phosphorylate identical substrate motifs [18], it is

currently very difficult to distinguish their activities. Moreover, at this moment there are no specific inhibitors that can discriminate between SGK and PKB. In conclusion, it is suggested that SGK functions complementary to PKB as an important integrator of cellular signaling [16].
The regulation of nuclear exclusion requires additional kinases besides PKB. In theory, all FoxO putative PKB motifs can be phosphorylated by SGK, but it appears that SGK prefers the third C-terminal PKB-motif [24]. The C-terminal PKB site is part of a stretch of 4 phosphoserines, which upon phosphorylation generate an acidic patch [23]. The third PKB site in FOXO1 is not exclusively phosphorylated by SGK [25]. In a mutant cell line able to activate PKB, but unable to activate SGK, the C-terminal PKB site in FOXO1 was still phosphorylated after insulin treatment, as in controls. Therefore, PKB may fully compensate for the loss of SGK in this cell-system, indicating that the in vivo situation is very complex.

2.3 FoxO phosphorylation by CK1

Casein kinase 1 (CK1) is a serine/threonine kinase of which at least 7 isoforms are identified [28]. CK1 has several physiological substrates including the M1 and M3 muscarinic receptors and FoxO proteins [29, 23]. All CK1 members contain a highly homologous kinase domain and a variable N- and C terminal domain [30] and its kinase activity is negatively regulated by auto-phosphorylation [31, 32]. Importantly, CK1 recognises and phosphorylates motifs that have been "primed", in other words, motifs that already contain a phosphorylated serine or threonine residue [33]. Agonists shown to induce CK1 kinase activity include DHPG [34] (a mGluR1 agonist) and DNA damage [35]. Insulin has no apparent direct effect on CK1 activity but it can be recruited to Nck, a small adapter protein that is recruited to IRS-1 after insulin stimulation [36]. Interestingly, it was reported that after insulin receptor activation, IRS-1 translocates from the membrane to cytosol and nucleus [37]. Possibly, this complex of IRS-1/Nck and CK1 phosphorylates nuclear proteins.
The second and third serine, adjacent to - and primed by the C-terminal PKB-site, are phosphorylated by CK1 [23]. Phosphorylation of the first CK1 site primes the second CK1 site. Mutation of the C-terminal PKB site to an alanine prevents the phosphorylation of both CK1 residues [23]. This sequential phosphorylation mechanism was further studied in PDK1 ES knockout cells, which lack PKB activation. In these cells no phosphorylation of the PKB and CK1 sites was detected under baseline and IGF-1 stimulated conditions [23]. This clearly demonstrates that CK1 phosphorylation of residue two and three of the serine stretch is dependent on initial phosphorylation of the C-terminal PKB site. This could be further substantiated by introducing a serine to aspartic acid mutant of the C-terminal PKB site into PDK1 ES knockout cells. In this case, the CK1 sites should be phosphorylated, proving PKB dependency on priming and PDK1 independent CK1 activity.

2.4 FoxO phosphorylation by DYRK

DYRK1A belongs to the dual specificity tyrosine phosphorylated and regulated kinase group of kinases. The DYRK group consists of DYRK1A/B/C, DYRK2, DYRK3 and

DYRK4A/B. DYRK1A and DYRK1B are nuclear whereas DYRK2 is cytosolic [38]. The intracellular location of DYRK3 and DYRK4A/B is at present unknown [38]. DYRKs are serine/threonine kinases that have tyrosine auto-phosphorylation activity [38]. DYRK kinase activity is dependent on a phosphorylation motif present in its activation loop, suggesting regulation by upstream kinases, which are at present unidentified [39]. Although some DYRK targets have been identified (ea. CREB and eIF2B and FoxOs) its intracellular function is unclear. A mutant form of DYRK in Drosophila results in a "minibrain phenotype" [40]. These animals have a smaller brain, due to a reduced number of neurons. In humans, DYRK1A has been mapped to the Downs Syndrome critical region. In mice, DYRK1A haploinsufficiency affects viability and causes developmental delay and abnormal brain morphology [41]. Taken together DYRKs appear to play a role in neuronal proliferation in CNS development.
A DYRK1A site, adjacent to the second CK1 site (see above and Figure 3), completes the stretch of serines that form an acidic patch upon phosphorylation. This serine residue appears to be constitutively phosphorylated [23]. Inhibitors of PI3K, and serine to alanine mutants of the PKB and CK1 residues do not influence its phosphorylation state [23]. This shows that that the phosphorylated residues of PKB and CK1 have no influence on the phosphorylation state of the DYRK1A residue. Mutation of the DYRK1A site to an alanine does result in an increased nuclear FoxO localisation under baseline conditions and increased transactivation of a FoxO reporter construct [42]. This indicates that regulation of the DYRK1A site is involved in the subcellular localisation of FoxO proteins, despite the fact that the site is independent of PKB and CK1 activity.

2.5 FoxO phosphorylation through the Ras-Ral pathway

Ras is a small GTPase that is activated by growth factors. Recruitment of Ras to growth factor receptors by adapter proteins results in activation of Ras by loading it with GTP. [43]. Activated Ras has been associated with transcription, DNA synthesis, differentiation and proliferation [43]. It was suggested that Ras activates specific guanine exchange factors (GEFs), RalGEFs, which activate Ral [44]. Moreover, it was shown that Ral is an important mediator of Ras induced proliferative signals [45]. Besides Ral activation, Ras activation also regulates activation of the classical mitogen activated protein kinase (MAPK) route and the PI3 kinase route. The Ras-Ral route appears to be an additional route besides the PI3-kinase and CK1 routes involved in FoxO phosphorylation [43].
The Ras-Ral signaling pathway influences FoxO transcription factors [46, 43]. Although the regulation of FoxO factors by Ral does not alter its intracellular localisation, it does influence FoxO transactivational capacity, as was shown for FOXO4 [43]. To date it is unclear whether or not Ral mediated phosphorylation of FoxOs is specific for FOXO4 or not, since it has only been documented for FOXO4. Importantly, sequence homology amongst FoxO family members is extremely high especially in regions encompassing the well described signaling motifs. The low degree of conservation of the Ral dependent phosphorylation sites is apparent, comparing human FOXO4 (447 TPVLT 451) to the corresponding region in mouse FoxO4 (TPVLA). The murine FoxO4 lacks the threonine corresponding to T451 in human FOXO4. This clearly indicates the low conservation of Ral-signaling to FoxO proteins in different species.

3 Cellular relocation mechanism of FoxO proteins

3.1 Nuclear transport machinery

The transport of FoxO proteins through the nuclear pore is dependent on active transport mechanisms. The nuclear pore complex is a large structure spanning the nuclear membrane and forms a physical barrier between the cytosol and nucleus. It has an estimated molecular weight of 125MDa. Proteins with a molecular weight up to 50kDa or 9 nm in diameter can diffuse freely through the aqueous channels of the nuclear pore complex. Larger molecules are transported through the nuclear core complex via active transport. Several variations on nuclear transport have been described [47, 48, 49].

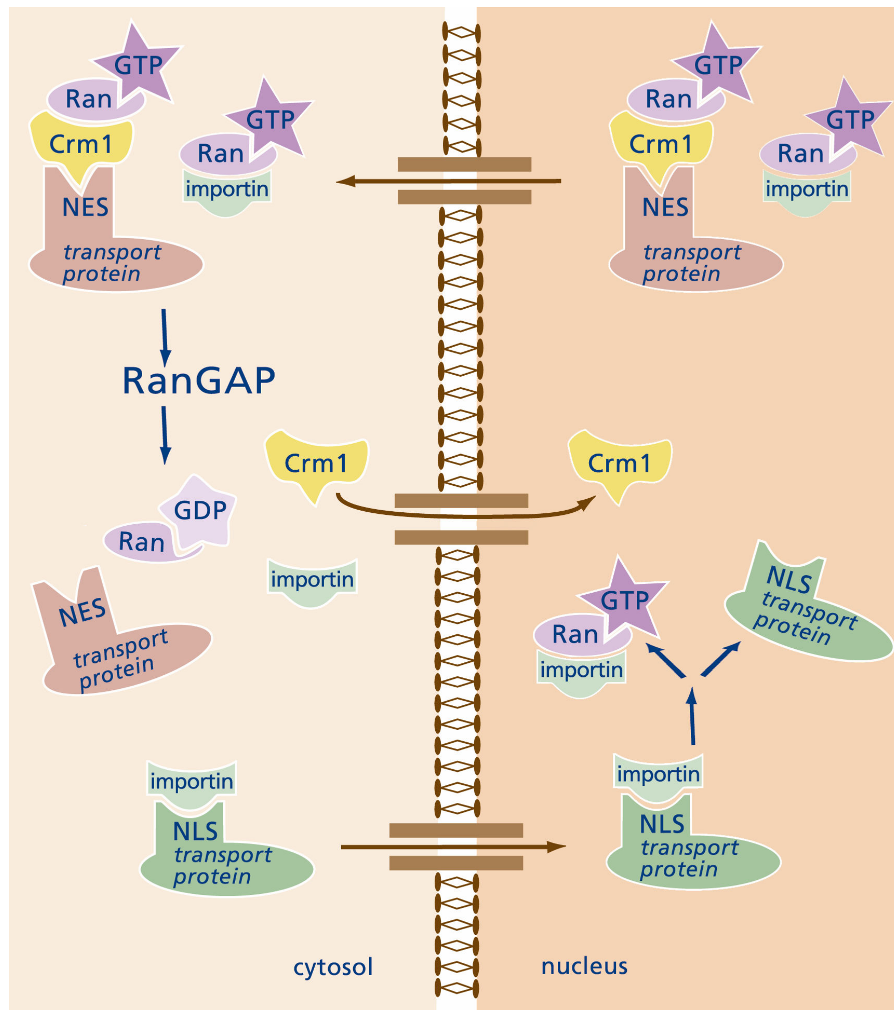

Figure 4: Representation of the molecular mechanisms that play a role in the relocation of proteins. A protein (transport protein) bound to the export receptor CRM1 and Ran-GTP is transported to the cytosol and disassembled by Ran-GAP and other proteins. A protein bound to an importin receptor is translocated to the nucleus where the complex is dissembled by Ran-GTP-binding to the importin receptor. The importin receptor (with bound Ran-GTP) is recycled to the cytosol. Transport protein: The protein that is transported.

The basic model is summarised here (Figure 4). Transport is regulated via specific adapter proteins and Ran, a small GTPase of the Ras family that is required for interaction with the nuclear pore complex [50]. Ran exists in a GDP or GTP bound form. The GDP bound form is mainly present in the cytosol whereas the GTP bound form is present in the nucleus. This gradient is the driving force for transport across the nuclear membrane and is maintained by cytosolic Ran-GAP and nuclear RCC1. In the cytosol Ran-GAP hydrolyses Ran-GTP to Ran-GDP. Whereas in the nucleus RCC1, a chromatin associated Ran-GEF, converts Ran-GDP to Ran-GTP.
Transport across the nuclear pore complex requires adapter proteins that mediate either import or export. These adapters are called importin or exportin receptors, respectively [48]. Importins and exportins recognise specific nuclear localisation signals (NLS) and nuclear export signals (NES) present in the transported protein. Several NES's are recognised by the evolutionary conserved exportin 1 (CRM1) protein. A protein bound to CRM1 is transported through the nuclear pore complex via an interaction with Ran-GTP. Within the cytosol the complex containing Ran-GTP is disassembled by Ran-GAP and RanBPs [47, 48, 49]. Nuclear import is mediated via importin receptors. In the nucleus the importin receptor binds to Ran-GTP, resulting is the release of the transported protein. The dimeric complex of the importin receptor and Ran-GTP is then recycled to the cytosol [49]. The presence of a NLS is a prerequisite for maintaining proteins in the nucleus whereas a NES maintains proteins in the cytosol. FoxO proteins however have both an NLS and an NES. Kinases and interactions with other proteins modulate the effectiveness of these NLS's and NES's, which forms the basis of FoxO shuttling.

3.2 Mechanism of FoxO relocation

3.2.1 CRM1

FoxO proteins accumulate in the nucleus, after treatment with Leptomycine B (LMB), a herbal fungicide that specifically inhibits CRM1 [20, 4]. This links CRM1-function to FoxO export. It was described that FoxO binds to CRM1 [20], although others reported that FoxO CRM1 interactions could not be detected [23]. The data indicate a direct interaction of FoxO to Ran [23]. The dramatic effect of LMB [20] suggests that an interaction of CRM1 to FoxO is apparent [4]. Moreover, an additional LMB sensitive NES in FOXO1 has been described [51]. Binding of CRM1 to FoxO proteins is not dependent on the phosphorylation status of the FoxO protein itself as has been shown for FOXO4. Binding of FOXO4 to CRM1 is phosphorylation state independent as both WT FOXO4 and a PKB-phosphorylation deficient FOXO4 mutant bound CRM1. The FOXO4 mutant, unable to be phosphorylated on its PKB sites, was still transported out of the nucleus, which was elegantly show with the use of cell fusion assays in which the donor and acceptor nuclei of the fused cells can be distinguished. In this system, export is measured by the accumulation of the FoxO mutant protein in the other nuclei. Since export of FoxO proteins seemed independent of phosphorylation the authors concluded that FoxO shuttling must be controlled through regulation of nuclear import [20]. Importantly, this particular study failed to examine the kinetics of export as phosphorylation results in a dramatic acceleration of FoxO export. The influence of phosphorylation on the efficiency of FoxO export has now been documented thoroughly

[52, 4, 53].

3.2.2 Nuclear localisation signals (NLS) in FoxO proteins

All FoxO proteins contain a sequence that conforms to a non-classical NLS. This NLS consists of three arginine residues present in the forkhead domain C-terminus, and three lysine residues located 19 residues downstream of the described arginine residues [20, 54]. Within this motif a PKB phosphorylation motif is present. The arginine residues are part of the RXRXXS PKB motif [19], in which the serine residue is phosphorylated. The basic region of the NLS is suggested to be essential for its function [20]. Phosphorylation of the serine residue in the PKB motif introduces a negative charge which might influence the NLS. Moreover, the phosphorylated serine might sterically hinder NLS-function. Mutation analysis confirmed that phosphorylation of this PKB motif inhibits NLS function, shifting FoxO to a cytoplasmic localisation [20]. Besides the blockade of the NLS by PKB mediated phosphorylation, FoxO proteins require additional factors for cytosolic retention. Recently, it was described that a second functional NLS is present in the N-terminal part of FOXO1 [51].

3.2.3 Relocation by 14-3-3 proteins

Transport of FoxO proteins requires 14-3-3 protein interaction [21]. Their name refers to a classification of brain proteins that were separated by DEAE-cellulose chromatography and gel electrophoresis [55]. 14-3-3s have a molecular weight of about 30kDa and have a U-shaped structure. Within the "U" 14-3-3s specifically recognise and bind phosphorylated serine or threonine residues. The proteins can form homo and heterodimers with other family members. 14-3-3 proteins control catalytic activity of the bound protein. It regulates interactions between the bound proteins and other molecules through sequestration or modification, and finally, influences the intracellular localisation of bound ligands [55, 56]. It was suggested that the N-terminal PKB motif and the PKB motif in the forkhead domain of FoxO proteins are involved in 14-3-3 binding [21]. 14-3-3 recognises RSXpSXP and RXXXpSXP motifs, where pS represent a phosphorylated serine residue. FoxO proteins contain only one optimal 14-3-3 binding site, overlapping with the N-terminal PKB motif. An optimal motif however, is not essential for 14-3-3 binding as it was demonstrated to bind to degenerated 14-3-3 motifs [55]. Interaction of 14-3-3 proteins with FoxO requires phosphorylation of the N-terminal PKB motif and the PKB motif in the forkhead domain. Phosphorylation of the N- and C- terminal PKB sites depends on the initial phosphorylation of the PKB motif located in the forkhead domain, which functions as a "gatekeeper" [52]. Substitution of the PKB site in the forkhead domain to an alanine completely abolishes the insulin-induced increase in total FoxO phosphorylation [57], whereas substitution of either the phosphorylation sites present in the N- and C-terminal parts does not. It has been shown that the amino acids between the second and third PKB motif are responsible for this hierarchical sequence of phosphorylation events [57]. Disruption of the N-terminal PKB site by mutating it to an alanine disrupts 14-3-3 binding and consequently inhibits nuclear export [57, 21]. Since the interaction of FoxOs with 14-3-3 proteins depends on phosphorylation it is logical that disruption of the gate-keeper of phosphorylation completely abolishes all 14-3-3

binding as does disruption of the optimal 14-3-3 binding site itself [55].

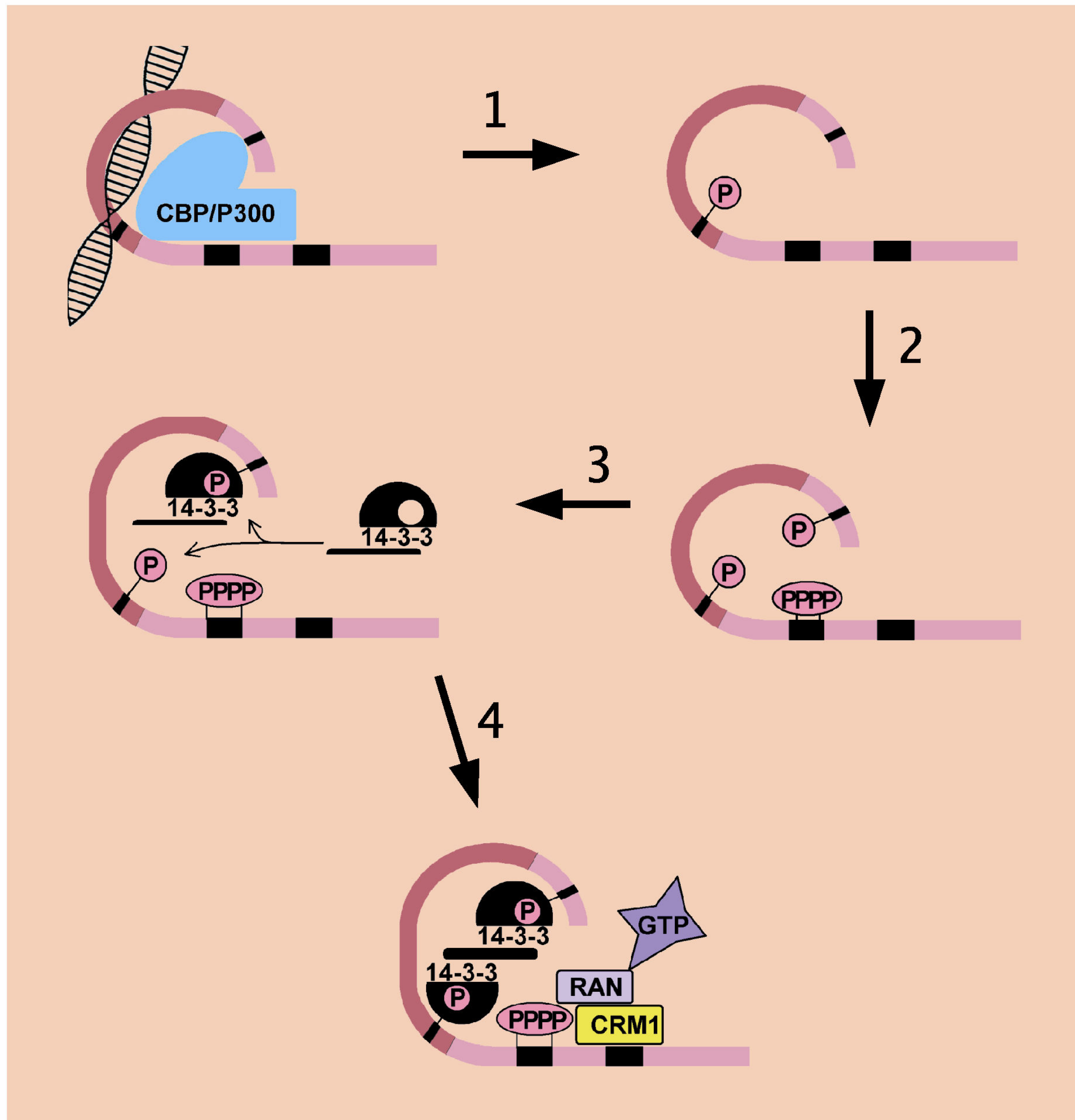

Figure 5: Schematic representation of the sequential phosphorylation events of FoxO proteins triggering nuclear exclusion. 1: Under conditions devoid of growth factors FoxO proteins bind to DNA and are transcriptionally active. FoxO proteins associate with components of the transcription machinery, including CBP/p300. Phosphorylation of the PKB site in the forkhead domain induces dissociation from DNA and blockade of the NLS. 2: First the N-, and C-terminal PKB sites are phosphorylated, additionally two CK1 sites become phosphorylated. The 4th serine, a target of DYRK1A, is also phosphorylated. 3: A 14-3-3 protein binds to the C-terminal PKB motif allowing subsequent binding of a second 14-3-3 protein that dimerises. This second 14-3-3 protein also interacts with the PKB motif in the forkhead domain. 4: CRM1 and Ran-GTP attach to the FoxO protein via interactions with the NES and possibly the stretch of 4 phosphorylated serine residues.

Although 14-3-3 monomers are very capable of binding to ligands, even phosphorylation independent [52], it appears that 14-3-3 dimerization is required for optimal phospho-ligand interaction [55]. Optimal regulation of Raf by 14-3-3 requires 14-3-3 dimers,

whereas 14-3-3 monomers, mutated to prevent dimerization, lack optimal Raf regulation [58]. It is suggested that dimerized 14-3-3 binds to ligands with a higher affinity. This was indicated by binding of a synthetic peptide containing a tandem 14-3-3 recognition site. These peptides bound to 14-3-3 proteins with 30 fold higher affinity compared to peptides with only one 14-3-3 recognition site [55]. It is suggested that a dimeric 14-3-3 protein binds to its ligand via a two step mechanism [55]. The first step involves the binding of one subunit of the dimeric 14-3-3 protein to a high-affinity binding site. The binding of 14-3-3 to a high affinity site would function as a gatekeeper of 14-3-3 binding. 14-3-3 binding to a high affinity site permits the binding of the other subunit to a low affinity site, which would not bind individually [55]. Recently, it was shown that a FOXO1 mutant relieved of its hierarchical phosphorylation sequence by truncation of the C-terminus, required both the N-terminal PKB motif and the PKB motif in the forkhead domain for optimal 14-3-3 binding [51]. Analytical gel filtration and sedimentation equilibrium experiments indicated that 14-3-3 optimally binds phosphorylated FOXO4 in a 2:1 molar stoichiometry [59]. These studies show that two 14-3-3 proteins bind one FoxO protein and that this requires phosphorylation of the two PKB-sites. Given the nature of 14-3-3 interactions, it is very likely that FoxO proteins itself have two 14-3-3 binding sites. Possibly, 14-3-3 binds the N-terminal 14-3-3 site first, as it is a perfect binding site and given the evidence of direct 14-3-3 binding to this site. Probably, the PKB motif in the forkhead domain defines the low affinity site.

3.2.4 Influence of phosphorylation on relocation

The phosphorylation of PKB, SGK, CK1 and DYRK1A sites within the FoxO protein is crucial for its localisation within the cell. The PKB site located in the forkhead domain acts as a trigger and the other sites function as fine-tuning of the shuttling behavior. Disruption of the stretch of four serine residues by mutating the PKB site to an alanine or by inhibition of PI3K with wortmannin does not inhibit FoxO binding to Ran and CRM1, although it dramatically impairs nuclear export [20, 23]. The stretch of four phosphorylated serines is suggested to form an acidic-patch, facilitating nuclear export [23]. Insertion of the four serines from FoxO3 into the corresponding region of FoxO6 rescues the ability of FoxO6 to shuttle efficiently from nucleus to cytosol, emphasising the importance of this region in FoxO relocation [4]. Both wild-type FoxO6 and the FoxO6-4SER chimeric protein are exclusively localised in the nucleus under serum-free conditions. This indicates that only the translocation efficiency is augmented by the serine stretch [4].

In summary, translocation from nucleus to cytosol can be divided into several sequential steps: 1) Phosphorylation of the PKB site in the forkhead domain leads to disruption of the NLS function and disruption of DNA binding (see for details below). Phosphorylation leads to accessibility of the N- and C-terminal PKB sites. 2) The N-and C-terminal PKB site are phosphorylated. Phosphorylation of the C-terminal PKB site by SGK or PKB leads to the phosphorylation of a stretch of 3 serines. A DYRK1A site completes the stretch of 4 phospho-serines. 3) The current phosphorylation state induces high-affinity binding of 14-3-3 proteins. 4) The phosphorylated FoxO protein in a complex with Ran and CRM1 is transported through the nuclear pore complex towards the cytosol. In addition, cytosolic and phosphorylated FoxO proteins are degraded by the ubiquitin

proteasome system [60, 61], providing the cell with a double negative regulation of FoxO factors [61]. Disruption of at least one of the above steps results in a disruption of nuclear export and accumulation of the FoxO protein in the nucleus. The exact mechanism of 14-3-3 binding and the mechanism through which the phospho-stretch increases nuclear export are at present unknown.

3.3 Shuttling and FoxO protein processing

The FoxO shuttling system was initially assumed to directly regulate FoxO transcriptional activity by altering its intracellular localization. In summary this is a two step model. First, activation of the PI3K pathway leads to translocation of FoxOs to the cytosol, by removing the transcription factor from the DNA and thereby terminating their transcriptional activity. Secondly, transcriptional activity is reinstated by deactivation of the PI3K-PKB pathway by chemical inhibition or removal of growth factors, allowing FoxOs to return towards the nucleus. In fact transcriptional regulation is partly independent of subcellular localisation and is not only dependent on the shuttling system. FoxO6 transcriptional activity is effectively regulated by growth factors to the same extent as FoxO1 and FoxO3, although FoxO6 is almost completely nuclear [4]. The remaining percentile of growth factor induced cytoplasmic shuttling, can never account for the decrease in FoxO6 dependent transcriptional activity. This is also in agreement with data that describe insulin dependent inhibition of FoxO1 activity in a export deficient mutant [62]. It is suggested that FoxO6 regulation by growth factors is provided through modulation of DNA binding. Probably, relocation to the cytosol serves additional purposes. Important clues are provided by studies investigating FoxO protein processing by the ubiquitin proteasome system. It was described that activated PKB results in a decrease of FOXO1 and FOXO3 protein levels [60]. The reduction in FOXO1 and FOXO3 protein levels could be decreased through the application of proteasome inhibitors. This suggests that the proteosome system has a role in the regulation of FoxO activity. In fact FOXO1 is ubiquitinated and degraded by the proteasome in response to insulin and this process is PI3K-PKB dependent [61]. It was shown, through the use of FOXO1 NES/NLS mutants and a constitutive nuclear FOXO1 mutant (triple PKB-site-alanine mutant), that phosphorylation of the PKB sites and cytosolic localization are required for optimal degradation of FOXO1. The degradation of FoxO factors adds an additional layer of negative regulation of FoxO activity that relies on the FoxO shuttling system. Since cytosolic localization is required for optimal degradation of FoxO1, this finding has intriguing consequences for FoxO6. Although not investigated, it can be assumed that FoxO6 is not degraded by the proteasome system as efficiently as its family members, making it the most stable protein of its family.

Besides processing by the ubiquitin system FoxOs are processed by proteases, diversifying the processing of FoxOs. FOXO3 is a substrate of caspase-3-like proteases [8]. Cleavage of FOXO3 by caspase-3-like proteases yields two fragments. An N-terminal fragment which encompasses the first two PKB sites and the forkhead domain and a C-terminal fragment which contains the transactivation domain. The C-terminal fragment is always cytosolic, presumably because it contains the NES. The intracellular localization of the N-terminal fragment is still dependent on the PKB phosphorylation sites. Dephosphorylation of the N-terminal fragment results in translocation to the

nucleus, where is possibly functions as a dominant negative. Interestingly, the sequence identified as the proteolytic cleavage site appears to be conserved in FOXO1 and FOXO4. An essential aspartic acid of the cleavage motif [63] is however not conserved in FoxO6 (DELD (position 304) in FOXO3, an the corresponding region DDYE in FoxO6, Fig 1 (j)). A similar proteolytic cleavage mechanism has been shown for FOXO1. Androgens initiate protease mediated cleavage (Figure 1 (r)) of a 70 kDa FOXO1 into a 60kDa N-terminal fragment and an C-terminal 10kDa fragment [9]. The C-terminal fragment contains the DNA binding domain and a large portion of the transactivation domain and functions as as a dominant negative [9]. Via this protease mechanism androgens are suggested to deregulate FOXO1 mediated processes. In conclusion, negative regulation of transcription by the FoxO shuttling system is reinforced by proteolytic processing of FoxO factors.

4 Transcriptional activity of FoxO proteins

The activity of FoxO proteins can be regulated by repositioning the protein in the cell. Also, direct regulation of transcriptional capacity by protein modification has been reported. These modifications can be found on the DNA-binding interface as well as in the transactivation domain. Finally, FoxO proteins interact and cooperate with other proteins. These interactions cause changes in FoxO transactivation but also influence other transcription factors [64].

4.1 Modification of the DNA-binding potential

The PKB phosphorylation motif in the forkhead domain is linked to the regulation of DNA binding [54]. Phosphorylation of this serine residue is not sufficient for relocation of the FoxO proteins although it is part of the NLS sequence and is involved in binding 14-3-3 proteins [51]. Earlier findings from several groups suggested that phosphorylation of this site disrupts transactivation without relocation of the protein [52, 62]. Gel-shift experiments have shown that the introduction of a negative charge in the PKB site (serine>aspartic acid mutation) is sufficient to disrupt DNA-binding [54]. Moreover, reporter gene studies showed that phosphorylation limits the transactivating potential of FOXO1 [54]. Interestingly, experiments performed by Obsil et al.[59] indicated that the affinity for DNA is not affected by phosphorylation of the first two PKB sites of FOXO4. However, a clear decrease in the affinity of phosphorylated FOXO4 for DNA is reported after binding of two 14-3-3 proteins. These conflicting results could be the result of the fact that the group of Obsil did not perform the studies on 14-3-3- and DNA-binding with full length FOXO4 but with a mutant protein lacking a large portion of the C-terminus. With the use of FoxO6, it was clearly demonstrated that transactivation is limited after phosphorylation by PKB without relocation of the protein from the nucleus to the cytosol [4]. In conclusion, DNA-binding is diminished by phosphorylation of the serine residue present in the C-terminus of the forkhead DNA-binding domain.
FoxO mediated expression of downstream genes can be mediated via direct FoxO binding to FoxO responsive elements in the target gene. These genes contain the optimal forkhead responsive element (FHRE) binding sequence or insulin response unit/sequence (IRU/IRS). Besides this mechanism, FoxOs however, have the capacity to regulate

downstream targets independently of DNA binding. In part this can be explained by the interactions of FoxO factors with other transcription factors such as nuclear hormone receptors (discussed below). DNA independent activity was elegantly shown by comparing expression profiles of two different cell lines, one containing a constitutively nuclear FOXO1 (triple PKB-site-alanine mutant, AAA) and one containing the same protein but DNA-binding deficient (HRAAA) [65]. HRAAA was generated by substituting a conserved histidine to arginine, in helix three of the forkhead domain. It should be noted that this mutant was only tested on binding to, - and non-activating a 3*IRS reporter. By comparing these two mutant forms of FOXO1 four different groups of downstream targets were classified. The first group of genes was activated by AAA and not by HRAAA. The second group was mainly activated by AAA and to a lesser extent by HRAAA. A third group encompassed targets activated mainly by HRAAA and not by AAA. Finally, a fourth group, with AAA and HRAAA downregulated targets. Interestingly, chromatin immuno-precipitations performed on genes from groups three and four still showed binding to HRAAA, suggesting that HRAAA interacts with the promoter independent of an IRS, presumably through interactions with other proteins. Moreover, it is plausible that this is also upholds for wild type FOXO1. Additionally, it was shown that the downregulation of genes in group four did not depend on the FOXO1 transactivation domain. However, the down-regulation of genes could be reversed by treating the cells with a histone deacetylase inhibitor, suggesting gene specific transcriptional repression [65]. In PTEN null LNCaP prostate adenocarcinoma cells [65] AAA induces cell death, but HRAAA does not. In PTEN null 786-O cells [65] AAA and HRAAA inhibit cell cycle progression and suppresses tumor formation. In PTEN null U87-MG glioblastoma cells AAA and HRAAA induce G1-arrest. This study clearly suggests that only the induction of apoptosis is differentially regulated by AAA and HRAAA. DNA-binding independent activity of FoxO can have a fundamental different activity profile compared to DNA-dependent FoxO activity.

4.2 Modification of transactivating domains

Studies using the C-terminus of FoxO1 fused to the GAL-4 DNA binding domain demonstrated that phosphorylation of FoxO1 can directly inhibit the transactivating potential of this domain [7]. Insulin induces phosphorylation of three regions: residues serine 319 (Fig1 (k)), serine 499 (Fig1 (p)) and residues in a stretch of 15 aa at aa 350-364 (Fig 1 (l)), which could explain the data describing the LY-294002 enhanced transcriptional activity in a triple or quadruple DAF-16 alanine mutant [66]. This points to other PI3-kinase dependent residues besides the classical PKB residues identified in all FoxO factors. Finally, the phosphorylation of human FOXO4 threonines 447 and 451 through activity of the Ras-Ral pathway augments transcriptional activity [43]. Taken together, the data suggest direct regulation of the transactivating potential without nuclear exclusion.

4.3 Interactions with co-activators

4.3.1 p300/CREB-binding protein

Interactions of forkhead proteins and other associated proteins, binding on the promoter of the insulin-like growth factor binding protein 1 (IGFBP-1) gene are described. This complex was induced by binding to an insulin responsive element (IRE) [67]. The FoxO interacting partner was identified as the p300/CREB-binding protein (CBP) [68, 22]. This protein plays an important role in integrating signaling events to the transcriptional machinery. The primary DNA-sequence of the IGFBP-1 promoter provides the integration of glucocorticoid and insulin signaling. The interaction of DAF-16, FOXO1 or FOXO3 with CBP enhances the glucocorticoid stimulated transcription, through possible interaction with the KIX- and E1A/SRC domain. Interestingly, FOXO4 does stimulate the IGFBP-1 gene but fails to enhance the glucocorticoid response. The interaction of FOXO4 and CBP is limited to the KIX domain of CBP[67], indicating that the glucocorticoid stimulated response is established though interaction of the CBP SRC-domain. However, the described transcriptionally inactive C-terminal truncation mutant of DAF-16 interacts with the SRC-domain and fails to interact with the KIX domain. The authors conclude that the enhanced glucocorticoid response relies on the interaction with the KIX domain [67]. This conclusion seems to be in conflict with the data derived from the FOXO4 experiments. In conclusion, it seems that both interaction domains of CBP are involved in mediating the enhanced glucocorticoid transcriptional activation.

4.3.2 CCAAT/Enhancer binding protein

A combination of in-vivo and in-vitro experiments show that FOXO1 interacts with CAAT/Enhancer-binding proteinβ (C/EBPβ). FOXO1 and C/EBPβ bind on a composite element present in the decidual prolactin (dPRL) proximal promoter [69]. Binding of C/EBPβ alone stimulates the dPRL promoter. After binding of FOXO1 the stimulation is more than additive. This suggests a functional interaction between these proteins. Experiments using GST fusion proteins and pull-down assays confirmed the interactions, which are DNA-binding independent. This demonstrates that the composite element is not required for the interaction. Transient transfection experiments with mutated dPRL promoter constructs, showed that deleting either part of the composite element reduces the transactivating potential [69]. This indicates that DNA-binding is important for the cooperative function of FOXO1 and C/EBPβ on the dPRL promoter.

4.3.3 DYRK1

In addition to the kinase activity described earlier involved in FoxO relocation, DYRK1A and B, but not DYRK2, can interact with FOXO1 and cooperate in activating the glucose-6-phosphatase (G6pc) gene [70]. A DYRK1 catalytic inactive mutant (Lys188Arg [71, 72]) was almost equally active in transactivating potential as compared to the wild-type protein. This indicates that kinase activity is not required for the cooperation with FOXO1 in transactivation of the G6pc gene. The functional interaction of these proteins depends on the binding of FOXO1 to its insulin responsive element (INR). Mutation of the INR element in the G6pc promoter abolishes the activation of the promoter by FOXO1/DYRK1. Taken together, the kinases DYRK1A and B can interact with FOXO1 and add in its transactivating potential through direct interaction on the G6pc promoter independent of the kinase activity of DYRK1.

4.3.4 PGC-1α

Proliferative activated receptor-gamma co-activator 1 (PGC-1α) is able to cooperate with FOXO1 in the activation of gluconeogenic liver genes (phosphoenolpyruvate carboxykinase1 (Pck1) and Glucose-6-phosphatase (G6pc)) [73]. The activation of these genes by PGC-1α depends on FOXO1, which was shown by using FOXO1 mutants [73]. Therefore, the insulin regulation of these genes depends on the tight regulation of FOXO1 and is based on the cooperation of FOXO1 with PGC-1α[73, 74] independent of DNA binding. Interestingly, the close homologue of PGC-1α, PGC-1β fails to interact with FOXO1 and does not enhance the FOXO1 mediated transcriptional activation of gluconeogenic genes [75]. The comparison of the biochemical properties of these two homologs might provide more inside into the molecular mechanism of PGC-1α cooperation with FOXO1.

4.3.5 FoxO-nuclear hormone receptor interactions

Besides regulation of transcription via direct interactions between FoxO and DNA, FoxO factors have a DNA binding independent effect on transcription via interactions with nuclear receptors. Nuclear receptors mostly function as ligand-dependent transcription factors. FoxO factors influence nuclear receptor transactivation by repressing or activating transcription, depending on the nuclear receptor involved. On the other hand, it was shown that nuclear receptors interacting with FoxO factors, function as inhibitors of FoxO mediated transcription [76, 77, 65].
Nuclear receptors interacting with FoxO factors include the estrogen receptor (ER) [76, 77], the progesterone receptor (PR) [76], the androgen receptor (AR) [78], the thyroid hormone receptor (THR) [76], the glucocorticoid receptor (GR) [76], retinoic acid receptor (RAR) [76], the peroxisome proliferator activated receptor (PPAR) [79], and hepatocyte nuclear factor-4 (HNF-4) [80]. Steroid receptors are retained in the cytoplasm and upon ligand binding they are released from their chaperones, dimerise, enter the nucleus and bind to specific response elements in the regulatory region of target genes [81]. Once bound to the specific response elements nuclear receptors facilitate the initiation of transcription.
In contrast to steroid receptors non-steroid receptors are mainly located in the nucleus. In the absence of ligand, they are associated with histone deacetylase-containing complexes tethered through co-repressors. This process results in chromatin compaction and silencing of the promoter regions of the target genes. Upon ligand binding, the co-repressor-binding interface is destabilised, which leads to their dissociation. Subsequently, nuclear receptors bound to their responsive elements facilitate recruitment of the transcription machinery (the general transcription factors and RNA Polymerase II) [81]. All FoxO factors contain an LxxLL domain located in the far C-terminal region of the protein (Figure 1). It is suggested that this domain facilitates interactions with nuclear receptors [82]. The mechanism underlying FoxO regulation of nuclear receptors and vice versa is very complex and is influenced by multiple pathways. First, interaction between nuclear receptors and FoxO factors can be dependent or independent of nuclear receptor ligand-binding [76]. As a typical example, estrogen enhances the binding of FOXO1 to

the estrogen receptor [76] whereas binding of FOXO1 to the RAR is ligand independent [76]. FOXO1 augments transactivation of the ER through an estrogen responsive element whereas the ER represses transactivation of FOXO1 through an insulin responsive element, indicating bi-direction function [77]. However, others have described that FOXO1 represses ER dependent transactivation [76]. The discrepancy between these two studies was explained by experimental differences in used cell-lines and promoters [77]. Second, FoxO phosphorylation can influence the interaction with nuclear receptors, as was shown for HNF-4 and FOXO1 [80]. The binding of HNF-4 to FOXO1 is negatively influenced by FOXO1 phosphorylation, and the repression of HNF-4 transactivation by FOXO1 is negatively influenced by insulin [80]. Moreover, repression of HNF-4 transactivation by a FOXO1 mutant, in which all PKB sites are substituted for alanines is not inhibited by insulin [80]. Since phosphorylation of FoxO factors influences their intracellular localisation, it is assumed that FoxO phosphorylation influences the interactions with steroid and non-steroid receptors. The repression of FOXO1 by the AR, is however independent of FOXO1 phosphorylation [78], demonstrating that the net result on transactivation depends on the particular nuclear receptor involved.
It appears that nuclear receptors somehow recruit FoxO factors to either augment or repress transcription. Whether, the functional consequence on transcription is stimulation or repression probably depends on the domains via which nuclear receptors and FoxO factors interact. Interactions between nuclear receptors and FOXO1, FOXO3 and FOXO4 have been documented, but to date nothing is known about the novel member FoxO6. As accounts for the other FoxO members, FoxO6 also possesses the LxxLL motif (Figure 1), which is implicated in the interaction with nuclear receptors [82]. Since FoxO6 is mainly nuclear [4], interactions with nuclear receptors could very well differ from the other FoxO proteins. This provides nuclear receptors with a co-factor that is continuously present.

4.3.6 FoxO acetylation

Two independent papers [5, 83] describe SIRT1 mediated deacetylation of FoxO factors. The homologue of SIRT1 in C. elegans, Sir2, positively regulates lifespan in a DAF-16 dependent manner [84], whereas in mammalian cells deacetylation of FoxO factors is a general mechanism that deactivates FoxO transcriptional activity [83]. On the other hand, others suggest that FoxO deacetylation regulates the balance between pro-apoptotic, - and cell cycle arrest genes [5]. Surprisingly, deacetylation experiments performed by two different groups lead to inconsistent results. Whereas a reduction of Bim by FoxO deacetylation is observed by both groups, a reduction of p27kip [83] and an increase in p27Kip expression is observed [5]. Earlier findings [6] support the latter experimental result, regarding p27kip. It is clearly described that acetylation of FOXO4 by CBP suppresses the activation of p27kip [6]. In addition to this, the FOXO4 lysine residues involved in the acetylation induced repression are mapped [6]. Several stress induced phosphorylation and acetylation sites are mapped (Figure 1) [5]. Functional analysis of these sites was however not performed. Interestingly, a possible stress induced acetylation site was also implicated in CBP mediated repression of FOXO4. The identified functional acetylated lysines are mainly located in the forkhead domain (Figure 1) [6], suggesting that acetylation could affect target DNA binding. Unfortunately this

was not investigated [6]. FoxO de-acetylation reduces apoptosis [5, 83] and increases G1 arrest [5]. This suggests that de-acetylation can shift the balance from pro-apoptotic processes to cell-cycle arrest and survival. Interestingly, the HRAAA FOXO1 mutant completely abolishes apoptosis, whereas G1 arrest was unaffected [65] (as described in section 4.1). Although speculative, acetylation and deacetylation may regulate the binding of FoxOs to target DNA, shifting the balance from DNA dependent - to DNA binding independent transcription. Interestingly, Sirt1 mediated de-acetylation of the tumor suppressor protein P53, thereby suppressing DNA binding activity [85], parallels the effect of de-acetylation of FoxO factors [5, 83]. This emphasizes the need to analyse the relationship between FoxO acetylation and DNA-binding activity. Besides acetylation, hydrogen peroxide induced stress leads to nuclear accumulation of FOXO3 under growth-factor rich conditions [5]. This stress induced translocation does not affect PKB phosphorylation itself and the phosphorylation state of the N-terminal FoxO PKB site. Apparently, stress overrules the PI3K-PKB pathway with respect to FoxO shuttling. It is interesting to examine 14-3-3 binding and the PKB phosphorylation site in the forkhead domain under these conditions, since both can have dramatic effects on the DNA binding capability of FoxOs.

4.4 In vivo functions

As shown in table 1 FoxO transcription factors have many downstream targets. They are associated with cell type specific effects on cell cycle, metabolism, DNA repair, protection against oxidative stress, and cell death. Since most of the downstream targets were identified in vitro, the in vivo extrapolation is problematic. However, data from C. elegans, Drosophila, and Mouse provides valuable information regarding the in vivo function of FoxO factors.

4.4.1 FoxO in C. elegans

DAF-16 is the FoxO homologue expressed in C. elegans and is regulated by a signaling pathway similar to the mouse insulin-PI3K-PKB pathway. DAF-16 is remarkably similar to mice FoxO proteins and can be partially substituted by FoxO3 [86]. Life-span extension, stress resistance, and arrest at the dauer diapause stage is accomplished by either inhibition or mutation of the insulin-PI3K-PKB pathway or direct activation of DAF-16 [87]. Besides cell autonomous inputs, DAF-16 also responds to environmental inputs. Starvation, heat and oxidative stress all activate DAF-16, whereas nutrient rich conditions deactivate DAF-16. In summary, DAF-16 responds to cues of a changing environment to reallocate resources at all stages of life [88].

4.4.2 FoxO in Drosophila melanogaster

Recently, a unique FoxO homologue in Drosophila was identified and named Drosophila FOXO (dFOXO) [113, 111, 112]. Ectopic expression of dFOXO results in a marked reduction in body size. This reduction is caused by a decrease in cell number [113, 111, 112] and cell size [113, 112]. The phenotype observed in starving-larvae resembles the dFOXO overexpression mutant [112]. This corresponds well to the fact that the insulin-

PI3K-PKB signaling cascade [113, 111, 112] and nutrients [113] negatively regulate dFOXO. However, dFOXO knock-out flies are viable and of normal size but more vulnerable to oxidative stress which suggests that dFOXO is not required for proper growth, but does provide protection against oxidative stress [113]. Microarray analysis identified d4E-BP, a translation inhibitor [113], as a dFOXO target whereas RNase protection assays identified d4E-BP and dInsR as dFOXO targets [111]. It is suggested that under stressful conditions such as nutrient deprivation dFOXO is activated and regulates growth via d4E-BP [113, 111]. The dInsR provides this system with negative feedback [111].

Table1: Selection of confirmed FoxO target genes

Survival genes:
Fas ligand (FasL) [89, 90, 91]
Transforming growth factor β-2 (TGF-β)[92]
Tumor necrosis factor related apoptosis-induced ligand (TRAIL)[93, 94]
Bcl-2 interacting mediator of cell-death (Bim)[95, 96, 97, 98, 99]
Growth arrest and DNA damage response protein 45 (Gadd45)[100]
Manganese superoxide dismutase (MnSOD)[101]
Cell cycle:
P27(kip) [102, 103, 96]
Polo-like kinase [104]
Cyclin B [104]
Cyclin D1/2 [65]
Cyclin G2 [105, 65]
Metabolism Genes:
Insulin-like growth factor binding protein-1 (IGFBP-1/3)[106, 46, 107, 65]
Glucose-6-phosphatase (G6pc)[108, 70, 73, 74]
Phosphoenolpyruvate carboxykinase (Pck1)[73, 74]
Tyrosine amino transferase[109, 89]
pyruvate dehydrogenase kinase 4 (PDK)[110]
Drosophila insulin receptor (dInR)[111]
Decidual prolactin gene (dPRL) [69]
Drosophila eukaryotic initiation factor 4E-binding protein (d4EBP) [111, 112, 113]
Lipoprotein lipase (LPL) [114]

4.4.3 FoxO in Mus musculus

FoxO1 homozygous null mutants die before birth due to several embryonic defects [115], due to incomplete vascular development [116]. Analysis of heterozygote null-mutants, indicated that FoxO1 is involved in pancreas growth, hepatic glucose metabolism and adipocyte differentiation[115, 117, 118]. A diabetic phenotype induced by diet or disruption of either the InsR or IRS-2, can be rescued by FoxO1 haploinsufficiency [115, 117, 118]. Moreover, a FoxO1 gain of function mutant induces diabetes [115]. The underlying mechanism involves FoxO1 mediated negative regulation of genes involved in insulin sensitivity in liver, adipocytes, and pancreatic beta cells [115, 117, 118]. Strikingly, FoxO mRNAs are regulated by nutritional and hormonal factors, as was

described for mice liver and skeletal muscle [119, 110, 114]. Food restriction or glucocorticoid treatment leads to an increase in FoxO1, FoxO3 and FoxO4 messenger. Re-feeding the mice reverses the starvation-induced increase in FoxO messenger[119, 110]. FoxO1 is also induced in skeletal muscle by streptozotocin-induced diabetes and treadmill running [114]. This implicates FoxOs in skeletal muscle energy metabolism. This is further strengthened by the fact that they regulate the expression of PDK4 and LPL, two enzymes involved energy utilisation [114, 110].
The mouse FoxO3 knockout do not have such a dramatic phenotype compared to FoxO1[120, 116], the FoxO4 knockout does not have any obvious abnormality [116]. Closer inspection of the FoxO3 knockout reveals hematologic abnormalities, a decreased glucose uptake in glucose tolerance tests [120] and a distinct ovarian phenotype due to premature follicular activation. FoxO3 is thus suggested to function at the early stages of follicular growth as a suppressor of follicular activation [120, 116].
Taken together, in-vivo studies implicate FoxO proteins in the homeostasis of metabolism. In C. elegans Drosophila and mouse, FoxOs respond to nutrients, growth factors and stress, in order to fine-tune cellular metabolism and optimally adapting the cell to an ever changing environment. Intriguing is the fact that FoxO1, FoxO3 and FoxO6 are all expressed in the central nervous system, a role in neuronal metabolism and stress defense is therefore not unlikely, since neurons have to last a lifetime.

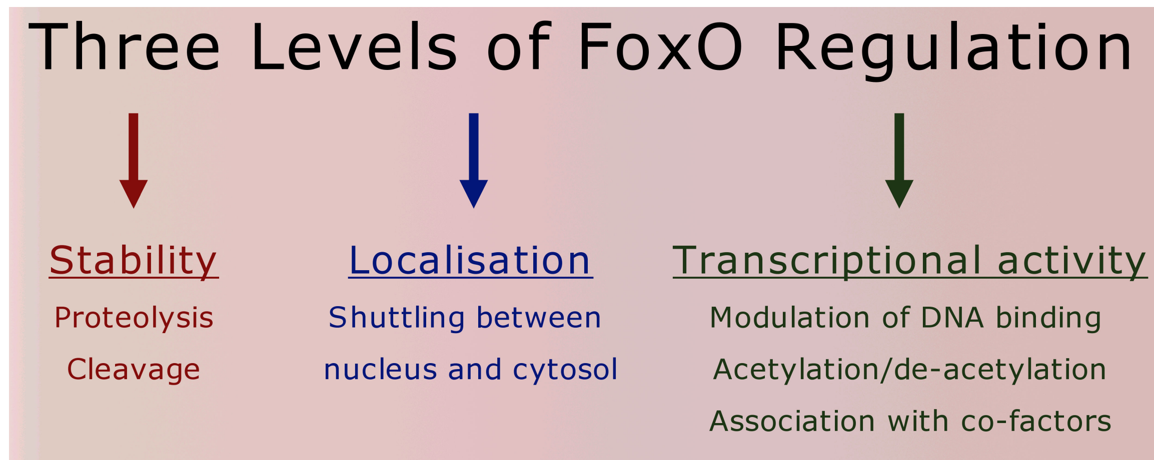

Figure 6: Schematic representation of different levels of FoxO regulation. FoxO factors are regulated by influencing protein stability, localisation and their transcriptional activity.

5 Conclusions

FoxO transcription factors consist of a family with currently 4 different members in mouse (Figure 1) [3, 4]. All FoxO members are regulated by multiple protein kinases, thereby serving as a transcriptional endpoint of several signaling cascades (Figure 2). Central to the regulation of FoxO members is a complex shuttling mechanism that regulates the intracellular FoxO localization (Figure 4,5). Phosphorylation on three highly conserved PKB phosphorylation sites result in FoxO sequestration in the cytosol by 14-3-3 proteins. A highly conserved motif that regulates the speed of nuclear export is not conserved in FoxO6 (Figure 1,3), resulting in a nuclear localization under all conditions

tested. Besides the regulation of intracellular localization, growth factors influence FoxO association with the general transcription machinery and DNA binding itself, providing the cell with an additional pathway in the regulation of transcription. FoxO association with nuclear receptors leads to either augmentation or repression of the nuclear receptor target genes, diversifying FoxO mediated transcriptional regulation. In summary, FoxOs are regulated at several levels and in different cellular compartments (Figure 6). In vitro and in vivo studies have identified an array of FoxO downstream targets (Table 1). These targets implicate FoxO function in processes such as cell cycle, cell death, metabolism, protection from oxidative stress and survival. In vivo studies ranging from C. elegans to mouse suggest that FoxO factors are involved in the integration of environmental cues to optimally adapt to changing environmental conditions. The importance of FoxO function is emphasised by their association with development of organs such as the pancreas [118] and the ovaries [120] and complex diseases such as diabetes[115]. The functional importance of FoxO1 and FoxO3 in-vivo, suggests important roles for FoxO4 and FoxO6 in cellular metabolism, although the FoxO4 knockout has no obvious phenotype[116]. Especially interesting is the possible in vivo function of FoxO6, since the protein is mainly nuclear and its messenger is widely expressed in the central nervous system. Therefore, a neuron specific FoxO6 function is conceivable, since neuronal development and maintenance is essential for an organ which must last a lifetime.

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Chapter 5

FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics

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FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics

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Forkhead transcription factors of the FoxO-group are associated with cellular processes like cell cycle progression and DNA-repair. FoxO function is regulated by protein kinase B (PKB) via the PI3-kinase/PKB survival pathway. Phosphorylation of serine and threonine residues in specific PKB-phosphorylation motifs leads to exclusion of FoxO-proteins from the nucleus, which excludes them from exerting transactivating activity. Members of the FoxO-group have three highly conserved regions containing a PKB-phosphorylation motif. This study describes the cloning and characterization of a novel forkhead-domain gene from mouse that appeared to be highly related to the FoxO-group of transcription factors and was therefore designated *FoxO6*. The *FoxO6* gene was mapped in region D1 on mouse chromosome 4. In humans, *FOXO6* is located on chromosomal region 1p34.1. Embryonic expression of FoxO6 is most apparent in the developing brain and FoxO6 is expressed in a specific temporal and spatial pattern. Therefore it is probably involved in regulation of specific cellular differentiation. In the adult animal *FoxO6* expression is maintained in areas of the nucleus accumbens, cingulate cortex, parts of the amygdala and in the hippocampus. Structure function analysis of FoxO6 compared to its group members show that the overall homology is high, but surprisingly a highly conserved region containing multiple phosphorylation sites is lacking. In transfection studies, FoxO6 coupled to GFP showed an unexpected high nuclear localization after stimulation with growth factors, in contrast to the predominant cytosolic localization of FoxO1 and FoxO3. We also show that nuclear export of FoxO6 is mediated through the PI3-kinase /PKB-pathway. Furthermore, we show, using a chimeric approach, that we can fully restore FoxO6's ability to shuttle between nucleus and cytosol. In conclusion, the data presented here gives a new view on regulation of FoxO-function through multiple phosphorylation events, and other mechanisms involved in the nuclear exclusion of FoxO-proteins.

Transcription factors of the forkhead family have an important role in development and function of an organism (1). Since the discovery of the winged helix structure (forkhead domain) in *Drosophila*, more than 90 genes containing the forkhead domain have been identified, in species ranging from yeast to humans(1). Daf-16, a forkhead transcription factor in *C. elegans* has been extensively studied for its role in controlling longevity and

dauer formation (2) . Transcriptional activity is negatively regulated via an insulin-like signal transduction cascade. In humans Daf-16, has four described orthologues, FOXO1 (FKHR), FOXO2, (AF6q21), FOXO3a (FKHRL1) and FOXO4 (AFX) **.** Together, these proteins form the FOXO-class of forkhead transcription factors in humans. Also in mice Daf-16 orthologues are identified and are designated FoxO1,FoxO3 and FoxO4 (3).

A subset of *FOXO*-genes has been associated with disorders like tumorogenesis and rhabdomyosarcomas. Genetic analysis of a type of acute lymphocytic leukemia revealed that the cause of the disorder is a translocation between Chromosome 11 and Chromosome X [t(X,11)]. This translocation involves fusion of the general transcription factor HTRX1 with the forkhead gene *FOXO4* on the X-chromosome (4). A form of rabdomyosarcoma is caused by a translocation between chromosome 2 or chromosome 1 and chromosome 13 [t(1,13) or t(2,13)], that leads to fusion of the *PAX7* or *PAX3* gene with the forkhead *FOXO1* (5,6). The fusion product turned out to be a stronger activator compared to PAX3 or PAX7, which function as inhibitors of myogenic differentiation of migrating limb myoblasts (7). Blockage of this terminal differentiation pathway by the PAX-FOXO1 fusion product is the direct cause of this disorder.

Since their discovery, FOXO-members have been subject of intensive investigation, especially their place in the PI3-kinase/PKB-pathway and the identification of the transcriptional targets. Binding of insulin-like substrates to the Insulin receptor leads via PI3-kinase to phosphorylation and activation of PKB. As demonstrated in mammalian cell-lines, PKB phosphorylates specific motifs within FOXO-proteins, inducing translocation to the cytosol, thereby preventing their transcriptional activity (8,9).

In order to elucidate cellular functions of FOXO-proteins, many studies focused on identification of their transcriptional targets. FOXO3a has been demonstrated to play an important part in cell cycle progression of fibroblast cells, by regulating expression of the mitotic genes *cyclin B* and *polo-like-kinase*. Interference with FOXO3a transcriptional activity induces defective cytokinesis, a delayed transition from M to G1 and finally accumulation of cells in the G2/M stage (10). FOXO3a triggers DNA repair through the Gadd45 protein, which was shown to be a direct transcriptional target of this forkhead protein (11). Recently FOXO3a has been shown to protect quiescent cells from oxidative stress by inducing transcription of MnSOD (12). Taken together, these findings indicate that FOXO-proteins are of crucial importance for the ability of a cell to respond to environmental changes. Processes of proliferation, differentiation and responsiveness to extracellular changes are highly relevant in the nervous system. The properties of FoxO proteins render them candidates to play an important role in neuronal regulatory processes. For this reason we eluded on the identification of FoxO proteins in the central nervous system. In this study we describe the cloning and characterization of a novel member of the FoxO-class and detail structural and functional properties related to gene regulation. This novel protein, FoxO6, clearly differs from FoxO1 and FoxO3 in its shuttling properties. Through mutation analysis and the generation of chimeric proteins this difference is identified as a domain absent in FoxO6 located just behind the forkhead domain in FoxO3 and FoxO1.

Experimental Procedures

PCR, cloning and sequencing. From adult C57/Bl6 mouse brain, we dissected the tissue in the ventral midbrain. Total RNA was isolated and subjected to RT-PCR for cDNA synthesis using reverse transcriptase Superscript II and both oligo(dt) and random hexamer primers. Degenerate primers (forward, 5'- MGGCTSAMHYTSKCBCAGAT-3'; reverse, 5'- TTGTGVCGRTAKGARTYCTTCCA- 3') were designed to identify (novel) members of a subset of the forkhead family of transcription factors. This set of primers amplifies part of the forkhead domain of members of the FoxO group. The annealing temperature was 45 ° C and PCR products were separated on a 2 % agarose-gel by gel-electrophoresis. Fragments of the expected length of 110 bp were purified (Qiagen PCR Purification Kit), ligated in pGemT easy (Promega) and transformed to *E.Coli* DH5alpha. Resulting colonies were subjected to colony PCR. Fragments of appropriate length were purified (Qiagen PCR-Purification Kit) and sequenced on a Beckman Coulter CEQ 2000 sequencer under standard conditions. In all other PCR reactions we used the Long range PCR kit (Roche) with the following modifications: denaturation and extension temperature were 98 ° C and 68 ° C respectively.

RNA probe synthesis. For the generation of a specific *FoxO6* RNA probe, EST clone IMAGp998p163044q2 was subjected to PCR and the amplified fragment was purified and sequenced. The fragment of 900 bp in length (200 bp coding sequence upstream the stopcodon and 700 bp 3'- UTR) did not contain the forkhead domain. T3- and T7- RNA polymerase were used in combination with a DIG RNA Labeling Kit (Roche) to synthesize a sense and antisense DIG labeled cRNA probe.

In situ hybridization. In situ hybridization was performed as follows. Cryostat sections cut at 16 µm, were thaw mounted onto Superfrost+ slides, dried and fixed for 10 minutes in fresh 4% paraformaldehyde in PBS. After washing with PBS, sections were acetylated for 10 minutes in a solution containing 245 ml H_2O, 3.3 ml triethanolamine, 438 µl HCl (37%) and 625 µl acetic anhydride. Sections were washed with PBS and prehybridized for 2 hours in a prehybridization solution (50% deionized formamide, 5*SSC, 5*Denhardt's solution, 250 µg/ml baker's yeast and 500 µg/ml sonificated salmon sperm DNA). Hybridization was performed overnight at 72 °C with 400 ng/ml DIG-labeled probe added to 150 µl hybridization solution each slide, covered with nescofilm. The nescofilm was removed in 2*SSC, and sections were placed in 0.2*SSC for 2 hours and washed in a solution containing 100 mM Tris/HCl, pH 7.4; 150 mM NaCl (buffer 1). Preincubation with 1.5 ml buffer 1 with 10% heat inactivated fetal calf serum (hiFCS) was performed for 1 hour at room temperature in a humidified chamber. Sections were incubated overnight at 4 °C with alkaline phosphatase-conjungated mouse anti DIG Fab fragment (Roche), 1:5000 diluted in buffer 1 with 1% heat inactivated fetal calf serum. Sections were washed the next day in buffer 1 and equilibrated with a solution containing 100 mM Tris/HCl, pH 9,5; 50 mM $MgCl_2$; 100 mM NaCl. Subsequently 200 µl NBT/BCIP solution (Roche) and 2.4 mg/10 ml final volume levamisole was added to a 100 mM Tris HCl, pH 9,5; 50 mM $MgCl_2$; 100 mM NaCl solution and the color reaction was performed in the dark for about 8 hours. The color reaction was stopped by adding

10 mM Tris/HCL, 5 mM EDTA, pH 8.0, slides were dehydrated with ethanol and mounted using entellan (Merck).

FoxO6-GFP translational fusion. Primers were designed to amplify the coding sequence of *FoxO1, FoxO3 and FoxO6*, introducing restriction sites, leaving the Methionine intact and removing the stopcodon. Both PCR products and the EGFP-N1 vector (Clontech) were cut with appropriate restriction enzymes and purified. After 1-hour ligation of the *FoxO1, FoxO3 and FoxO6* cDNA fragments into the EGFP-N1 vector, the resulting constructs were transformed to *E.Coli* DH5 alpha. Colonies were subjected to colony PCR, and products were sequenced. A colony carrying the correct construct was selected, grown and plasmids were purified (Qiagen). The final construct encoded FoxO1, FoxO3 or FoxO6, immediately followed by EGFP. Mutations of either Thr26 or Ser184 to alanine residues were generated using site directed mutagenesis. Resulting mutant DNA-fragments were ligated in EGFP-N1 and sequenced. For the construction of the chimeric FoxO6[4Ser] we undertook a PCR based strategy using the FoxO6-GFP construct in which we replaced FoxO6 AA 243-259 for FoxO3 AA 303-327. In a similar way we constructed FoxO6[NES2], in which we replaced FoxO6 AA 314-355 for FoxO3 AA 381-433. For the PCRs we used FoxO6 sequence based primers with FoxO3-sequence overhang, and vice versa. Both constructs were sequenced.

Cell culture and transfection of HEK-293 cells. HEK-293 cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% (v/v) hiFCS, 100 units/ml penicillin, 100 units/ml streptomycin and 2 mM L-Glutamine in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were seeded in 12 well plates, and grown for 24 hours on glass coverslips. Cells were transfected with calcium phosphate precipitates containing 1.9-µg/well plasmid DNA. (0.12 µg target construct / 1.78 µg pBlueScript carrier DNA).

Forkhead translocation protocol. Twenty hours after transfection cells were serum starved for 24 hours. Translocation was induced by replacing the serum free medium with medium supplemented with hiFCS (10% (v/v)), insulin (100nM) or IGF-1 (1 ng/ml). After 2 hours of incubation cells were fixed using 4 %PFA in PBS for 10 minutes at room temperature. Slides were embedded in Dabco-mowiol and analyzed by fluorescent-microscopy. In experiments using inhibitors, cells were preincubated with either PD98059 (25µM), LY294002 (25µM) (TOCRIS) or Leptomycin B (2ng/ml) for 1 hour. Subsequent stimuli were in the presence of inhibitors.

Luciferase assays. Cells were grown in 6 well plates and transfected with 5µg of plasmid DNA/well, including, 1µg 6xDBE-Luc (kindly provided by BM Burgering), with or without 0.3µg FoxO-GFP or empty vector and the appropriate amount of carrier plasmid. After transfection, cells were lysed and total GFP fluorescence was measured in 96well plates using a FujiFilm FLA-5000 image reader to normalize the samples for transfection efficiency. Linearity of the measurements was checked with an EGFP standard curve. Luciferase activity of each sample was measured and corrected for total FoxO-GFP. Each experiment was at least performed in triplicate.

Results

Isolation and characterization of FoxO6 mRNA. We used a degenerate PCR-strategy to screen for (novel) members of the FoxO-group of forkhead transcription factors expressed in the mouse ventral midbrain. Primers were designed to amplify part of the forkhead domain, a region with high sequence homology. Using this strategy we cloned PCR fragments encoding FoxO1, FoxO3 and FoxO4. Interestingly, we cloned a PCR fragment that showed high similarity with these genes, but differed in 10 out of 110 bp compared to its closest family member. Database analysis of this sequence in mouse genomic DNA databases led to characterization of the putative 3'- and 5'- part of a novel gene of the FoxO-group of transcription factors. Initially 7 mouse-derived ESTs from mouse tissue were identified, originating from the 3'- region (BI686281, AA656491, BF581745, AI593097, D21486, AI425281, BF461725), and only recently a mouse brain-derived EST was released originating from the 5' region (CA316065). Based on genomic DNA-sequence information, primers (forward; *gcgggaccatggctgcgaagc*, reverse; *acttcaaccatccctcccagac*) were designed to amplify the total coding region from mouse ventral midbrain cDNA. The resulting PCR fragment was cloned and sequenced. Primary sequence analysis revealed that the amplified cDNA contained a large open reading frame predicted to encode a 559 amino acid protein. The presence of a forkhead domain and overall similarity to FoxO1, FoxO3 and FoxO4, identified the protein as a novel member of the FoxO-class of forkhead transcription factors (Figure 1). Since FoxO5 is already designated in zebrafish (3), we named this gene *FoxO6*. Noteworthy are the recently submitted "genome scan" gene predictions (XM284000, XM143959) based on genomic and EST sequence data. These predictions are incomplete and incorrect for the fact that part of the genomic sequence of *FoxO6* is not yet present in the databases. Comparison of the deduced amino acid sequence of FoxO6, FoxO1, FoxO3 and FoxO4, demonstrated that FoxO6 is 34% identical to FoxO1, 38% identical to FoxO3, and 36% identical to FoxO4 over their shared lengths. Within the forkhead domain this identity is increased to 90% for FoxO1, 89% for FoxO3 and 90%for FoxO4 (Figure 1).

Chromosomal structure and localization. In the murine genome *FoxO6* is located on chromosome 4, region D1 between chromosomal markers 1283756 and X59556 (within 20 Kb of marker 1283756), according to the MGSC v3-database of the Sanger Institute. Mouse genomic database analysis revealed that the open reading frame of *FoxO6* is divided by a large intron of approximately 18 KB long, resulting in 2 putative exons of 414 and 1266 bp in length. A polyadenylation signal (AATAAA) is found 818 bp downstream from the stop codon. This 3'- end corresponds to 3'- EST sequences which indicates that the FoxO6 mRNA contains a 3'- UTR of at least 818 bp long and that this is in fact the last exon of the *FoxO6* gene. The startcodon (GGCGGGACC**ATG**G) of the mapped FoxO6 amino acid sequences lies within a proper Kozak sequence. In addition, the 5' EST contains no upstream startcodons in either frame. These facts and the homology to FoxO1, FoxO3 and FoxO4 indicate that the mapped methionine is the correct startcodon. Based on the 5' EST, *FoxO6* contains a 5' - UTR of at least 98 bp. Comparison of mouse *FoxO6* to human genomic databases revealed that the human *FoxO6*-orthologue is located at chromosomal region 1p34.1. Within this regions several diseases have been mapped, but no clear indication for FoxO6 dysfunction related disease

could be identified. In the human EST database four different 3'-ESTs were found, originating from brain tissue and tumor-cell lines. (AI361654, AI341823, M85901, AA927741) All human ESTs showed approximately 95% sequence identity to mouse *FoxO6*.

Expression pattern of FoxO6 in murine tissue. In order to elucidate the possible function of FoxO6 we examined the spatial and temporal expression pattern in murine tissues. In-situ hybridizations using DIG-labeled probes specific for *FoxO6*-transcripts was performed in adult mouse brain (Figure 2). In rostral sections the *FoxO6* transcript was detected in the ependyma, the medial part of the anterior olfactory nucleus and diffuse in the cingulate cortex (Figure 2A). More caudal, expression was detected in the shell of the nucleus accumbens, the claustrum, the dorsal endopiriform nucleus and the cingulate cortex (Figure 2B). The transcript was dominantly present in the hippocampus, especially CA1 and CA3 areas, and to a lesser extent in the dentate gyrus and CA2 area (Figure 2C and D). Furthermore, the transcript was detected in the posteroventral part of the medial amygdaloid nucleus, portions of the amygdalo- hippocampal area, and dorsal and ventral endopiriform nuclei. In E12.5 embryos, a high level of expression of *FoxO6* was detected in the trigeminal ganglion and tissue surrounding the lateral portion of the fourth ventricle that forms the cerebellum (Figure 3A). The olfactory epithelium showed high amounts of the transcript, as well as the dorsal root ganglia along the embryo's spine (Figure 3B). Lower amounts of the transcript were found in striatal areas and in the neopallial cortex, that forms the cerebral cortex. The level of expression in the olfactory epithelium and the dorsal root ganglions was sustained in embryos of E14.5 and E18.5, whereas expression in the trigeminal ganglion and developing cerebellum was diminished and expression in the striatal area was slightly increased. In addition, embryos of E14.5 showed a markedly increased expression in the neopallial cortex (Figure 3C). Expression in the neopallial cortex was most apparent in the outermost layer which represents the layer of cells that migrated most recently (Figure 3D). In the cerebral cortex of E18.5 mouse, the transcript was still abundantly detected. This was also the case in the developing hippocampal areas (Figure 3E), especially the inner layer that also contains the most recently migrated cells. In the periphery, the FoxO6 transcript was detected in the thymus (Figure 3F), the cortical region of the kidney (Figure 3G), the whiskers and dents (data not shown). These data show that the *FoxO6* gene is dominantly present in the developing and adult murine brain, indicative for a function of FoxO6 during development and in the adult functional central nervous system.

FoxO6 lacks a region containing a PKB-, CK1- and DYRK1A-phosphorylation motif. In FoxO1, FoxO3 and FoxO4, three PKB phosphorylation motifs (13) have been reported (8). The first PKB phosphorylation motif is located in the region just downstream the startcondon, a second in the forkhead domain, and a third in a region just downstream the forkhead domain. (Figure 4A). The first and second regions containing a PKB-phosphorylation motif are present in FoxO6 as well. Strikingly, the third region containing a motif for PKB catalyzed phosphorylation is absent in FoxO6. Besides a PKB-phosphorylation motif, this region contains a stretch of 3 additional serine residues, present in the other members of the FoxO group (Figure 4B). In FOXO1, Ser 319 is substrate for PKB, Ser322 and Ser325 are phosphorylated by CK1 (14), and Ser329 is phosphorylated by DYRK1A (15). Although homology of FoxO6 to FoxO3 and O1 is

high just upstream this region, the conserved PKB site including the stretch of serine residues is not conserved. Noteworthy is the fact that a third Arg-Xaa-Arg-Xaa-Xaa-Thr motif is found in the far C-terminus of FoxO6, in a region that shows no similarity to the other FoxO proteins and Daf16. In addition, no CK1 or DYRK1A motifs are found in this region. Therefore it is not sure whether the threonine residue in this region is a natural substrate for PKB.

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FoxO6     ------MAAKLRAHQVDVDPDFAPQSRPRSCTWPLPQPDLAG--------------------DEDGA
FoxO1     MAEAPQ--------V.ET....E.LP.Q........R.EFNQSNSTTSSPAPSGGAAANPDAAASL.
FoxO3     MAEAPASPVP.SPLE.EL..E.E............QR.E.QASPAKPSGETAADSMIPEEDD...DE
FoxO4     MDPENKKS.TGA.AIL.L....E.............R....T------------------------

FoxO6     LGAG-----------------------------VAEGSEDCG-----P----------ERRATAPAM
FoxO1     SASAVSTDFMSNLSLLEESEDFARAPGCVAVAAA.AA.RGLCGDFQG.EAGCVHPAPPQPPP.G.LS
FoxO3     D.G.RASSAMVIGGGVSSTLGSGLLLEDSAML-L.P.GQ.L.SGPASAAGALSGGTPTQLQPQQ.LP
FoxO4     ------------------------------EPHEPSEVEPSLGQKV.TEGHSEPILLPS.LPE..G

FoxO6     APAP-------PLGAEVGPLRKAKSSRRNAWGNLSYADLITKAIESAPDKRLTLSQIYDWMVRYVPY
FoxO1     Q.P.VPPSAAAAA.PLA.QP..TS.....................SAE.........E...KS...
FoxO3     Q.Q.-------GAAGGS.QP..CS.-...............R....S...........E....C...
FoxO4     G.Q.------GI...-.TGP..GG.-.......Q...E..SQ......E.....A...E....T...
                        ><
FoxO6     FKDKGDSNSSAGWKNSIRHNLSLHTRFIRVQNEGTGKSSWWMLNPEGGKTGKTPRRRAVSMDNGAKF
FoxO1     ........................SK.......................S..S.....A....NS..
FoxO3     ........................S..M.............II..D...S..A..........SN.Y
FoxO4     ........................SK..K.H..A...........D...G..A.....A...SSS.L

FoxO6     LTIKGKASKKKQ--LHLPERSPDDSPPGAPVPGPLSASAKWAASPASHASDDYEAWADFRGSRRP--
FoxO1     AKSR.R.A...AS-.QSGQEG.G...-.--------.QFS..P...G..SN..FDN.ST..PRTSSNA
FoxO3     TKSR.R.A...AA-.QAAPE.A....---------.QLS..PG..T.RS..ELD..T...SRTNSNA
FoxO4     .RGRS.GP...PSV.PA.PEGATPRS-------..GHF...SS..CPRNREEADV.TT..PRSSSNA

FoxO6     ------LLGEAAELEDDEALEALAPSSPLMYPSPASALSPALGARCPGELPRLAELGGPLGLHGGGV
FoxO1     STISGR.SPIMT.QD.LGDGDVHS------------LVY.PSA.KMAST..S.S.ISN.ENMEN--L
FoxO3     STVSGR.SPILAST.L.DVQDDDG.L..ML.S.-SAS...SVSKP.TV.....TDMA.TMN.ND.LA
FoxO4     STVSTR.SPMRP.S.VLAEE.MP.----------S---ASSYAGGV.PT.SEDL..LDG.N.AS---

FoxO6     AGLPDALLDGAQDAYGPRARAGTPSYFGS-----CKASAYGG--GGG----FGPPALGSLRRLPMQT
FoxO1     LDNLNL.SSPTSLTVSTQSSP.SMMQQTPCYSFAPPNTSLNSPSPNYSKYTY.QSSMSP.PQM....
FoxO3     EN.M.D...NIALPPSQPSPP.GLMQR..SFPYTA.S.GL.SPT.SFNSTV...SS.N...QS....
FoxO4     ---.HS..SRSGLSGFSLQHP.LAGPLH.----------------------Y.ASLF.PI-------

FoxO6     IQENKQASFVQAAAPFRPGALPALLPPPPPAPRPGPLLGAPGELALAGAAAAYPGKGAAPYAPPAPS
FoxO1     L.DS.SSYGGLNQYNCA..L.KE..TSDS.PHNDI-MSPVDPGV.QPNSRVLGQNVMMG.NSVMPAY
FoxO3     ....R-PATFSSVSHYGNQT.QD..ASDSLSHSDVMMTQSDPLMSQ.ST.VSAQNARRNVMLRND.M
FoxO4     ---DGSL.AGEGCFSSSQSLEAL.TSDT..P.ADVLMTQVDPI.SQ.PTLLLLG.MPSSSKLGTGV.

FoxO6     RSALAHPIS---------------------LMTLPGEAGAAGLAPPAHAAAFGGP---PGG----LL
FoxO1     G.QAS.NKMMNPSSHTHPGHAQQTASVNGRTPPHVVNTMPHTS.MNRLTPVKTPLQVPLSHPMQMSA
FoxO3     M.FA.Q.TQGSLVNQNLLHHQHQTQGALGGSRA.SNSVSNM..SDSSSLGSADHQQQS.ASQSMQT.
FoxO4     LCPTPLEGP-----------------------------.PSN.V.NLSVM.PPPV-----M----AG

FoxO6     LDALPG-PYAAAAAGPLGAGPDRFPADLDLDMFSGSLECDVESIILNDFMDSDEMDFNFDSALPPP-
FoxO1     .GRYSSVSSCNGYGRMGVLHQEKL.S...G-..IER.D..M....R..L..G.TL.....NV..NQ-
FoxO3     S.S.S.SSLYS.S.NLPVM.H.K..S.......N......M....RSEL..A.GL......LISTQN
FoxO4     APIPKVLGTPVL.SPTEDSSH..M.Q......YMEN....MDN..-S.L..GEGL....EPDP*

FoxO6     ---P-PGLAGAPPPN--QSWVPG*
FoxO1     -------SFPHSVKTTTH...S.*
FoxO3     VVGLNV.NFTGAKQASS......*
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Figure 1. Amino acid sequences of the mouse FoxO6 protein compared to FoxO1, FoxO3 and FoxO4. FoxO6 is 34% identical to FoxO1, 38% identical to FoxO3, and 36% identical to FoxO4 over their shared lengths. Within the forkhead domain this identity is 90% for FoxO1, 89% for FoxO3 and 90% for FoxO4. The N- and C-terminal regions have an overall high degree of similarity. The forkhead domain is underlined, conserved amino acids are indicated as dots, and gaps are indicated as hyphens. The junction of the two exons in the *FoxO6* gene is indicated by ><.

Translocation of FoxO6 is dramatically decreased compared to FoxO1 and FoxO3. Previous studies in mammalian cell lines have shown that in response to stimulation with insulin-like growth factors, PKB phosphorylates FOXO-proteins. This results in translocation of the forkhead protein from the nucleus to the cytosol (8). To test whether FoxO6 responds in a similar manner to growth factor stimulation, we transfected human embryonic kidney-cells (HEK- 293) with FoxO1-, FoxO3- and FoxO6-GFP-constructs. Twenty-four hours after transfection FoxO1 and FoxO3 displayed a predominant cytosolic localization in virtually 100% of transfected cells for FoxO1 and approximately 80% for FoxO3. In strong contrast to FoxO1/FoxO3, FoxO6 was fully localized in the nucleus 24 hours after transfection (Figure 5, 1st column). Subsequent serum starvation for 20 hours resulted in a predominant nuclear localization for FoxO1 and FoxO3, although some cytoplasmic fluorescence was still apparent. FoxO6 however had an exclusive nuclear localization (Figure 5, 2nd column). When stimulated with serum, IGF-1 or insulin, FoxO1 and FoxO3 were excluded from the nucleus and showed a predominant cytosolic localization (Figure 5, 3rd, 4th and 5th column). Under these conditions FoxO6 displayed a predominant nuclear localization. Although translocation of FoxO6 was significantly less as compared to FoxO1 and FoxO3, a general increase in cytoplasmic fluorescence was detected. This indicates that some protein export from the nucleus had occurred.

Translocation of FoxO6 is mediated by a PI3-kinase-dependent mechanism. To assess whether nuclear export of FoxO6 is regulated in a PI3-kinase-dependent manner, we preincubated cells with LY294002, a PI3-kinase inhibitor, before treatment with either IGF-1 or insulin. PI3-kinase inhibition resulted in a significant decrease in cytosolic localization of FoxO6 in cells treated with either IGF-1 or insulin (Figure 6). Besides the PI3-kinase pathway, IGF-1 and insulin can activate the MAPK-pathway as well. Cells preincubated with PD98059, an inhibitor of the MAPK pathway, displayed no difference in IGF-1/insulin induced translocation. These findings clearly indicate that translocation of FoxO6 upon IGF-1 or insulin stimulation is mediated by the PI3-kinase pathway. These results are in perfect agreement with results from similar studies done with other FoxO proteins (16,17,18).

Mutation of Thr26 or Ser184 blocks nuclear exclusion of FoxO6. Mutation analysis in FOXO1 has shown that substitution of Thr24 or Ser256 by alanine residues (mimicking a non-phosphorylated state) results in a blocked nuclear exclusion (16,19). As stated before, the regions containing Thr24 and Ser256 in FOXO1 are highly conserved in all members. Thr26 and Ser184 are the equivalent residues in FoxO6 and are therefore potentially phosphorylated by PKB as well, resulting in nuclear export. To verify this possibility, we constructed mutant FoxO6 proteins, where either the Thr26 or the Ser184 residue was substituted by an alanine. Both mutant FoxO6 proteins displayed no IGF-1-induced increase in cytosolic localization, in contrast to the wild type protein (Figure 7). This clearly demonstrates that each PKB phosphorylation motif is required for nuclear exclusion of the FoxO6 protein

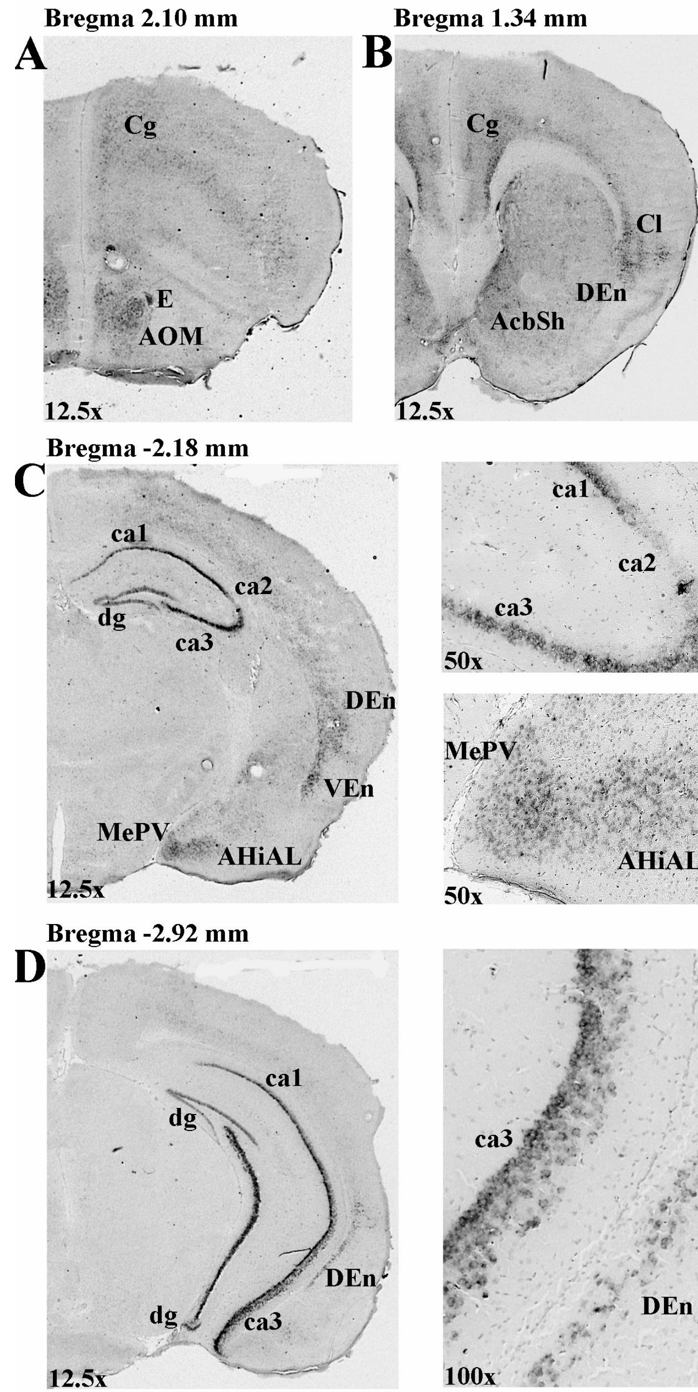


Figure 2. Expression of FoxO6 in the mouse brain. In situ hybridization on coronal sections of adult mouse brain, using a DIG-labeled RNA-probe specific for *FoxO6* mRNA is shown. In rostral sections (A and B) expression was detected in the medial part of the anterior olfactory nucleus (AOM), the ependyma (E), the cingulate cortex (Cg), claustrum (Cl), the dorsal endopiriform nucleus (DEn), and the shell of the nucleus accumbens (AcbSh). More caudal (C and D), expression was detected in hippocampal areas CA1, CA2 and CA3 (ca1/2/3), dentate gyrus (dg), posteroventral part of the medial amygdaloid nucleus (MePV), anterolateral part of the amygdalohippocampal area (AHiAL) and ventral and dorsal endpiriform nucleus (VEn, Den). Control experiments with sense probes of the same sequence did not give any signal.

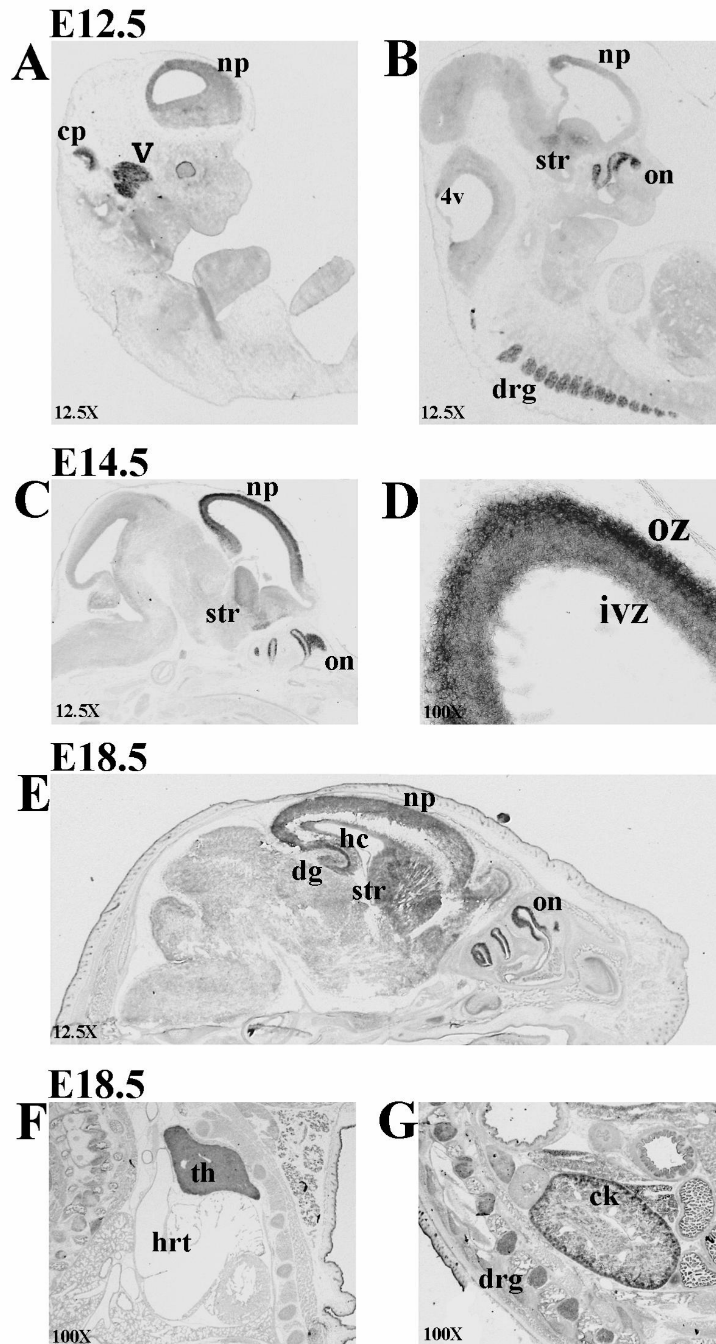
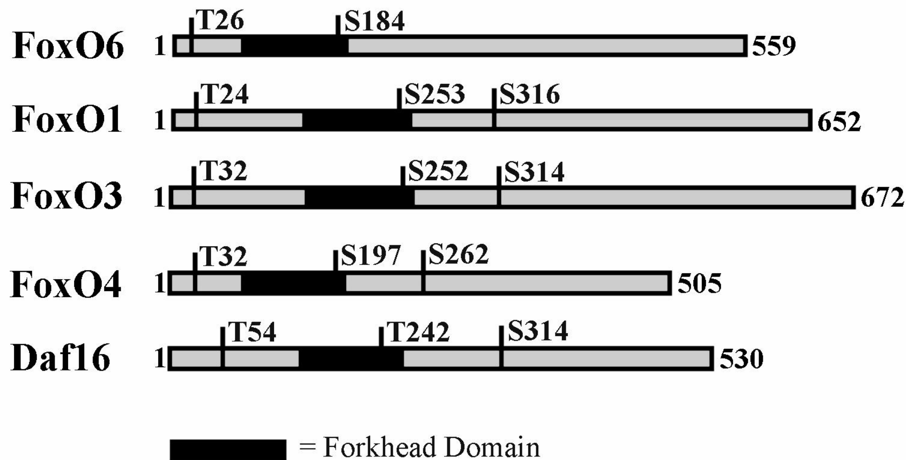


Figure 3. Expression of the FoxO6 gene in the mouse embryo. In situ hybridization for *FoxO6* on embryonic mouse sagital sections from stage E12.5 to E18.5 is shown. In E12.5 embryo's (A and B), *FoxO6* was expressed in the neopallial cortex (premordial cerebral cortex) (np), the trigeminal ganglion (V), the cerebellum primordium (premordial cerebellum) (cp), the olfactory neuroepithelium (on), striatum (str) and the dorsal root ganglions (drg). At E14.5 increased expression in the neopallial cortex (C), most prominent in the outer zone (oz) of the cortex is detected (D). (ivz; interventricular zone of neopallial cortex). In E18.5 embryos additional expression was detected in hippocampus (hc) and dentate gyrus (dg) (E), the thymus (th) (F) and the cortex of the kidney (ck) (G). (hrt; heart). Control experiments with sense probes of the same sequence did not give any signal.

A



B

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FoxO6 (Thr26)   QSRPRSCTWPLPQ
FoxO1 (Thr24)   LPRQRSCTWPLPR
FoxO3 (Thr32)   LPRQRSCTWPLQR
FoxO4 (Thr32)   LPRQRSCTWPLPR

FoxO6 (Ser184) TPRRRAVSMDNGT
FoxO1 (Ser253) SPRRRAASMDNNS
FoxO3 (Ser252) APRRRAVSMDNSN
FoxO4 (Ser197) APRRRAASMDSSS

FoxO6          DFR---------------
FoxO1 (Ser316) TFRPRTSSNASTISGRLS
FoxO3 (Ser314) DFRSRTNSNASTVSGRLS
FoxO4 (Ser262) TFRPRSSSNASTVSTRLS
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Figure 4. Phosphorylation motifs of the mouse FoxO6 protein compared to other mouse FoxO members and DAF16. A) Positions of conserved regions containing putative PKB-phosphorylation sites in mouse FoxO proteins. All members contain PKB phosphorylation sites in the forkhead domain and in the N-terminal region. Note the missing third conserved region containing a PKB phosphorylation site C-terminally from the forkhead domain in FoxO6. B) Comparison of FoxO1, FoxO3, FoxO4 and FoxO6. The proteins all contain the first two highly homologous regions containing a putative PKB phosphorylation site. The third conserved region is missing in FoxO6. Phosphorylated serine or threonines are underlined. The additional CK1 phosphorylated serines are in italics (first and second additional serine is phosphorylated by CK1, the third additional serine is phosphorylated by DYRK1A. The position of the forkhead domain is shown in black.

FoxO6 functions as a transcription factor. To investigate whether FoxO6 is indeed a functional transcriptional activator, we analyzed its activity on a luciferase reporter

construct containing six optimal DAF-16 binding elements (6DBE). In this experiment we compared FoxO6 activity to FoxO1 and FoxO3 in order to assess whether FoxO6 functions similar to these other two proteins. Under the experimental conditions used, the basal activity of the 6DBE reporter construct is very low as was found after transfection of the EGFP vector.

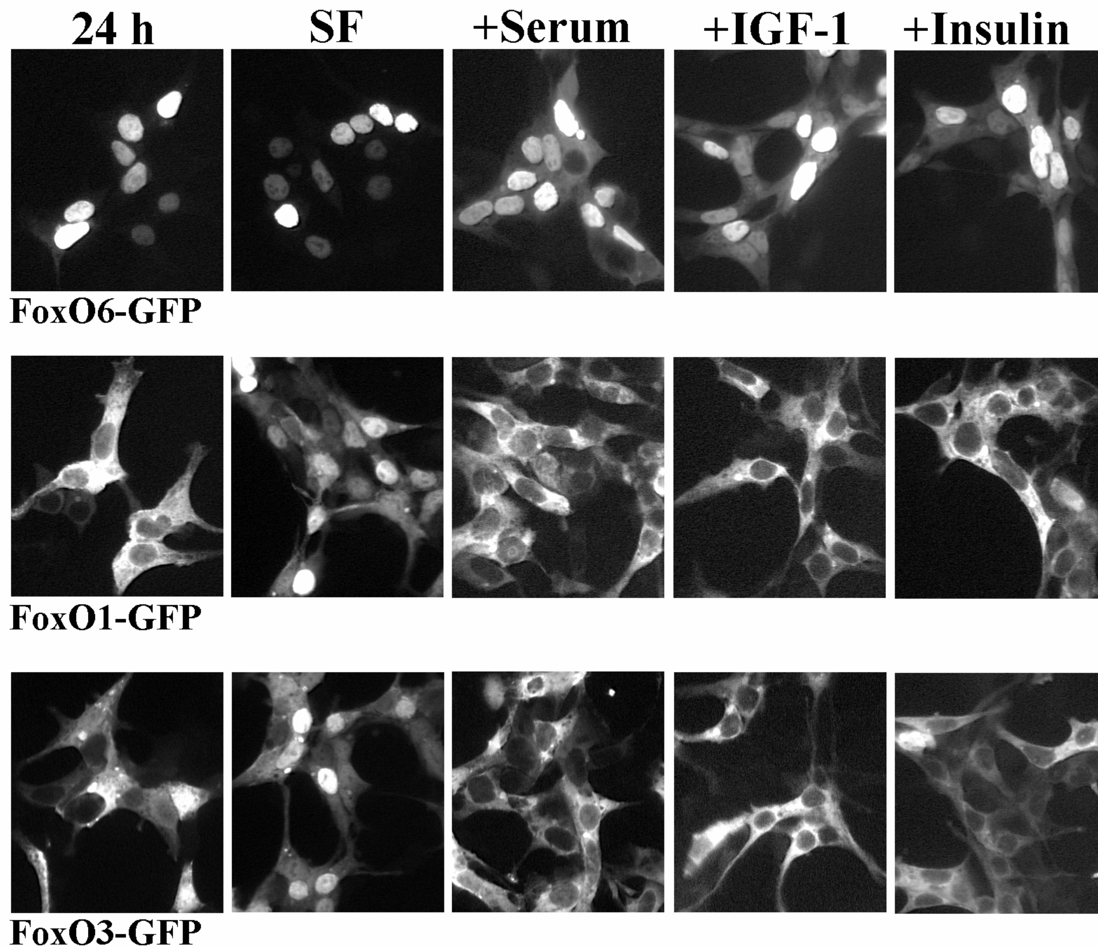

Figure 5. Translocation properties of FoxO1, FoxO3 and FoxO6. Cells were transfected with expression vectors for FoxO-GFP. 24 hours after transfection (24 h), cells were serum starved for 24 hours (SF) and subsequently treated with 10%hiFCS (Serum), IGF-1 or insulin. FoxO6 (upper panel) has a predominantly nuclear localization under all conditions, and shows an increase in cytosolic fluorescence when treated with growth factors. FoxO1 and FoxO3 (middle and lower panels) relocalize to the nucleus when cells are serum-starved, and can completely be shuttled back to the cytoplasm when treated with growth factors. Experiments using a control construct expressing GFP alone, resulted in an even distribution of fluorescence through the whole cell (data not shown).

The data on the FoxO activity showed that FoxO3 had the highest activity, FoxO6 activity was intermediate and FoxO1 showed the lowest level of transcriptional activation (Figure 8A). Since this experiment was performed under serum free conditions the localization of all different factors is mainly nuclear. Previous studies have shown that FoxO transactivation is highly dependent on the phosphorylation status of the serine

located in the DNA binding domain. Phosphorylation of this serine is described to reduce DNA binding and thereby transactivation (20). FoxO6 has a nuclear localization under serum and serum free conditions. Therefore, we compared FoxO6-WT and Ser184 mutants to investigate possible differences in transactivation as a consequence of the phosphorylation state of Ser184 (Figure 8B). FoxO6-WT had low transactivation properties as compared to the FoxO6-184Ala under serum conditions and could be reduced even further by mutating FoxO6-Ser184 to an aspartic acid (FoxO6-184Asp). Serum starvation increased FoxO6-WT activity to a comparable level as found for the FoxO6-184Ala mutant. These data indicate that indeed the phosphorylation state of Ala184 is essential in regulating the transactivating properties of FoxO6 independent of its subcellular localization.

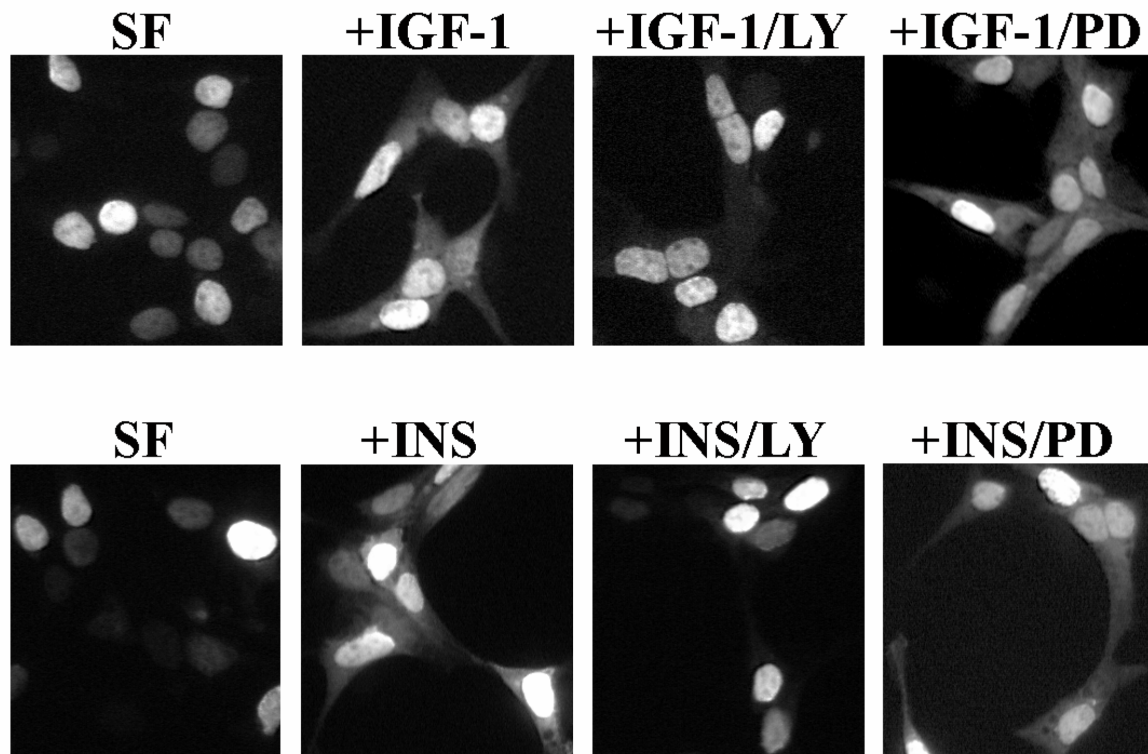

Figure 6. Nuclear export of FoxO6 is PI3-kinase dependent. IGF-1 or insulin-induced translocation of FoxO6 was inhibited by the specific PI3-kinase inhibitor (LY), but not by the specific inhibitor of MAPK activation (PD). Conditions were as described in the legend of Figure 5.

Shuttling of FoxO6 can be restored through insertion of a Fox03 derived phosphorylation domain. Here we address why FoxO6 is largely retained in the nucleus after growth factor stimulation in contrast to FoxO1 and FoxO3. In our view, there are two structural differences that potentially underlie FoxO6's remarkable distinct translocation efficiency (Figure 9). The first domain is a nuclear export signal (NES) described by Brunet et al. (21). They report that in FOXO3a, two NESs are crucial for nuclear export. FoxO6 has a putative (optimal) NES corresponding to the first in FOXO3, but lacks convincing similarity to the second NES. The second domain that is absent in FoxO6 as compared to the other FoxO proteins, as mentioned above, contains a stretch of four serine residues, which have been shown to be phosphorylated by the kinases PKB, CK1 and DYRK1A.

Mutational analysis of FOXO1 by Rena et al. (14) revealed that mutation of Ser319, Ser322 or Ser319/Ser322/Ser325/Ser329 to alanine residues results in a decreased speed of nuclear export.

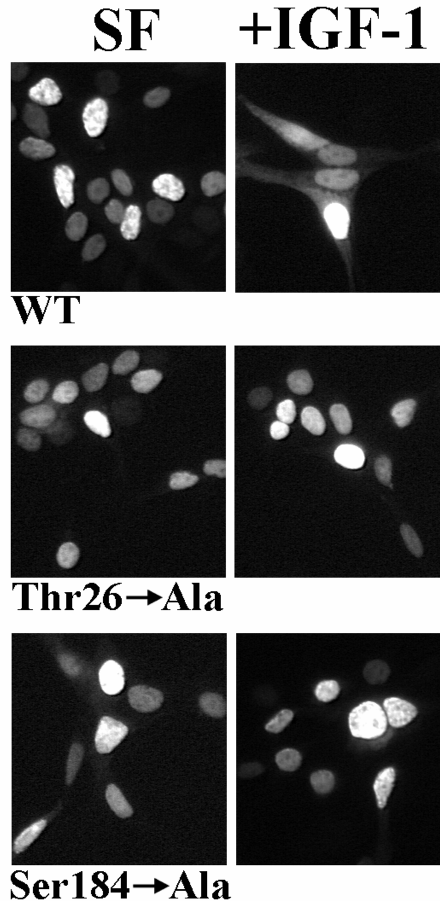

Figure 7. Mutation of PKB phosphorylation sites within FoxO6 abolishes IGF-1-induced nuclear export. Wild type FoxO6 (WT). Mutation of threonine 26 to alanine (Thr26->Ala) and mutation of serine 184 to alanine (Ser184->Ala) block translocation in response to IGF-1.

To test whether the absence of a second NES or the stretch of phosphorylation sites could explain the impaired nuclear export of FoxO6, we constructed two chimeric proteins (Figure 9). In the first chimeric protein, FoxO6[NES2], we replaced part of FoxO6 for the FoxO3 sequence containing the second NES. In the second chimeric protein, FoxO6[4Ser], part of the FoxO6 sequence was replaced by the domain containing the stretch of serine residues as present in FoxO3. Both chimeric constructs were transfected and localization was monitored at three different stages; 24 hours after transfection, deprived of serum for an additional 20 hours, and subsequent insulin stimulation. The subcellular localization of FoxO6[NES2] was similar to wild-type FoxO6. (Figure 10A, 2nd and 3rd row). Interestingly, localization of FoxO6[4Ser] was significantly altered compared to wild-type FoxO6. After transfection, cells showed both nuclear and cytosolic FoxO6[4Ser]. This resembled FoxO3 localization rather than FoxO6 (Figure

10A, 1st, 2nd and 4th row). Serum starvation led to an exclusive nuclear localization of FoxO6[4Ser] and subsequent insulin stimulation resulted in a surprisingly high level of cytosolic staining in all cells. Insulin induced FoxO6[4Ser] cytosolic relocation was significantly reduced by PI3-Kinase inhibition, indicating that nuclear export is PI3-kinase mediated. In addition, we monitored cellular relocalization upon insulin stimulation in combination with Leptomicin B (LMB), an inhibitor of Crm1- and NES-dependent nuclear export (Figure 10B)(21). The data from this experiment showed that FoxO6[4Ser] was exclusively localized in the nucleus, indicating that insulin-stimulated nuclear export of the FoxO6[4Ser] chimeric protein is a Crm1-and NES-dependent process.

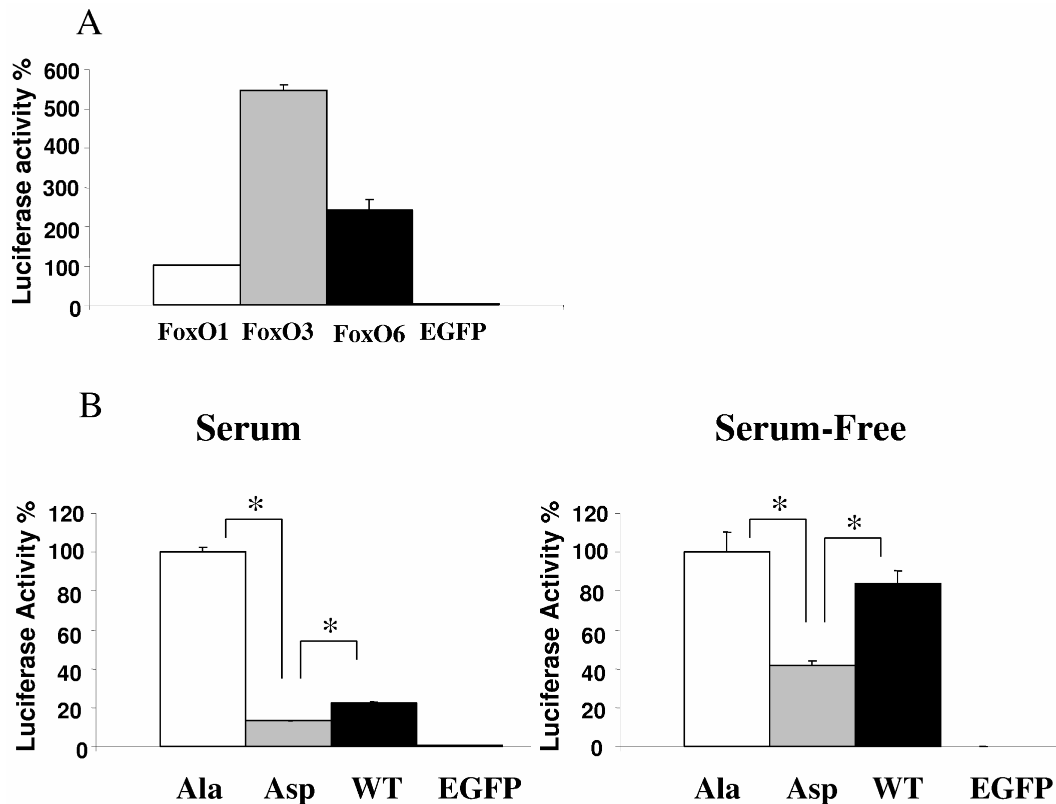

Figure 8. Comparison of FoxO transcriptional activity on a 6DBE containing luciferase construct. A) FoxO1 activity was set at 100% and compared to FoxO3 and FoxO6 and the EGFP vector. Cells were serum starved for 20-24h before measurement of luciferase activity. B) Activity of FoxO6 wild type (WT), FoxO6-GFP in which Ser184 was replaced with an alanine (Ala), FoxO6-GFP in which Ser184 was replaced with an aspartic acid (Asp) or EGFP. The Ser184 to alanine mutant was set to 100% since this protein exhibits constitutive DNA binding activity. Cells were grown for 20-24h in serum or serum free conditions. Statistical analysis was performed using a Student's t-test.

Data obtained from the insulin-induced relocalization to the cytosol was quantified by counting cells on a representative area of the slide and monitoring FoxO localization in each individual cell (Figure 11). FoxO3 was present in about 50% of the cells exclusively in the nucleus (Figure 11; "N") and in about 50% both in the nucleus and the cytosol (Figure 11; "C/N") under serum free conditions. After the application of insulin this shifts largely (~50% of the cells) to an exclusive cytosolic localization (Figure 11;

"C"). FoxO6 was almost exclusively localised in the nuclear compartment under serum free conditions and was present in about 20% of the cells both in the nucleus and in the cytosol (20% C/N) after insulin treatment. No clear differences between the FoxO6[NES2] chimera and the wild-type FoxO6 protein was observed. Under serum free conditions, the FoxO6[4Ser] mutant matched wild type FoxO6, but showed a dramatic shift towards an exclusive cytosolic localization after insulin treatment. In sharp contrast to the wild-type FoxO6, the FoxO6[4Ser] chimera was exclusively located in the cytosol in approximately 30% of the cells.

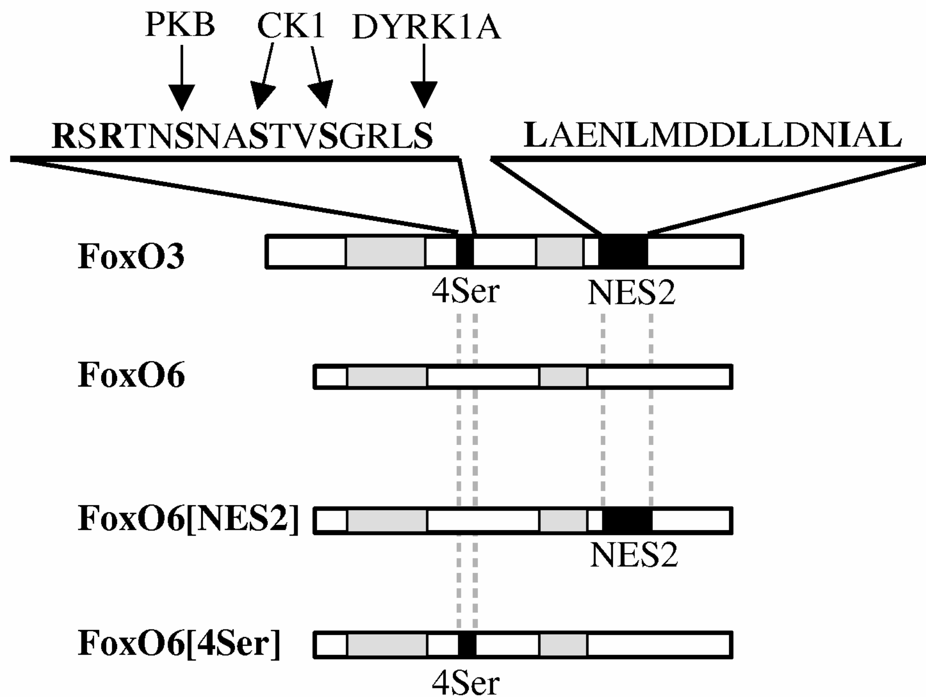

Figure 9. Schematic representation of the conserved FoxO domains used for the construction of chimeric FoxO6-FoxO3 proteins. FoxO3 contains two nuclear export sequences (NES) located at the C-terminal part of the protein. The second FoxO3 NES, which is absent in FoxO6, was placed in the corresponding region of FoxO6 (FoxO6[NES2]). FoxO3 contains a region containing four serine's including the third conserved PKB phosphorylation site. This region, absent in FoxO6, was placed in the corresponding region of FoxO6 (FoxO6[4Ser]).

Discussion

Here we describe the identification, expression and functional characterization of a novel gene of the *FoxO*-group of forkhead transcription factors in mouse, which we designated *FoxO6*. In-situ hybridization experiments showed that the *FoxO6* gene was predominantly expressed in a restricted manner in the developing and adult brain, especially cortical and hippocampal structures. In the adult brain, besides some positive

cells scattered through the cerebral cortex, cortical expression was limited to the endopiriform nuclei. The endopiriform nucleus is a limbic related structure and is thought to play a part in the acquisition of conditioned fear (22) and is associated with temporal lobe epileptogenesis (23). It has been reported that this cortical structure is connected to the claustrum, amygdala and the anterior olfactory nucleus (23). Connections between the endopiriform nucleus and hippocampal structures were found as well. Interestingly, *FoxO6* is expressed in all these structures in the adult animal suggesting that FoxO6 expression is consistent with a functional neuronal network.

The similarity of FoxO6 to the other members of the FoxO-class, FoxO1, FoxO3 and FoxO4 ,was highest in the forkhead domain, whereas other regions were highly to moderately conserved. Remarkable is the homology in two regions that have been reported to be involved in phosphorylation by PKB via the PI3-kinase/PKB pathway. Strikingly, a third conserved region containing a stretch of four phosphorylation sites as found in the other FoxO proteins including Daf16, is not present in FoxO6. Furthermore, in FoxO6 there is reasonable sequence conservation in regions implicated in transactivation as described by So and Cleary (24). With the use of a reporter construct we have shown that FoxO6 is indeed a fully functional transcription factor, that can be compared to FoxO1 and FoxO3, in its transactivating property. Experiments showed that there are differences in transcriptional activity amongst individual FoxO proteins.

To investigate possible differences in translocation kinetics between FoxO6 and other FoxO proteins, we included FoxO1 and FoxO3. Our data show that FoxO1 and FoxO3 are mainly located in the cytoplasm under serum conditions, whereas FoxO6 was mainly located in the nucleus. The high nuclear level of FoxO6 could imply transcriptional activity under these conditions, in contrast to FoxO1 and FoxO3. Serum starvation resulted in a predominant nuclear localization of all FoxO proteins. Subsequent stimulation with growth factors resulted in a predominant cytosolic localization of FoxO1 and FoxO3 proteins, which is conform data from other groups (16,17). Although the cytosolic level of the FoxO6 protein was slightly increased after stimulation, indicating the ability of FoxO6 to translocate, FoxO6 protein was still predominantly localized in the nucleus. Apparently, some intrinsic property of FoxO6 significantly influences the quantity or ratio between nuclear import and export.

To address the structural properties of FoxO6 that underlie the distinct translocation properties, we first determined whether the PKB motifs in FoxO6 are functionally conserved and whether the observed translocation was mediated through the PI3-kinase/PKB pathway. In all FoxO proteins, including FoxO6, two regions containing a PKB-phosphorylation motif are conserved.
In FOXO1, PKB-catalyzed phosphorylation of Ser256 is thought to be critical for phosphorylation of Thr24. These phosphorylated amino acids form a motif for binding of 14-3-3 proteins, that in turn stimulate nuclear exclusion and cytoplasmic retention by masking a nuclear localization signal (NLS) (19,21,25). Inhibition of PI3-kinase, or mutation of these PKB-phosphorylation motifs in FOXO1, results in a blocked nuclear exclusion (19). These studies clearly indicate that PI3-kinase/PKB-mediated phosphorylation of these residues is critical for translocation of the forkhead protein from

nucleus to cytosol. In this study we show that this is also the case for FoxO6. We showed that blockade of PI3-kinase or mutation of Thr26 or Ser184 all cause the inhibition of FoxO6 nuclear export. This indicates that the regions containing Thr26 and Ser184 in FoxO6 are indeed functionally conserved PKB phosphorylation sites.

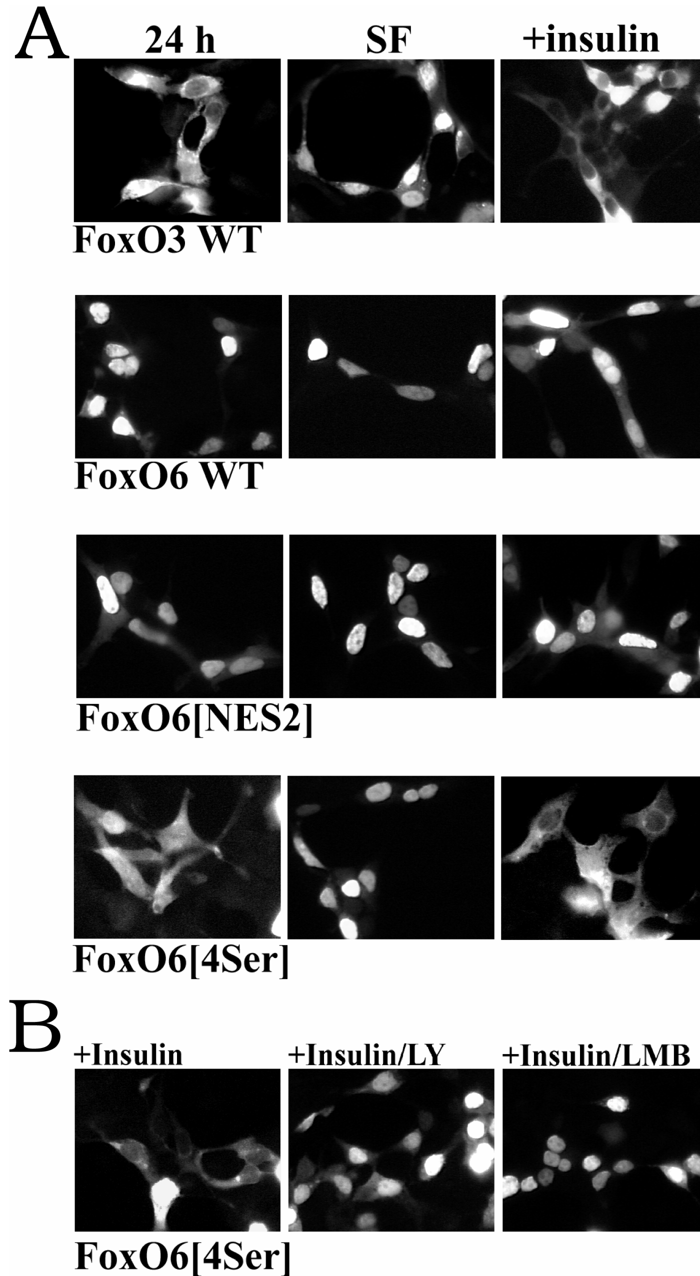

Figure 10. Translocation of chimeric FoxO6-FoxO3-GFP proteins in cells. A) Wild-type FoxO6 and FoxO3 were compared to the FoxO6 mutants: FoxO6[NES2] and FoxO6[4Ser](se Figure 9). After transfection cells were grown for an additional 24h in serum containing medium (24h). Subsequently cells were grown in serum free medium for 20h (SF) before treatment with 100 nM insulin (+insulin). B) Subcellular localization of FoxO6[4Ser] after treatment with insulin with or without the inhibitors LY (PI3-kinase inhibitor) and Leptomicine B (NES/Crm-1 nuclear export inhibitor).

The subcellular localization of FoxO6 is mainly nuclear and can be influenced mildly by growth factor stimulation. The transactivation capacities are however very sensitive to the presence of growth factors. Under growth factor deprived conditions the wild-type FoxO6 protein has comparable activity to the FoxO6-184 serine to alanine mutant protein, which cannot be phosphorylated in its DNA binding domain and thus has constitutive DNA-binding activity. The FoxO6-184Asp mutant protein, which mimics the phosphorylated state, did also display increased transactivational activity after growth factor deprivation but still far less compared to the wild-type FoxO6 protein. This can be explained by a reduction in DNA-binding activity as is shown extensively for FOXO1 (20). Interestingly, under growth factor conditions the wild-type FoxO6 protein still has the capacity to transactivate, which is probably a direct result of its prominent nuclear localization. Although, FoxO6 is mainly nuclear localized its transcriptional activity is still tightly regulated, probably through phosphorylation of the Ser184.

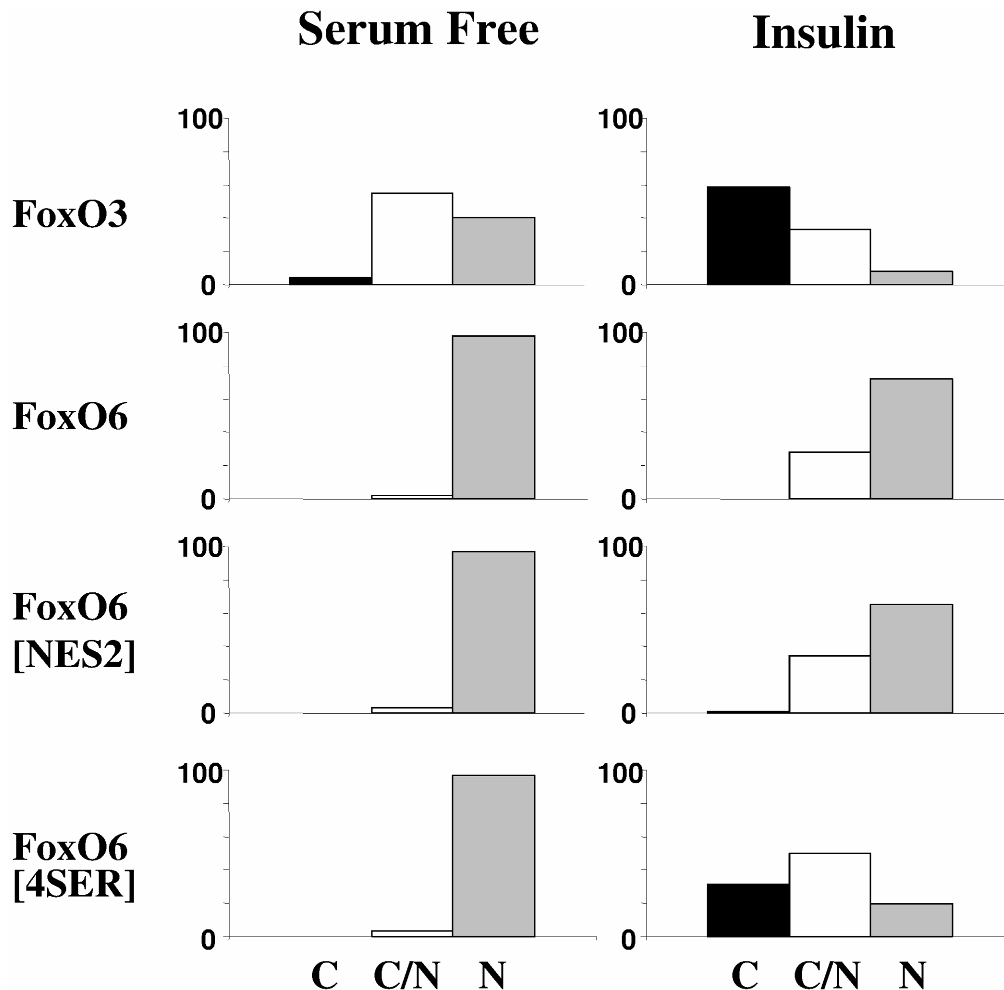

Figure 11. Quantification of FoxO protein relocation in response to insulin treatment. Cells were divided into three categories: cells with predominant cytosolic FoxO localization (C), distributed between cytosol and nucleus (C/N) or mainly nuclear (N). At least 100 cells were counted in a representative area of the slide. The percentage of cells present in each category is shown relative to the total of all three categories.

Two domains, implicated in nucleo-cytoplasmic shuttling in all known FoxO proteins, are absent in FoxO6. The first domain is a region that functions as a NES as present in the C-terminal part of FOXO3 (21). The second domain is the stretch of four phosphorylation sites just downstream the forkhead domain, highly conserved in all other FoxO-members. Recent findings with FOXO1 concern the PKB-catalyzed phosphorylation of serine 319 in this region. This event primes CK1-mediated phosphorylation of serine residues 322 and 325 (14). Serine residue 329 in FOXO1 is a substrate for phosphorylation by DYRK1A in a PI3-kinase-independent manner (15). Studies using FOXO1 with artificial mutations in this conserved region, show that substitution of Ser319 and Ser329 by alanine residues, results in an increased nuclear localization in the absence of growth factors (15,19). Furthermore, phosphorylation of Ser319, Ser322 and Ser325 together with Ser329 form an acidic patch that functions as a NES (14). Indeed, mutation analysis of FOXO1 by Rena et al. (14), revealed that mutation of Ser319, Ser322 or Ser319/Ser322/Ser325/Ser329 to alanine results in a decreased speed of nuclear export. The same study shows no decrease in speed of nuclear export for the Ser329 to alanine mutant.

In the present study, using an artificial chimeric FoxO6 protein, we succeeded in re-establishing nuclear export to a level comparable to FoxO3. The chimeric protein FoxO6[4Ser] consists of FoxO6 in which we replaced part of the gene for the corresponding part of FoxO3 containing four phosphorylation sites as described above. This chimeric protein was highly comparable to FoxO3 in its overall localization. Another chimeric protein FoxO6[NES2] in which we placed the second FoxO3 NES domain showed no significant changes in cytoplasmic relocation. These results clearly indicate that the absence of an important functional domain in FoxO6, results in a dramatically reduced nucleo-cytoplasmic shuttling. The consequential distinct localization of FoxO6 suggests a different timing and/or duration of transcriptional activity for this transcription factor. The data presented here widen the understanding of the regulation of subcellular relocation of FoxO proteins and their transactivating potential, which may provide new insights in FoxO mediated processes.

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Chapter 6

FoxO6 transcriptional activity is regulated by Thr26 and Ser184, independently of nucleo-cytoplasmic shuttling

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To be submitted

FoxO6 transcriptional activity is regulated by Thr26 and Ser184, independently of nucleo-cytoplasmic shuttling

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Forkhead members of the O class (FoxO) are transcription factors crucial for several cellular processes. Well described is their regulation of metabolism, cell cycle, cell death and survival. FoxO mediated transcriptional activity is negatively regulated by PI3K-PKB signaling, trough phosphorylation of three conserved residues resulting in the dissociation from DNA, association with 14-3-3 proteins and translocation from the nucleus to the cytosol. In contrast to FoxO1, FoxO3, and FoxO4, FoxO6 lacks a stretch of four serine residues, including a conserved PKB-site that enhances nuclear export. Nucleo-cytoplasmic shuttling of FoxO6 is consequently dramatically impaired, and FoxO6 is mainly nuclear. Here we analyzed the role of the two remaining PKB sites, Thr26 and Ser184, in the regulation of shuttling and transcription. Our results show that FoxO6 transcriptional activity is inhibited by growth factors, independently of shuttling. In contrast to FoxO1 and FoxO3 the growth factor induced decrease in FoxO6 activity was dependent on the N-terminal PKB site (Thr26), but not the PKB site in the forkhead domain (Ser184). Ser184 however regulates phosphorylation of Thr26, implying that both the N-terminal PKB site and the PKB site in the forkhead domain have separate functions.

In relation to the observed shuttling independent regulation of FoxO6 activity we analyzed effects of stress. Hydrogen peroxide negatively regulates FoxO6 mediated transcriptional activity in a growth factor independent manner, thereby providing a parallel pathway in the regulation of FoxO6. Stress and survival factors determine FoxO6 transcriptional activity without the requirement of shuttling to the cytosol.

Forkhead members of the “O” class (FoxO) are transcription factors that have been implicated in a multitude of biological processes including the cell cycle, protection against stress, cell death and cellular survival (van der Heide L.P., 2004). Their transcriptional activity is under the negative control of insulin/insulin-like signaling via the PI3K-PKB pathway (Brunet A., 1999; Kops G.J., 1999; Rena G., 1999). Activated PKB phosphorylates multiple FoxO residues resulting in the translocation of FoxO proteins from the nucleus to the cytosol, thereby terminating its ability to induce transcription of target genes (van der Heide L.P., 2004).

To date, the FoxO group has four mammalian members: FoxO1, FoxO3, FoxO4, and FoxO6. The degree of homology been these four members is extremely high, especially in the forkhead domain, which contains the DNA-binding interface. All FoxO members contain an N-terminal PKB motif, a PKB motif in the forkhead domain, and a C-terminal PKB motif (Fig1). The C-terminal PKB recognition sequence is not conserved in FoxO6 (Jacobs F.M.J., 2003). Interestingly phosphorylation of the C-terminal PKB residue in FoxO1 primes two neighboring CK1 sites (Rena G., 2002, 2004). A constitutively phosphorylated DYRK1A site is located adjacent to the second CK1 site (Woods Y.L., 2001). Together this stretch of four phosphorylated serines facilitates nuclear export (Rena G., 2002; Jacobs F.M.J., 2003; Rena G., 2004). Since FoxO6 lacks all four serine residues it is mainly nuclear under all conditions tested (Jacobs F.M.J., 2003). Insertion of the C-terminal PKB site, the CK1 sites, and the DYRK1A site rescues its in-ability to shuttle from nucleus to cytosol upon growth factor addition (Jacobs F.M.J., 2003). Shuttling is considered as the main negative regulator of FoxO mediated transcriptional activity, although there is substantial data pointing to a shuttling-independent regulation of transcription activity (Jacobs F.M.J., 2003; Tsai W.C., 2003).

Growth factor-induced FoxO shuttling is under the control of a hierarchical sequence of phosphorylation events which orchestrates interactions with 14-3-3 chaperones and the nuclear export machinery (van der Heide L.P., 2004). When deprived of growth factors, FoxO factors are bound to the DNA and are transcriptionally active. Under these conditions the N-and C-terminal PKB sites are inaccessible and in a non-phosphorylated state (Nakae J., 2000; Rena G., 2001). Insulin stimulation results in PKB-mediated phosphorylation of the PKB site in the forkhead domain, disrupting DNA binding and making the N-and C-terminal PKB sites accessible to phosphorylation (Guo S., 1999; Nakae J., 2000; Rena G., 2001; Zhang X., 2002). In addition to functioning as a gate-keeper of phosphorylation, the PKB motif in the forkhead domain is required for growth factor-induced inhibition of transcription, presumably by regulating phosphorylation of the other PKB sites (Zhang X., 2002).
PKB-mediated phosphorylation of the N-terminal PKB site creates a docking motif for 14-3-3 proteins (Brunet A., 1999; Rena G., 2001; Cahill C.M., 2001). It has been suggested that a 14-3-3 dimer requires stable binding to the phosphorylated N-terminal PKB site before the other half of the dimer can bind to the phosphorylated PKB motif in the forkhead domain (Obsil T., 2003; van der Heide L.P., 2004), which is not an optimal 14-3-3 binding motif by itself. The binding of a 14-3-3 dimer to a FoxO protein has several consequences as it blocks an intrinsic FoxO NLS (Brownawell A., 2001, Brunet A., 2002), prevents DNA binding (Cahill C.M., 2001, Obsil T., 2003) and mediates translocation to and sequestration of FoxO factors in the cytosol (Brunet A., 2002).

The C-terminal PKB site is not involved in 14-3-3 binding, but it is subject to the hierarchical phosphorylation sequence (Nakae J., 2000, Rena G., 2001). Phosphorylation of the four residues containing the C-terminal PKB site increases the rate of export by mediating interactions with Ran-GTP and CRM1, possibly in cooperation with a nuclear export sequence located further downstream (Rena G., 2002).

Whereas growth factor application results in cytosolic accumulation of FoxO proteins, stress results in the opposite as it induces translocation into the nucleus (Brunet A., 2004). However the mechanism behind stress-induced nuclear import is unclear.
As mentioned before FoxO6 is mainly nuclear under all conditions tested (Jacobs F.M.J., 2003).We have previously shown that the shuttling impairment can be rescued by inserting a stretch of four serine residues, including the C-terminal PKB site (Jacobs F.M.J., 2003). However, whether the role of the remaining two PKB residues in FoxO6 regulation is comparable to the other FoxOs remains unknown. Interestingly, FoxO6 does have a non-conserved optimal PKB motif in the C-terminal part. This non-conserved PKB motif is located immediately adjacent a nuclear export sequence. The exact role of this motif is at present unknown. Using mutation analysis we studied the role of the two conserved and the non-conserved PKB motif in translocation and transcriptional activity. In addition, we studied the transcriptional activity of FoxO6 under growth factor deprived and rich conditions and studied its response to hydrogen peroxide induced stress. Our results show that transcriptional activity of FoxO6 is efficiently regulated without shuttling to the cytosol. Intrinsic transcriptional activity and translocation can be impaired by mutating Thr26 or Ser184, or by the application of hydrogen peroxide. Both PKB residues have individual roles in the regulation of translocation and transcriptional activity, which strongly suggests that FoxO6 is regulated differently as compared to its family members.

Materials and Methods

FoxO-GFP Translational Fusion. FoxO1, FoxO3, FoxO6 and the FoxO6[4Ser] mutant were obtained as described previously (Jacobs 2003). Mutations of Thr-26, Ser-184, and Thr338 to an alanine or aspartic acid residue were generated using site-directed mutagenesis using the same approach as described (Jacobs F.M.J., 2003). .

Cell Culture and Transfection of HEK-293 Cells. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) hiFCS, 100 units/ml penicillin, 100 units/ml streptomycin, and 2 mM L-glutamine in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were seeded in 12-well plates and grown for 24 h on glass coverslips. Cells were transfected with calcium phosphate precipitates containing 1.9 µg/well plasmid DNA. (0.12 µg target construct/1.78 µg pBlueScript carrier DNA).

Forkhead Translocation Protocol. Twenty hours after transfection cells were serum-starved for 24 h. Translocation was induced by replacing the serum-free medium with medium supplemented with hiFCS (10% (v/v)) or insulin (100 nM). After 2 h of incubation cells were fixed using 4% PFA in phosphate-buffered saline for 10 min at room temperature. Slides were embedded in Dabco-Mowiol and analyzed by fluorescent-microscopy.

Luciferase Assays. Cells were grown in 6-well plates and transfected with 5 µg of plasmid DNA/well, including 1 µg 6x DBE-Luc (kindly provided by B. M. Burgering) or 1 µg of the glucose-6-phopsphatase-Luc or mutated glucose-6-phopshatase-Luc (both

kindly provided by A. Barthel) with or without 0.3 µg FoxO-GFP or empty vector and the appropriate amount of carrier plasmid. After transfection, cells were lysed and total GFP fluorescence was measured in 96-well plates using a FujiFilm FLA-5000 image reader to normalize the samples for transfection efficiency as previously described (Jacobs F.M.J., 2003). Each experiment was at least performed in triplicate.

Western Blotting. Cells were grown in 6-well plates and transfected with a total of 5 µg of plasmid DNA/well as described above. Cells were subsequently grown for 24h in the presence of serum before cells were starved for 24h. Serum starved cells were treated for 1h with or without DMEM containing 10% FCS before harvesting with lysis buffer containing 50mM TRIS, 1mM EDTA, 1mM EGTA, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 100mM sodium fluoride and 1mM sodium vanadate on ice. Insoluble material was removed from the sample by centrifugation at 12,000 g for 10s. Concentrated SDS sample buffer containing 66mM Tris/HCL pH 6.8, 3% (w/v) SDS, 5% (v/v) glycerol, 0.001% (w/v) bromophenol blue and 2% (v/v) β-mercapthoethanol was added to the samples before samples were heated for 15min at 100°C. Protein samples were separated by 9% SDS-PAGE. After electrophoresis protein was transferred to nitrocellulose membranes (Amersham) using a Biorad wet Blotting apparatus according to manufacturer's instructions. Protein transfer and blotting efficiency was checked with ponseau-S. Blots were blocked for 1h at room temperature in PBS containing 0.05% Tween-20 (PBS-T) and 5% milkpowder Anti phospho-Thr24/Thr32 FKHR/FKHRL1 (FOXO1/FOXO3) antibody (Cell Signaling Technologies) was diluted 1/1000 in PBS-T and incubated overnight. Secondary anti-rabbit antibody-HRP conjugate was diluted 1/50000 in PBS-T and incubated for 45min before visualisation with ECL detection substrate (Amersham) and HyperFilm (Amersham).
After detection of phopsho-Thr26 blots were stripped with PBS containing 2% SDS and 100mM 2-mercapthoethanol for 10min. Blots were blocked as described above before incubation with anti-GFP diluted 1/10000 in PBS-T for 1h. The remainder of the protocol is as described above.

Results

FoxO6 contains three putative RxRxxS/T PKB phosphorylation motifs (whereby the x represents any amino acid and the S/T denotes the residue phosphorylated by PKB), two are conserved amongst all other FoxOs. The two conserved PKB motifs are located at the N-terminus and in the forkhead domain; the third FoxO6 specific PKB motif is located in the C-terminus (fig1). To explore the role of each individual PKB motif in FoxO6 nucleo-cytoplasmic shuttling we created alanine and aspartic acid mutants, to mimic a non-phosphorylated or phosphorylated state respectively and subsequently studied their response to growth factors.

Thr26 regulates translocation.

We serum starved FoxO6-GFP-transfected cells for 24 hours to inactivate the PI3K-PKB pathway before treatment with either insulin or fetal calf serum (serum). Subsequently we

analyzed the intracellular FoxO6-GFP localization. Serum starvation resulted in a nuclear localization of wild-type FoxO6-GFP whereas insulin or serum treatment resulted in an increase in cytosolic fluorescence as described (Jacobs F.M.J., 2003) (Fig2). Mutation of Thr26 or Ser184 to Ala completely disrupted insulin or serum induced translocation to the cytosol which is confirmatory with the described effect of IGF-I (Jacobs F.M.J., 2003). Mutation of Ser184 to an Asp resulted in a decreased level of cytosolic fluorescence as compared to wild type FoxO6, after the addition of growth factors, but was still clearly present (Fig2). Surprisingly, mutation of Thr26 to an Asp abolished the effect of insulin or serum on nucleo-cytoplasmic FoxO6 shuttling, since it remains nuclear. A double mutation of Thr26 and Ser184 to Asp did not shuttle and remained nuclear, which suggests that the mutation of Thr26 to an aspartic acid is dominant over the mutation of Ser184 to an Asp (Fig2).

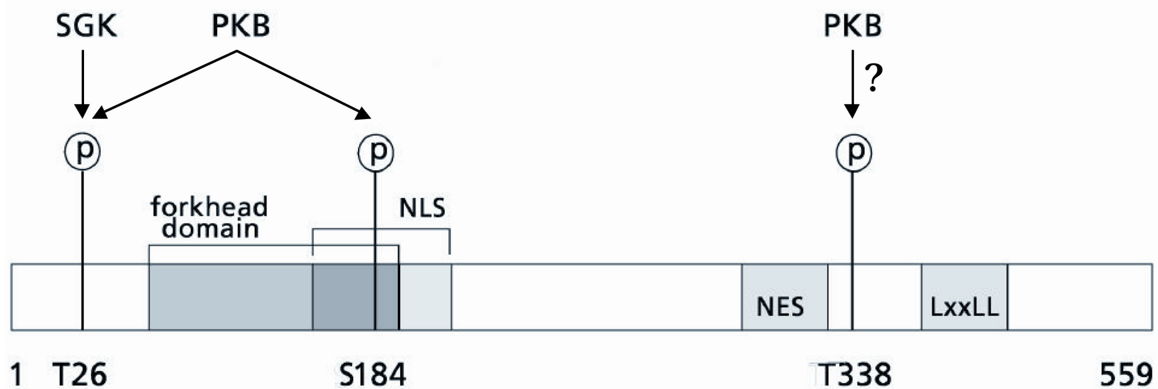

Figure 1. Schematic linear structure of FoxO6. FoxO6 contains 2 conserved PKB motifs and 1 putative PKB motif. FoxO6 contains an N-terminal PKB site (Thr26) which is preferentially phosphorylated by SGK and a PKB site in the forkhead domain (Ser184) which is phosphorylated by PKB. Phosphorylation of Ser184 possibly obscures the nuclear localization sequence (NLS), which is located around the PKB motif. In the FoxO6 C-terminal region a putative optimal PKB motif is present (Thr338) next to a nuclear export sequence (NES). In the C-terminal region FoxO6 contains an LxxLL motif implicated in nuclear receptor interactions.

A FoxO6 specific putative PKB motif is located next to the putative NES. Possibly phosphorylation of Thr338 influences the functionality of this NES. To asses the role of Thr338 in growth factor induced translocation we analyzed the Ala and Asp mutants of this site. Mutation of Thr338 to an Asp or Ala did not affect growth factor induced translocation from nucleus to cytosol, nor did the serum starved condition differ from wild-type FoxO6-GFP (Fig2).

Growth factor inhibition of transcriptional activity is independent of shuttling

To analyze whether shuttling from nucleus to cytosol has a role in regulating transcriptional activity we compared wild-type FoxO6 to a FoxO6-FoxO3 chimeric protein which has a superior shuttling ability (jacobs F.M.J., 2003). Both wild-type FoxO6 and the FoxO6-4Ser mutant have a comparable reduction in luciferase activity

after the application of growth factors. Interestingly the FoxO6-4Ser mutant is mainly cytosolic as wild type FoxO6 is nuclear (Fig3). These data indicate that shuttling is not required for regulation of FoxO6 transcriptional activity.

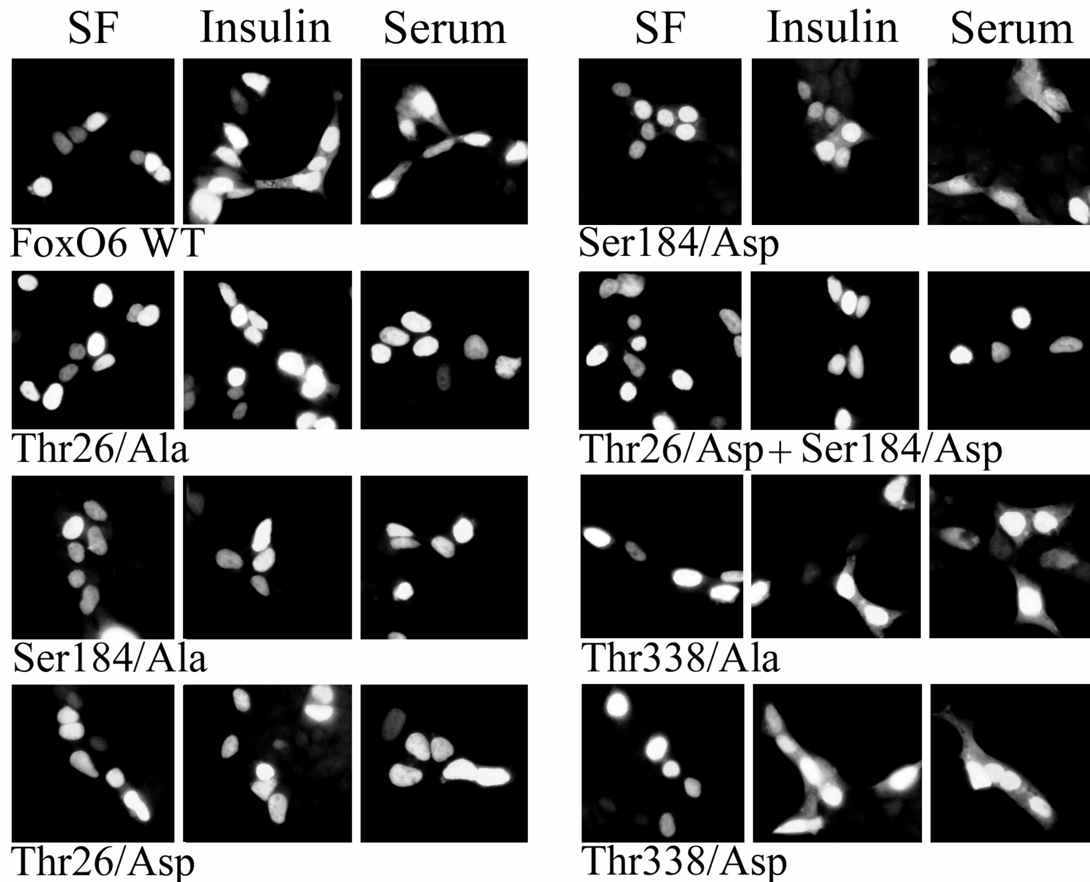

Figure 2. Mutation of Thr26 ablates growth factor induced FoxO6 translocation.
Cells were transfected with FoxO6-GFP or FoxO6 mutants fused to GFP. After transfection cells were serum starved and subsequently treated with insulin or serum. Wild-Type FoxO6 translocates to the cytosol after insulin or serum treatment which can be seen by the increase in cytosolic fluorescence. Mutation of Thr26 to an Ala, Ser184 to an Ala or Thr26 to an Asp completely prevents translocation. The mutation of Ser184/Asp does not prevent growth factor induced translocation, whereas a double mutation of Thr26 and Ser184 to Asp does prevent translocation. Mutants of Thr338 to an Ala or Asp were indistinguishable from wild-type FoxO6.

FoxO6 activates the Glucose-6-phosphatase promoter

Since FoxO6 is predominantly nuclear under all conditions tested, we were able to study the transcriptional activity of several FoxO6 mutants.
We analysed the transcriptional activity of the mutants described in fig2 on two different reporters fused to luciferase. We used an artificial reporter containing 6 optimal FoxO binding elements and a glucose-6-phosphatase reporter construct to investigate FoxO6 activity

Luciferase levels in the experiment using wild-type FoxO6 compared to the empty GFP vector reveals that FoxO6 is capable of activating the glucose-6-phosphatase reporter (Fig4). Addition of serum greatly reduced the activity of wild-type FoxO6 on both the 6DBE and the G-6-Pase reporter.

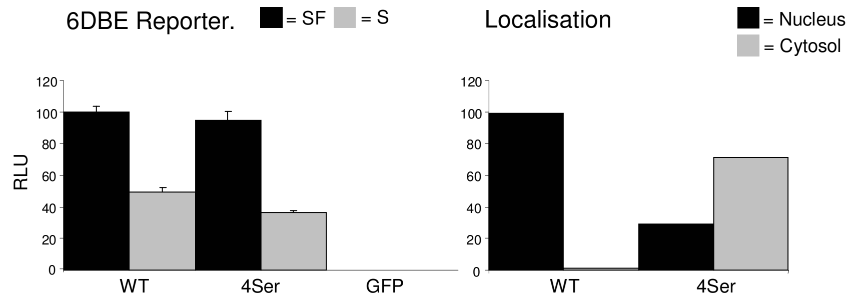

Figure 3. Growth factor-induced inhibition of transcriptional activation is independent of FoxO6 translocation (Left panel). The activity of FoxO6 was compared to a FoxO6-FoxO3 chimerical protein that has the ability to shuttle extensively. Both wild-type FoxO6 (WT) and the chimerical protein (4Ser) had comparable amounts of transcriptional activity on the 6DBE reporter under serum and serum-free conditions (Right panel). The intracellular localization of WT and 4Ser was quantified under serum treated conditions. WT was almost exclusively localized in the nucleus whereas the 4Ser mutant was mainly localized in the cytosol (Jacobs FMJ., 2003). Cytosolic localization was determined as having equal as or more fluorescence in the cytosol than in the nucleus. At least 100 transfected cells were counted.

Ser184/Ala does not inhibit growth factor sensitivity

Mutation of Ser184/Asp greatly reduced the activity of FoxO6 under serum free conditions and could be further reduced by serum addition (Fig4). Ser184/Ala resulted in a higher activity compared to wild-type FoxO6, and could be reduced by the addition of serum, which is in contrast to studies performed by others who claim that mutation of the corresponding Ser in FoxO1 abolishes growth factor induced FoxO inhibition of transcription (Rena G., 2001; Zhang X., 2002). It is clear that this does not apply to FoxO6.

Ser184 serves as a gate-keeper of phosphorylation

Mutation of the PKB site in the Forkhead domain to an Ala in FoxO1 abolishes growth factor sensitivity by preventing phosphorylation of the N and C terminal PKB sites (Nakae J., 2000; Rena G., 2001). The lack of phosphorylation could explain the insensitivity to growth factors. Using a phospho-specific antibody we investigated the effect of Ser184 mutation on Thr26 phosphorylation (Fig5). Growth factor addition results in a large increase in Thr-26 phosphorylation of wild type FoxO6, whereas no phosphorylation is observed under serum free conditions. Ser184/Ala indeed greatly diminishes phosphorylation of Thr26 in response to growth factors, but this is also the case when Ser184 is mutated to an Asp. No phosphorylation of Thr26 could be measured when cells were transfected with Thr26/Ala, which confirms antibody specificity.

Clearly, mutation of Ser184 influences phosphorylation of Thr26, but does not abrogate the transcriptional inhibition by growth factors as shown earlier (Fig4).

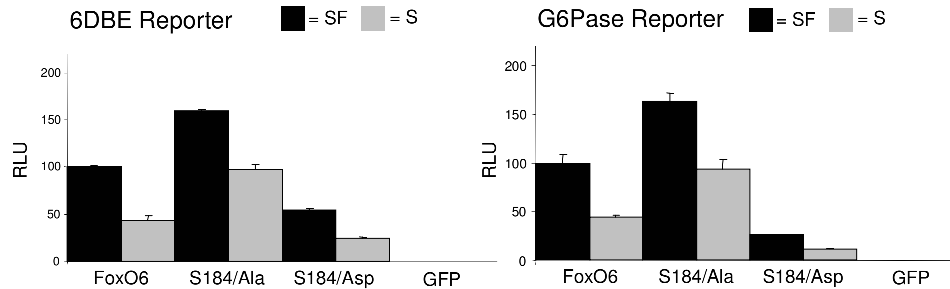

Figure 4. FoxO6 wild-type or alanine or aspartic acid mutants of Ser184 were transfected together with a 6 times optimal FoxO binding site reporter fused to luciferase (6DBE) or the glucose-6-phosphatase promoter fused to luciferase (G6Pase).
Serum application (S) resulted in a decrease in transcriptional activity of wild-type FoxO6 and S184 mutants. Both 6DBE and G6Pase reporters used showed comparable patterns of activity in response to FoxO6 and FoxO6 mutants. The Ser184/Ala mutant displayed higher activity under serum and serum free conditions as compared to wild-type FoxO6, the Ser184/Asp mutant lower activity. The GFP control does not contribute to the effects measured.

Thr26 regulates growth factor sensitivity

The N-terminal PKB site has been implicated in 14-3-3 binding which is a requirement for translocation and complete dissociation from the DNA. Interestingly, a Thr26/Ala mutation resulted in an increased activity of FoxO6 under serum free conditions as compared to wild-type (Fig6). The Thr26/Ala mutant could not be inhibited by the addition of growth factors and the mutation of Thr26 to an aspartic acid had the same result as the Thr26/Ala mutation. Apparently, Thr26 mediates growth factor inhibition of transcriptional activity regulated by the PKB site in the forkhead domain of FoxO1.

Thr338 has no apparent function in transcriptional activity

Next, we analysed the effect of Thr338 to alanine or aspartic acid mutation on FoxO6 mediated transcriptional activity. Thr338 displayed no apparent function in translocation induced by serum or insulin, it is however located in the putative transactivation domain, which suggests that it may mediate effects directly on transcriptional activity. Both alanine and aspartic acid Thr338 mutants showed a growth factor induced reduction in luciferase activity comparable to wild type (Fig7), although under serum free conditions the activity of both mutants was slightly higher on both the 6DBE and G-6-Pase reporters. In conclusion, Thr338 has no clear function in the regulation of FoxO6 mediated transcriptional activity.

Hydrogen peroxide inhibits FoxO6 activity

Under conditions of stress, FoxO3 is acetylated and translocates to the nucleus, even under growth factor rich conditions (Brunet A., 2004; Motta M.C., 2004; van der Horst

A., 2004). Acetylation reduces the activity of FoxO3 and FoxO4 on several reporters (Fukuoka M., 2003; Motta M.C., 2004; van der Horst A., 2004), but the effect of hydrogen peroxide itself on FoxO mediated transcriptional activity has not been studied. Overnight application of hydrogen peroxide resulted in a reduction of FoxO6 mediated luciferase activity (Fig8). A synergy between the effect of hydrogen peroxide stress and the Ser184/Asp mutant could be observed but this effect was not consistently found. Multiple experiments performed in triplicate revealed that the hydrogen peroxide induced reduction in FoxO6 mediated luciferase activity varied from 17% to 57% (average reduction 34.8 %, SD = 15.7 from 6 experiments performed in triplicate). An oxidation of FoxO6 by hydrogen peroxide is unlikely to account for the varying results as we corrected directly for GFP fluorescence. Oxidation would result in a relative increase in FoxO6 activity, which is not observed (not even at concentrations of 1mM hydrogen peroxide (data not shown).

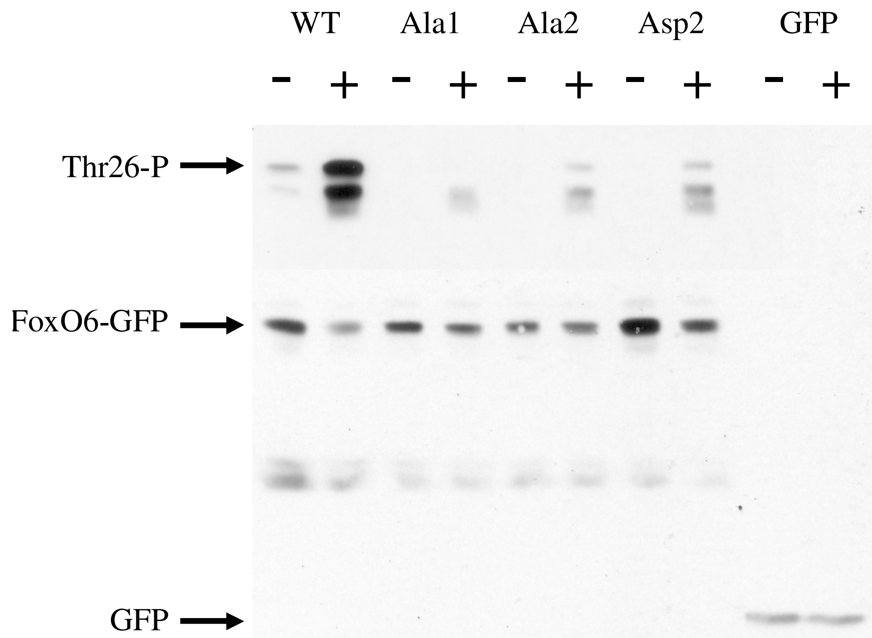

Figure 5. The PKB site in the forkhead domain functions as a gatekeeper of phosphorylation. Cells were transfected with wild-type FoxO6 or alanine or aspartic acid mutants of Thr26 and Ser184. Cells were serum starved for 24h before treatment with serum for 1h. FoxO6 proteins were analyzed for their phosphorylation on Thr26 using a phospho-specific antibody. Serum application induced a large increase in phosphorylation of Thr26 as compared to serum free conditions. The antibody is specific for Thr26 as a mutation of Thr26 to an alanine (Ala1) is no longer recognized by the antibody. Mutation of Ser184 to an alanine greatly diminished phosphorylation of Thr26. Mutation of Ser184 to an aspartic acid however also greatly diminished phosphorylation of Thr26. An increase in phosphorylation can still be observed in both Ser184 mutants after the application of serum. The specific band indicating Thr26 phosphorylation is indicated by an arrow. As a control the same blot was stripped and subsequently analyzed for its content of FoxO-GFP using a specific GFP-antibody (arrow).

Hydrogen peroxide does not influence translocation

Since it has been shown that stress induces translocation of FOXO3 to the nucleus, we wondered if our varying results may be due to translocation of FoxO6, although unlikely,

since the activity of the FoxO6-4Ser mutant did not differ from wild-type FoxO6. We analysed the effect of hydrogen peroxide on translocation of FoxO1, FoxO3 and FoxO6 on time points 1,4,8,12,24 hours after hydrogen peroxide application in serum-free and serum containing medium. At all time-points tested we observed no difference between hydrogen peroxide treated cells and controls (data not shown). These data clearly indicate that the effects of hydrogen peroxide on transcriptional activity are independent of translocation.

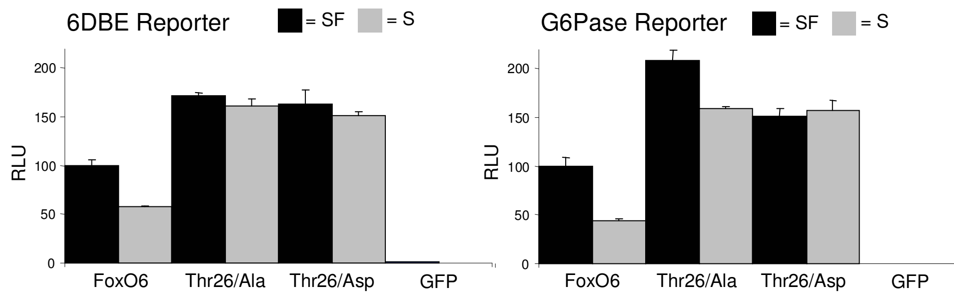

Figure 6. Mutation of Th26 impairs growth factor induced inhibition of FoxO6 transcriptional activity. As compared to wild-type FoxO6, mutation of Thr26 to an alanine or aspartic acid disrupted the growth factor induced decrease in transcriptional activity. Activity on 6DBE and G6Pase reporters was comparable.

Transcriptional activity of FoxO6 is dependent on FoxO binding sites

FoxO1 activity on the glucose-6-phosphatase reporter depends on a stretch of multiple insulin response units (IRUs) (Ayala J.E., 1999, Schmoll D., 2000). Mutation of the IRUs within this promoter diminishes FoxO1 activity on this promoter (Schmoll D., 2000). To address whether FoxO6 activated the G-6-Pase reporter identical to FoxO1, we monitored FoxO6 activity on wild type and mutated G-6-Pase reporter constructs. FoxO1, FoxO3 and FoxO6 indeed have lower activity on the mutated promoter indicating that binding to the IRUs is necessary for maximal activity (Fig9). It is striking that FoxO1 displays the lowest transcriptional activity on this reporter compared to FoxO3 and FoxO6. Although the mutant promoter contains mutated binding sites, FoxO6 still retains the ability to activate this reporter comparable to FoxO1 on the wild-type promoter. To further demonstrate the DNA binding dependence of FoxO6 in activating the mutated G-6-Pase reporter we used FoxO6 mutants of Ser184, since this residue has been implicated essential for DNA binding. Both alanine and aspartic acid mutants of S184 displayed diminished activity on the mutant G-6-Pase reporter as compared to the wild-type reporter (Fig8). This indicates that FoxO6 DNA binding is necessary to activate this mutant G-6-Pase promoter. Interestingly, the S184/Asp mutant still has residual activity on the mutant G-6-Pase reporter, indicating that mutation of the IRUs and inhibition of FoxO6 DNA binding is not enough to eliminate activity completely.

Discussion

FoxO shuttling and transcription is under the control of PI3K-PKB signaling. FoxO phosphorylation of specific residues by PKB and or SGK triggers the dissociation from the DNA, recruitment of 14-3-3 proteins and translocation to the cytosol (van der Heide

L.P., 2004). Here, we analysed the role of two conserved PKB sites and a C-terminal non-conserved site. The data presented above indicates that the N-terminal PKB site and the PKB site in the forkhead domain have distinct roles in the regulation of translocation and FoxO6 mediated transcription. The non-conserved FoxO6 specific C-terminal PKB motif does not appear to be functional in these activities as mutants of Thr338 did not differ from wild-type FoxO6 in these experimental conditions.

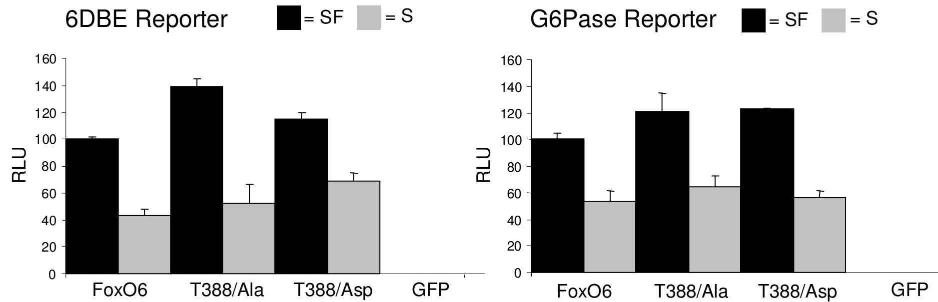

Figure 7. Thr338 is not involved in regulation of FoxO6 transactivation activity.
Alanine or aspartic acid mutants of Thr338 were analyzed for their transcriptional activity on a 6DBE or G6Pase luciferase construct.
The Thr388 alanine mutant (T338/Ala) displayed higher activity on the 6DBE and G6Pase reporter as compared to FoxO6 wild-type and activity of T338/Ala could be decreased by serum application. An aspartic acid mutant of Thr338 (T388/Asp) displayed higher activity under serum conditions on the 6DBE but not the G6Pase reporter. Overall the Thr388 mutants behaved similarly to wild-type FoxO6 in response to serum application and serum starvation.

Regulation of FoxO6 transcriptional activity does not require nucleo-cytoplasmic shuttling

Shuttling has been suggested as the main regulator of FoxO transcriptional activity. By physically removing the FoxO transcription factor from the nucleus it can no longer be transcriptionally active. However, by comparing a mutant FoxO6-FoxO3 chimera to wild-type FoxO6, we clearly show that shuttling is not the main regulator of FoxO6 function. Apparently, phosphorylation of PKB residues in FoxO6 is sufficient to suppress transcriptional activity without removal of the protein from the nucleus. This suggests that all the components needed for the negative regulation of FoxO6 are present in the nucleus or can be recruited there after growth factor stimulation.

Ser184 functions as a gatekeeper of FoxO6 phosphorylation

The PKB site in the forkhead domain has been shown to regulate the phosphorylation of the N and C-terminal PKB sites in FOXO1 and has therefore been referred to as the gatekeeper of FoxO phosphorylation (Guo S., 1999; Nakae J., 2000; Rena G., 2001; Zhang X., 2002). By preventing phosphorylation of the PKB site in the forkhead domain FOXO1 does not respond to growth factors and its transcriptional activity can not be inhibited. In analogy, the “gatekeeper” hypothesis also applies to FoxO6. A S184 to alanine mutation indeed disrupts phosphorylation of Thr26, but surprisingly does not completely inhibit the growth factor induced decrease in transcriptional activity as reported by others (Nakae J., 2000; Rena G., 2001).

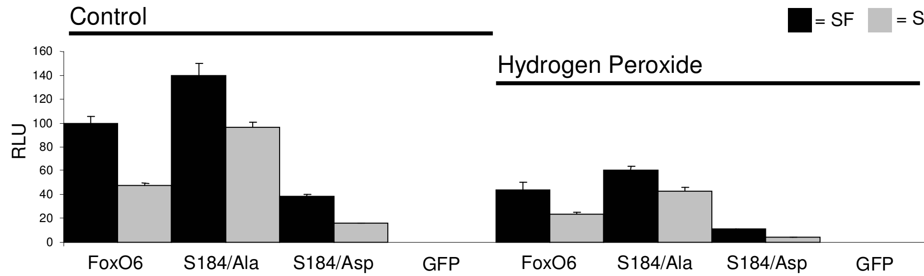

Figure 8. Hydrogen peroxide decreases FoxO6 mediated transcriptional activity.
Cells were transfected with FoxO6 or mutants of Ser184 and treated with or without serum and hydrogen peroxide. Hydrogen peroxide decreased activity of FoxO6 and the mutants irrespective of serum stimulation.

Thr26 mediates growth factor sensitivity

Phosphorylation of the PKB site in the forkhead domain inhibits FOXO1-DNA binding (Zhang X., 2002). Accordingly, the Ser184/Asp FoxO6 mutant has the lowest transcriptional activity (Fig4). A major reduction of the affinity for DNA, besides phosphorylation of the PKB site in the forkhead domain, however, requires FoxO 14-3-3 binding (Obsil T., 2003) (Fig10). This indirectly implicates 14-3-3 as the main negative regulator of FoxO transcriptional activity. The 14-3-3 mediated decrease in DNA binding however requires binding of a 14-3-3 dimer, which binds the N-terminal PKB site and the PKB site in the forkhead domain (Obsil T., 2004) (Fig10). Therefore the Ser184/Ala mutant can not stably bind a 14-3-3 dimer. However, residual phosphorylation of Thr26 may suffice to regulate FoxO6 transcriptional activity.
The negative regulation of FoxO mediated transcription by phosphorylation of Thr26 is further strengthened by the observation that the growth factor induced decrease in transcriptional activity could be efficiently blocked by mutating Thr26 to an alanine. As a consequence, transcriptional activity remained as high as under serum free conditions. Surprisingly, the Thr26/Asp mutant was indistinguishable from the Thr26/Ala mutant. Possibly the aspartic acid may not fully mimic a phosphorylated residue and thus resemble the non-phosphorylated state. This may also explain why the Ser184/Asp mutant is not as efficiently phosphorylated on Thr26 after growth factor addition as compared to wild-type FoxO6. Secondly, for Thr26 to function properly it may have to be susceptible to alternating phosphorylation and de-phosphorylation events. In correspondence to this, FOXO1 mutants of the N-terminal PKB site do not display growth factor induced shuttling and accumulate in either cytosol or nucleus (Nakae J., 2000). However it is not known whether FOXO1 mutants of the N-terminal PKB site can still shuttle at all between nucleus and cytosol. Experimental approaches using leptomycin B to inhibit CRM1 dependent nuclear export would solve this issue.
A third explanation why the Thr26/Asp can not be regulated by growth factors could reside in the mechanism of hierarchical FoxO phosphorylation. If indeed the phosphorylation of the PKB site in the forkhead domain has to occur before phosphorylation of the N-terminal PKB site, a Thr26/Asp mutation may structurally disrupt FoxO6, resulting in growth factor insensitivity.

Stress decreases FoxO6 mediated transcriptional activity

Overnight treatment of hydrogen peroxide resulted in a decrease in FoxO6 mediated transcription (Fig8). The reduction in FoxO6 mediated transcription did not involve a modulation of the PKB site in the forkhead domain as alanine and aspartic acid mutants did not prevent the decrease in transcription. What has to be noted is that hydrogen peroxide treatment always resulted in a decrease in FoxO6 activity, but that this decrease was not consistent in magnitude. Possibly cellular sensitivity to peroxide stress is influenced by multiple variables such as cellular passage and confluency (Bello R.I., 2003, Naderi J., 2003). Stress has been shown to influence the intracellular localisation of FOXO3, but we did not observe alterations in the intracellular localisation of FoxO1, FoxO3 or FoxO6 in response to stress.

Stress has been shown to down-regulate FOXO3 transcriptional activity (Leong M.L.,2003). In this particular study stress also induced SGK, which could partly have accounted for the decrease in transcriptional activity by phosphorylation of the N and C terminal FOXO3 PKB sites. The hydrogen peroxide induced reduction in FOXO3 mediated transcription is however far greater than the effect on translocation, which confirms an SGK independent reduction in activity (Leong M.L., 2003).

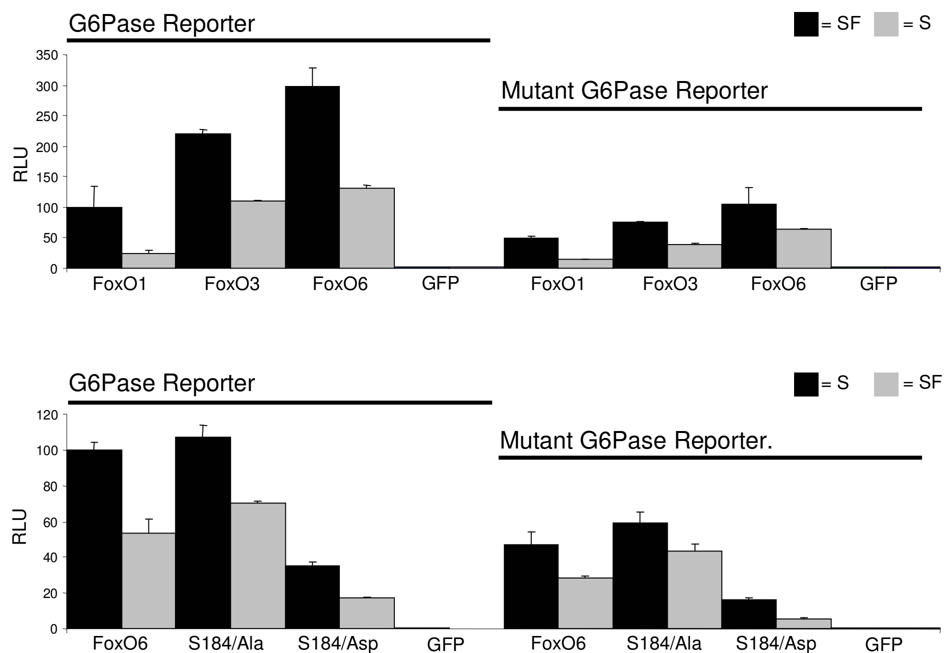

Figure 9. FoxO6 requires optimal binding sites and DNA binding activity for transcriptional activity. (Upper two panels) FoxO1, FoxO3 and FoxO6 were transfected together with wild type and mutant G6Pase promoter-luciferase construct. Serum application decreased FoxO activity on both wild-type and mutant promoters. Activity on the mutant G6Pase reporter was however lower compared to the wild-type promoter. (Lower two panels) Wild-type FoxO6 and mutants of Ser184 were transfected together with the wild-type or mutant G6Pase reporter. Activity of FoxO6 was greatly reduced on the mutant reporter and was further reduced by mutating Ser184 to an aspartic acid (S184/Asp).

Stress regulates FoxO6 in a growth factor independent manner

The effect of hydrogen peroxide on FoxOs appears independent of growth factors, since stress did not influence the intra-cellular FoxO localization and mutation of the PKB site

in the forkhead domain could not prevent the stress induced decrease in FoxO6 mediated transcription. Thus stress has the ability to influence FoxO mediated transcription independently of the PI3K-PKB pathway and independently of translocation.

FoxO6 transcriptional activity depends on intact binding sites and FoxO6-DNA binding

FoxO1 activity on the G-6-Pase promoter is dependent on the presence of IRUs (Schmoll D., 2000). Mutation of the IRU disrupts FoxO1 mediated transcription of the G-6-Pase gene (Schmoll D., 2000; van Groote-Bidlingmaier F., 2002). We compared the efficacy of FoxO6 mediated transcription to FoxO1 and FoxO3 on the mutant G-6-Pase promoter to asses the importance of intact binding sites. Surprisingly, FoxO6 was a very potent activator of the wild-type G-6-Pase reporter as compared to FoxO1. FoxO3 and FoxO6 were relatively comparable. Mutation of the IRUs present in the reporter diminished activity of all three FoxOs to similar extent, pointing out that FoxO6 needs binding sites to induce transcription. The potency of FoxO6 in inducing the G-6-Pase reporter is highlighted by the fact that FoxO6 activates the mutated G-6-Pase reporter as efficiently as FoxO1 activates the wild-type reporter.

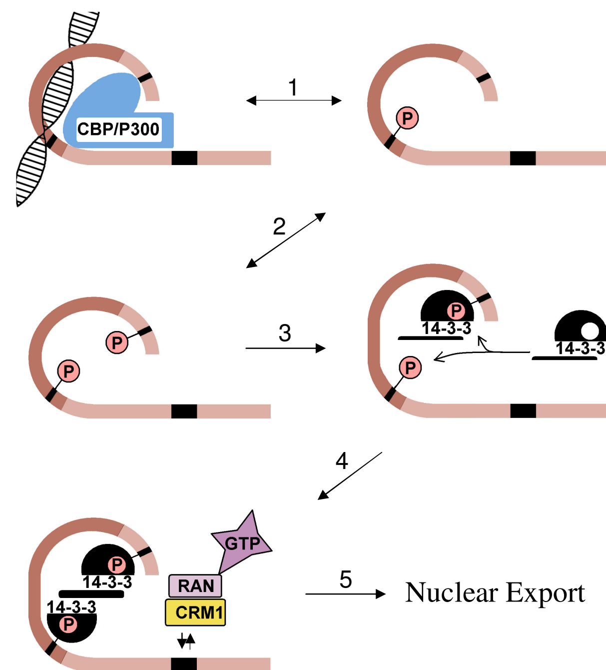

Figure 10. Hypothetical scheme describing different stages in FoxO6 nuclear export.
1. FoxO6 is bound to the DNA and transcriptionally active. Treatment with insulin or serum results in the phosphorylation of Ser184 in the forkhead domain. Phosphorylation of Ser184 disrupts the interaction with DNA and/or cofactors. 2. Phosphorylation of Thr26 can occur after Ser184 is phosphorylated. 3. A 14-3-3 dimer attaches itself to phosphorylated Thr26 and Ser184. The 14-3-3 dimer attached to FoxO6 stabilizes a conformation with can not interact with DNA and/or cofactors. 5. FoxO6 is exported out of the nucleus in a CRM1 dependent manner. A weak interaction with CRM1 and Ran-GTP may underlie the small extent of FoxO6 nuclear export.

To asses whether the remaining FoxO6 activity on the mutated promoter does indeed require FoxO6 DNA binding we used mutants of the PKB site in the forkhead domain. A Ser184/Asp mutation further decreased residual FoxO6 activity on the mutated promoter, pointing out that FoxO6 requires DNA binding to activate transcription of this reporter. The residual activity observed, can be explained by the fact that not all binding sites have been mutated. Immediately adjacent to the stretch of three optimal IRUs an additional IRU is present, which was not mutated.

In conclusion, we have elucidated the individual role of Thr26 and Ser184 in mediating growth factor signals to FoxO6. Ser184 regulates phosphorylation of Thr26, but Thr26 is the major residue mediating the regulatory effects of growth factors on translocation and transcriptional regulation. Surprisingly, growth factor regulation of FoxO6 mediated transcriptional activity does not require translocation to the cytosol and is efficiently regulated in the nucleus. Possibly, translocation to the cytosol provides a second mode of regulation by other processes such as ubiquitination and proteolytic breakdown (van der Heide 2003). Taken together, FoxO6 integrates growth factor and stress signals to determine the extent of its transcriptional output, independently of translocation to the cytosol.

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Chapter 7

Summary and Discussion

Insulin stimulates glucose uptake in peripheral organs but also has functions in the central nervous system. Insulin and its receptor are expressed in the central nervous system and are implicated in neuronal-metabolism and survival. Besides a role in cellular physiology, brain insulin has been implicated in several diseases such as diabetes and Alzheimer's disease. The aim of this thesis was to explore how insulin modulates intracellular signaling routes and how these routes relate to long lasting changes in synaptic plasticity. Particularly, we focused on the PI3K-PKB pathway and the ERK1/2 pathway. Moreover, we focused on a novel FoxO transcription factor, FoxO6, identified in mouse brain, as a transcriptional end-point of insulin signaling. Here the results of the experiments are summarized and discussed in relation to functions of insulin in the brain.

In peripheral cells, insulin has been described to activate PKB and ERK1/2. We addressed the issue whether this also applied to cells of neuronal origin (chapter 2). Insulin application to Neuro2a cells resulted in PKB phosphorylation in a dose dependent, and time independent manner. The phosphorylation of PKB, through insulin application, induced its catalytic activity measured through the phosphorylation of GSK3beta, a well described PKB target. Surprisingly, insulin-induced PKB phosphorylation was accompanied by a dose-dependent and time-independent decrease in ERK1/2 phosphorylation. Further investigation into the mechanism underlying ERK1/2 dephosphorylation revealed that this process was PI3K dependent.

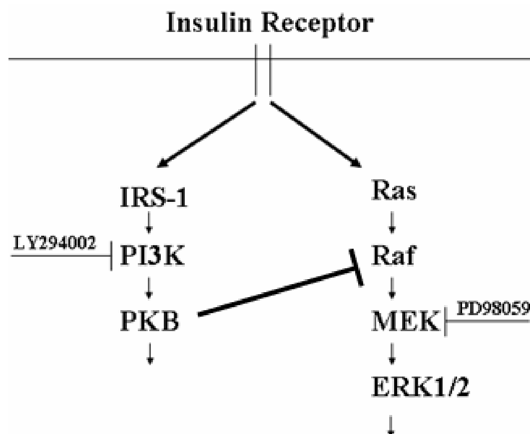

Figure 1. Insulin signaling recruits insulin receptor substrate 1 (IRS-1) to the insulin receptor. This triggers phosphatidyl-inositol-kinase (PI3K) recruitment and activation of PI3K mediated PKB activation. Insulin also activates extracellular regulated kinase 1 and 2 (ERK1/2) by sequentially activating Ras, Raf , and MEK. The PI3K pathway inhibits the ERK1/2 pathway after insulin application, possibly via an interaction between PKB and Raf. LY294002 prevents ERK1/2 inhibition by preventing PKB activation. PD98059 prevents ERK1/2 activation which confirms that it is activated by MEK.

Inhibition of the PI3K pathway, by LY294002, during insulin stimulation resulted in an increase in ERK1/2phosphorylation (Fig 1), indicating that insulin normally activates PI3K and ERK1/2. However, downstream crosstalk in the signaling pathway normally inhibits ERK1/2 phosphorylation (Fig 1). Prolonged insulin application desensitized the PI3K route to a secondary insulin stimulus; as a result the inhibition on ERK1/2 phosphorylation was also decreased. Prolonged insulin application did not affect ERK1/2 activation by insulin as insulin application in combination with PI3K inhibitors resulted in normal ERK1/2 phosphorylation (Fig 1). Apparently, prolonged insulin application only results in a desensitization of the PI3K route, suggesting that this occurs downstream of the insulin receptor, after the bifurcation between PI3K and ERK1/2 signaling.

We showed that PI3K plays an important part in the integration of neuronal insulin signaling in vitro, but it is unclear whether this is also the case in vivo (chapter 2). To answer this question, we investigated the effects of insulin ex-vivo (chapter 3). Insulin application to hippocampal slices resulted in an induction of long term depression (LTD) of hippocampal CA1 neurons. Investigation into the mechanism underlying insulin-mediated LTD, revealed that insulin required activation of NMDA receptors. Since LTD induction occurred at a test frequency, which normally does not induce LTD, we stimulated at other frequencies to asses whether the sensitivity of the neuronal network was changed. Insulin application to hippocampal slices indeed shifted the frequency response curve of synaptic plasticity to the left, thereby allowing NMDA receptor-dependent LTD and LTP induction at lower stimulation frequencies (Fig 2).

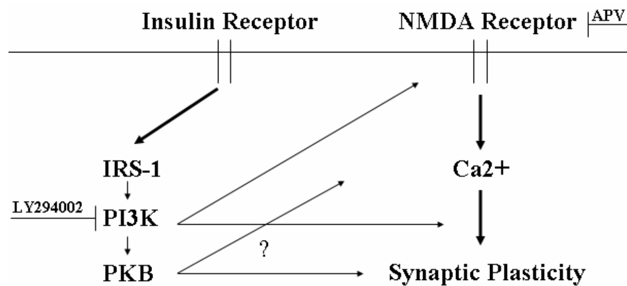

Figure 2. Insulin facilitates NMDA receptor mediated synaptic plasticity in a PI3K dependent manner. The facilitation of synaptic plasticity can be prevented by blocking PI3K or the NMDA receptor. It is unknown whether the effects of insulin on synaptic plasticity require PKB activation.

Since we have previously observed that PI3K is an important mediator of insulin signaling (Chapter2), we investigated if PI3K signaling was involved in mediating the electrophysiological effect of insulin. Insulin application to hippocampal slices induced PKB phosphorylation within 15 min and the insulin mediated LTD induction could indeed be blocked by the PI3K inhibitor LY294002 suggesting that PI3K is necessary for LTD induction to occur (Fig 2). In summary, insulin facilitates the induction of regular LTD.

Insulin regulates the activation of several intracellular kinases which can have immediate effects for example opening a potassium channel, or long lasting effects by modulating transcription factors. We searched for FoxO transcription factors in the CNS as they have been described as transcriptional end-points of insulin-PI3K-PKB signaling (Chapter 5).
The FoxO family of transcription factors consisted of three known members in mouse, FoxO1, FoxO3 and FoxO4. We identified a fourth member from ventral midbrain tissue and named it FoxO6.
In situ hybridization experiments revealed that the FoxO6 transcript was expressed throughout the brain, but was dominantly expressed in the hippocampus, especially CA1 and CA3 areas, and to a lesser extent in the dentate gyrus and CA2 area.
Since FoxO factors translocate to the cytosol upon PI3K-PKB activation we tested whether FoxO6 also translocated in response to PI3K-PKB activation. When compared to FoxO1 and FoxO3, FoxO6 was predominantly nuclear under all conditions tested, whereas FoxO1 and FoxO3 shuttled to the cytosol after treatment with insulin or other growth factors. FoxO6 did shuttle to the cytosol to a limited extent. FoxO6 translocation was PI3K-PKB dependent as a specific PI3K inhibitor could prevent translocation. In

addition, mutations of the PKB sites also prevented growth factor induced translocation of FoxO6. Analysis of the FoxO6 sequence reveals that FoxO6 lacks a region which is conserved in the other FoxO factors (Fig 3). This region contains four sequential phosphorylation sites: a PKB site, two CK1 sites, and a DYRK1A site. Insertion of this stretch from FoxO3 into the corresponding region of FoxO6 restored the ability of FoxO6 to shuttle between nucleus and cytosol comparable to FoxO3. The significance of the results in this chapter is the discovery of a fourth FoxO transcription factor which differs from the known three members by its restricted PKB-mediated shuttling properties. This factor, FoxO6, is likely to be a novel player in insulin and growth factor signal transduction in the hippocampus.

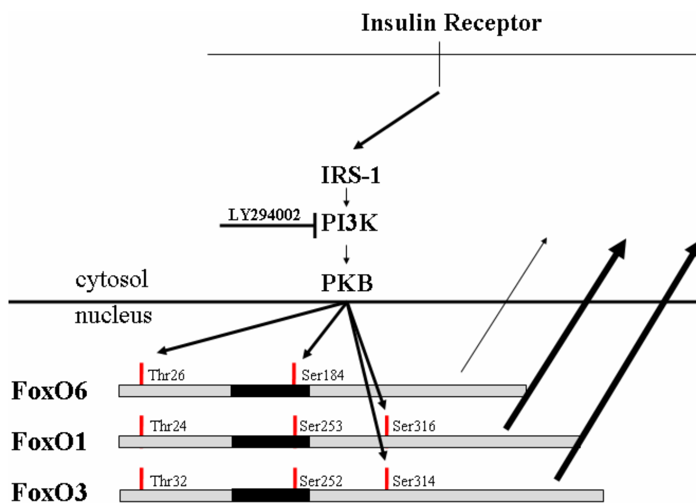

Figure 3. Phosphorylation of FoxO factors by PKB resulted in translocation to the cytosol. Export of FoxO6 is small (narrow arrow) compared to FoxO1 and FoxO3 (thick arrow), due to the absence of a region containing the C-terminal PKB site. Inhibition of PI3K with LY294002 prevented insulin- induced translocation to the cytosol. IRS-1; insulin receptor substrate 1, PI3K; phosphatidyl-inositol-3-kinase, PKB; protein kinase B. The black bar in the FoxO proteins represents the forkhead domain.

Translocation of FoxO1, FoxO3, and FoxO4 to the cytosol is triggered by phosphorylation of three PKB sites (Chapter 4, 5). Phosphorylation of these sites recruits chaperones that are required for transport through the nuclear pore complex and sequestration in the cytosol. FoxO6 has two (Thr26 and Thr184) of the three conserved PKB motifs. The C-terminus contains a non-conserved optimal PKB phosphorylation site (Thr338). Since FoxO6 remains almost completely nuclear under all condition tested, but did display some phosphorylation-dependent shuttling, we investigated the role of the remaining two PKB sites in translocation and transactivation (chapter 6). Using mutation analysis we showed that translocation depended on the phosphorylation state of both Thr26 and Ser184. The non conserved Thr338 PKB site had no apparent function in translocation. Growth factors are suggested to inhibit FoxO activity by initiating translocation from the nucleus to the cytosol, thereby physically removing the transcription factor from the DNA. Growth factors did indeed inhibit FoxO6 activity on a reporter construct containing six optimal binding sites fused to luciferase, but the inhibition was not due to translocation. A FoxO6-FoxO3 chimeric protein (Chapter 5) had the same transcriptional activity as wild-type FoxO6 and was inhibited by growth factors to the same extent as wild-type FoxO6 (Chapter 6). Mutation analysis revealed that Thr26 is presumably necessary to mediate inhibition of transcriptional activity by growth factor stimulation whereas Ser184 controls baseline levels of transcriptional activity (Fig 4). Besides growth factors, stress also reduced the activity of FoxO6 on

reporter constructs. The reduction in activity induced by stress was independent of the phosphorylation state of Ser184, indicating that stress utilizes additional PI3K-independent pathways in the

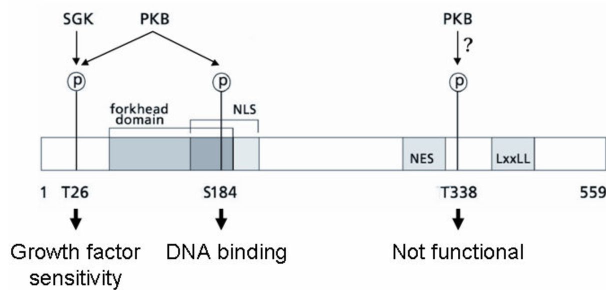

Figure 4 Mutation analysis revealed thatThr26 (T26) mediates growth factor sensitivity whereas Ser184 (S184) presumably mediates DNA binding. Thr338 (T338) located between a nuclear export sequence (NES) and a motif involved in the interactions with nuclear receptors (LxxLL), has no apparent function in translocation and transactivation

regulation of FoxO6. Further analysis of FoxO6-mediated activity on the glucose-6-phosphatase promoter revealed that FoxO6 activity depends on intact FoxO binding sites. In summary, FoxO6 transcriptional activity is regulated by phosphorylation of Thr26 and Ser184 independent of translocation to the cytosol. Apparently, tight transcription control by FoxO factors is possible without a requirement of cytoplasmic shuttling.

Overall, insulin regulates intracellular cross-talk, facilitates synaptic plasticity and controls the activity of FoxO transcription factors. All three aspects discussed may have implications for the correct development and maintenance of the central nervous system.

A role for insulin in neuro-development and neurogenesis?

In Chapter 2 we described insulin induced crosstalk between PI3K and ERK1/2 signaling. We did not investigate what the result of this crosstalk is for cellular metabolism, but studies in non-neuronal systems, describing this crosstalk, suggest that insulin may have an important role in regulating the balance between proliferation and differentiation. A study performed in MCF-7 breast cancer cells revealed that IGF-I also induced crosstalk between PI3K and ERK1/2 (Moelling K., 2002). In this study it was shown that PKB/Akt phosphorylates Raf, an upstream ERK1/2 activator, and causes its inactivation. Interestingly, inhibition of Raf by PKB shifts the cellular response from cell-cycle arrest to proliferation (Zimmermann S., 1999). They postulate that the PI3K route regulates proliferation and survival whereas the ERK1/2 route regulates growth arrest and differentiation. Since the system desensitizes in response to prolonged high concentrations of insulin, the dose and presence of insulin are carefully integrated into a cellular response. If this system would be functional in developing neurons, insulin could control the spatio temporal patterns of neuronal proliferation and differentiation. A variant of this system could also control neuronal axon guidance. Insulin receptors on the growth cone of migrating neurons (Song J., 2003) could sense a gradient of insulin and would be able to respond by proliferating/migrating further or differentiating into a functional neuron when the context is appropriate. Interestingly, deletion of the insulin receptor in drosophila results in abnormal axon guidance and targeting in the *Drosophila* visual system (Song J., 2004) suggesting that insulin signalling is important, in vivo, for proper neuronal growth, outgrowth and differentiation. The correct deposition of an

extracellular insulin gradient would have to be a prerequisite for insulin to control neuronal migration. It is however unknown if such an insulin gradient is present in the central nervous system or how this gradient would be created. Interestingly, insulin mRNA is expressed locally in the hippocampal CA1 and CA3 regions and could be an important factor in neuronal development and maintenance (Devaskar S.U., 1994).
IGF-1 administration directly to the brain or peripheral blood stream increases the rate of hippocampal neurogenesis as measured by 5-bromo-2-deoxyuridine incorporation (Aberg M.A., 2000; Anderson M.F., 2002). Since IGF-1 has been shown to act on the same intracellular signaling components as insulin (Moelling K., 2002), it could well be that insulin may also affect hippocampal neurogenesis, especially since its receptor is also expressed in the hippocampus (Fig 5). Since neurogenesis must be under tight control to prevent proliferation, crosstalk between PI3K and ERK1/2 may control the rate of stem cell proliferation and quiescence.

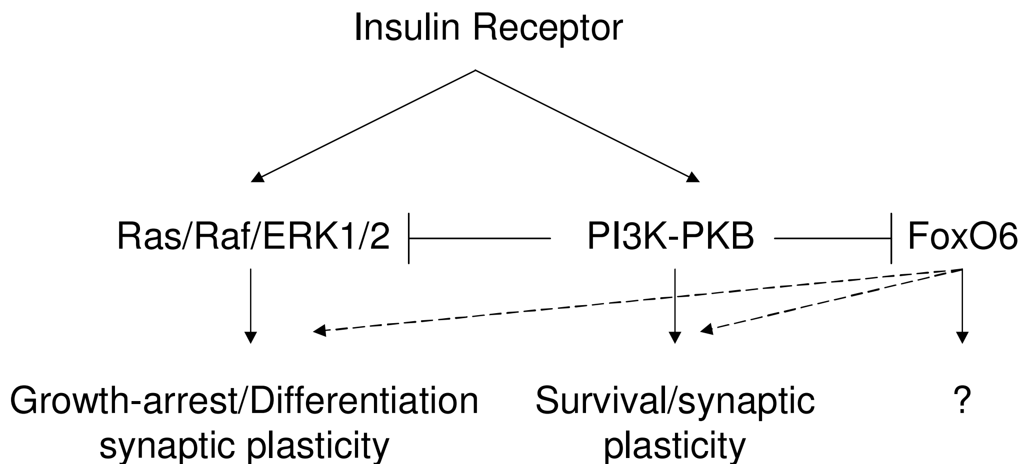

Figure 5. Possible actions of insulin signaling in the central nervous system. Activity of the insulin receptor regulates PI3K/PKB and Ras/Raf/ERK1/2. PI3K has multiple outputs as it negatively regulates ERK1/2 (chapter 2) and FoxO6 (chapter 5), influences synaptic plasticity (chapter 3) and stimulates survival. FoxO6 may be involved in learning and memory and growth arrest and differentiation.

A role for insulin in neuronal survival and synaptic plasticity?

In this thesis it was shown that the PI3K pathway facilitates NMDA receptor dependent changes in synaptic plasticity. If indeed the effects of insulin are mediated mainly by PI3K, other compounds acting on this route should all have the same effect on synaptic plasticity (Fig 5). Two of such examples are leptin (Shanley L.J., 2001), and BDNF (Yamada K., 2003). They both act via PI3K signaling and modulate synaptic plasticity. Leptin is a satiety hormone involved in feeding behavior and facilitates NMDA receptor-mediated signaling in a PI3K-dependent manner (Shanley L.J., 2001), similarly to insulin. BDNF also acts on PI3K signaling and is associated with the consolidation of spatial memory (Yamada K., 2003). Besides influencing synaptic plasticity, insulin, leptin, and BDNF are very potent in neuroprotection at the cellular level, presumably by activating the PI3K-PKB pathway (Wu X., 2004; Maroni P., 2003; Zhu D., 2002). This

data led to the hypothesis that synaptic plasticity and survival are linked (chapter 3). If this is correct, signals that act on neuronal survival and the PI3K route should alter synaptic plasticity, and synaptic activity should induce survival, since they both converge intracellularly on the PI3K-PKB pathway. Indeed, it was shown that LTP induction, or activation of the NMDA and mGluR receptor, results in an activation of the PI3K-PKB pathway (Sanna P.P., 2002; Hou L., 2004), and that this contributes to neuronal survival (Zhu D., 2002). Importantly, this suggests that the absence of synaptic activity can be largely compensated by growth factors acting on the PI3K-PKB route which has implications for development and disease (treatment). It has been found that Alzheimer's patients treated with insulin have a short term improvement in memory (Craft S., 1999) which may be explained by the effect of insulin on synaptic plasticity. On the other hand the increased neuronal degeneration observed may reflect a loss of insulin signaling due to the reduction in the amount of insulin receptors (Frolich L., 1998). Possibly, insulin or insulin-like factors could be modified in a treatment paradigm to delay the symptoms of Alzheimer's disease. Diabetes patients seem to suffer from high amounts of insulin as patients with high insulin levels have the greatest risk of developing dementia (chapter 1). Prolonged high levels of insulin may not favor synaptic plasticity and neuronal survival by inducing a general desensitization to insulin and insulin-like compounds. Since we observed in vitro, that insulin selectively desensitized the PI3K pathway, this may underlie the risk of developing dementia. Stimulating synaptic activity may compensate the insulin induced desensitization by stimulating PI3K. Although speculative, this may explain why memory training games as an exercise for elderly are effective in delaying the onset of dementia and enhancing cognitive functions (Arkin S.M., 2003).

FoxO6 is a transcriptional end-point of insulin signaling. FoxO6 is expressed at high levels in the hippocampus suggesting that it is negatively regulated by brain insulin.
Since FoxO factors have many putative downstream targets, FoxO6 may have multiple cellular functions and could be implicated in all effects attributed to insulin discussed earlier (chapter 4 and this chapter).

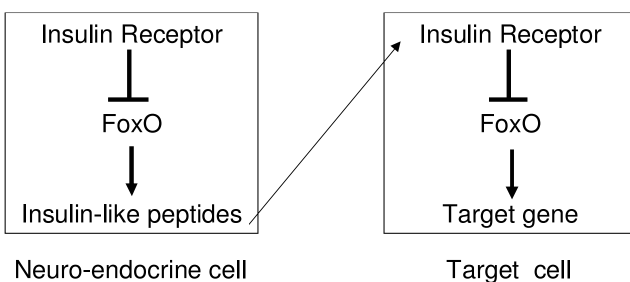

Figure 6. Brain insulin affects target cells non-autonomously by regulating the autonomous expression and secretion of insulin-like peptides. These insulin-like peptides diffuse to their receptors elsewhere in the organism. Once bound the secreted insulin-like factor inhibits FoxO function in that particular cell.

Studies across model systems have established that besides cell autonomous functions, insulin-like signaling can operate non-autonomously from a limited set of cells or a specific tissue. An insulin-like signaling pathway controls *Caenorhabditis elegans* aging, metabolism, and development. Mutations in the *daf-2* insulin-like receptor gene or the downstream *age-1* phosphoinositide 3-kinase gene extend adult life-span two- to threefold. Restoration of insulin like signaling only in neurons, muscle or intestine, revealed that Insulin-like signaling in neurons alone was sufficient to specify wild-type

life-span, but muscle or intestinal signaling was not (Wolkow C.A., 2000). In Drosophila, activated dFOXO in the head fat body is sufficient to increase both male and female lifespan, increase resistance to oxidative challenge and to alter whole-animal lipid metabolism (Hwangbo D.S., 2004). These findings identify the nervous system as a central regulator of animal longevity in *C.elegans* and *Drosophila* and suggest important roles for FoxO factors in regulating metabolism in mice and man.

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Samenvatting

Insuline stimuleert de opname van glucose in perifere organen, maar heeft ook functies in het centrale zenuwstelsel. Insuline en bijbehorende receptor worden tot expressie gebracht in het centrale zenuwstelsel en zijn betrokken bij neuronale stofwisseling en overleving. Naast een rol in cellulaire fysiologie speelt herseninsuline een rol in verschillende ziektes, zoals suikerziekte (diabetes) en Alzheimer. Dit proefschrift heeft tot doel te onderzoeken hoe insuline intracellulaire signaleringsroutes moduleert en hoe deze routes verband houden met blijvende veranderingen in synaptische plasticiteit. Onze aandacht ging daarbij in het bijzonder uit naar het PI3K-PKB route en het ERK1/2 route. Voorts ging onze aandacht uit naar een nieuwe FoxO transcriptie factor, FoxO6, geïdentificeerd in muizenhersenen, als een transcriptioneel eindpunt van insuline signalering. De resultaten van de experimenten zijn hier samengevat en besproken in relatie tot de functies van insuline in de hersenen.

Insuline heeft de eigenschap PKB en ERK1/2 te activeren in perifere cellen. Wij stellen de vraag aan de orde of dit ook geldt voor cellen met een neuronale oorsprong (hoofdstuk 2). Toepassing van insuline op Neuro2a cellen leidde afhankelijke van dosis en onafhankelijk van tijd tot PKB fosforilatie. De fosforilatie van PKB, door toepassing van insuline, verhoogde zijn katalytische activiteit, gemeten dmv de fosforilatie van GSK3beta, een goed omschreven PKB substraat. Insuline-geïnduceerde PKB-fosforilatie ging verrassenderwijs gepaard met een dosis-afhankelijke en tijd-onafhankelijke afname van ERK1/2 fosforilatie. Nader onderzoek naar het mechanisme dat ten grondslag ligt aan ERK1/2 defosforilatie liet zien dat het proces afhankelijk is van PI3K. Remming door LY294002 van de PI3K route, gedurende insuline stimulering, resulteerde in een toename van ERK1/2 fosforilatie, hetgeen aantoont dat insuline normaal gesproken PI3K en ERK1/2 activeert. Normaal gesproken remt stroomafwaartse crosstalk in de signalerings route ERK1/2 echter (fig 1). Verlende stimulatie met insuline desensibiliseert de PI3K route met als gevolg dat de remming op ERK1/2 fosforilatie ook afnam. Verlengde stimulatie met insuline had geen effect op ERK1/2 activering door insuline, aangezien stimulatie met insuline in combinatie met PI3K remmers in normale ERK1/2 fosforilatie resulteerde (fig 1.). Verlengde toepassing van insuline zorgt klaarblijkelijk alleen voor desensibilisering van de PI3K route, hetgeen doet vermoeden dat dit zich stroomafwaarts van de insuline receptor voordoet, na de bifurcatie tussen PI3K en ERK1/2 signalering.

We hebben aangetoond dat PI3K in vitro een belangrijke rol speelt in de integratie van neuronale insuline signalering, maar het in onduidelijk of hetzelfde geldt in vivo (hoofdstuk 2). Teneinde die vraag te beantwoorden hebben we de effecten van insuline ex-vivo onderzocht (hoofdstuk 3). Toepassing van insuline op plakjes van de hippocampus resulteerde in remming van lange termijn depressie (LTD) van hippocampale CA1 neuronen. Onderzoek naar het mechnisme dat ten grondslag ligt aan insuline-gemedieerde LTD, onthulde dat insuline om activering van NMDA receptors vraagt. Aangezien LTD remming plaatshad bij een testfrequentie die normaal gesproken niet leidt tot LTD remming, hebben we bij andere frequenties gestimuleerd teneinde te achterhalen of de sensitiviteit van het neurale netwerk was veranderd. Toepassing van insuline op plakjes van de hippocampus bleek de frequentie-respons curve van

synaptische plasticiteit naar links te verschuiven, zodat NMDA receptor afhankelijke LTD en LTP bij lagere frequenties kan plaatsvinden (fig 2).
Aangezien we eerder konden zien dat PI3K een belangrijke bemiddelaar voor insuline signalering is (hoofdstuk 2), onderzochten we of PI3K signalering een rol speelt in het overbrengen van de elektrofysiologische effecten van insuline. Toepassing van insuline op plakjes van de hippocampus stimuleerde PKB fosforilatie binnen 15 minuten en opwekking van de insuline gemedieerde LTD kon inderdaad worden geblokkeerd door de PI3K remmer, hetgeen doet vermoeden dat PI3K noodzakelijk is bij opwekking van LTD (fig 2). Samengevat: insuline bevordert de opwekking van normale LTD.

Insuline reguleert de activering van verschillende intracellulaire kinases, die onmiddellijke gevolgen kunnen hebben voor bijvoorbeeld het openen van een kalium kanaal of blijvende effecten bij het moduleren van transcriptie factoren. We hebben naar FoxO transcriptie factoren in de CNS gezocht, die bekend staan als transcriptionele eindpunten van insuline-PI3K-PKB signalering (hoofdstuk 5). De FoxO transcriptie factoren-familie heeft voor zover bekend drie leden in de muis, te weten FoxO1, FoxO3 en FoxO4. Wij determineerden een vierde lid en noemden het FoxO6.
Experimenten met in-situ hybridisatie lieten zien dat het FoxO6 transcript weliswaar werd uitgedrukt door de gehele hersenen, maar dat het overwegend in de hippocampus werd uitgedrukt, met name in de CA1 en CA3 gebieden en in mindere maten in de dentate gyrus en het CA2 gebied.
Aangezien FoxO factoren bij PI3K-PKB activering naar de cytosol translokeren hebben we getest of FoxO6 ook translokeert bij activering van PI3K-PKB. Vergeleken met FoxO1 en FoxO3 was FoxO6 onder alle geteste condities hoofdzakelijk nucleair, daar waar FoxO1 en FoxO3 na behandeling met insuline of andere groeifactoren naar de cytosol pendelden. FoxO6 pendelde in beperkte mate naar het cytosol. FoxO6 translokatie was afhankelijk van PI3K-PKB omdat een specifieke PI3K remmer translokatie kon verhinderen. Mutaties van de PKB residueen verhinderden daarbij ook door groei factoren veroorzaakte translokatie van FoxO6. Analyse van de FoxO6 sequentie laat zien dat FoxO6 een regio ontbeert die in de andere FoxO factoren bewaard is (fig3). In deze regio bevinden zich vier sequentiële fosforilatie residueen: een PKB residue, twee CK1 residueen en een DYRK1A residue. In de overeenkomstige regio van FoxO6 invoegen van dit stuk van FoxO3, herstelde het vermogen van FoxO6 om te pendelen tussen kern/nucleus en cytosol, net als in FoxO3. Het belang van deze resultaten ligt in de ontdekking van een vierde FoxO transcriptiefactor die afwijkt van de bekende drie leden van de familie door zijn beperkte PKB-mediated pendel eigenschappen. Deze factor, FoxO6, is naar alle waarschijnlijkheid een nieuwe speler in insuline en groeifactor signaal transductie in de hippocampus.

Translokatie van FoxO1, FoxO3 en foxO4 naar de cytosol wordt veroorzaakt door fosforilatie van drie PKB plaatsen (hoofdstuk 4,5) Fosforilatie van deze plaatsen trekt begeleiders aan die nodig zijn voor transport door het nucleaire poriën complex en sekwestratie in het cytosol. FoxO6 heeft twee (Thr26 en Thr 184) van de drie geconserveerde PKB motieven. De C-terminus bevat een niet-geconserveerde optimale PKB fosforilatie site (Thr338). Aangezien FoxO6 bijna volledig nucleair blijft onder alle geteste condities, maar wel enige van fosforilatie afhankelijke pendeling liet zien,

onderzochten we de rol van de overblijvende twee PKB residueen in translokatie en transactivering (hoofdstuk 6). Door gebruik te maken van mutatieanalyse toonden we aan dat translokatie afhing van de fosforilatie staat van zowel Thr26 als Ser184. De niet-geconserveerde Thr338 PKB-site had geen aanwijsbare functie in translokatie. Wij wijzen erop dat groeifactoren de activiteit van FoxO6 remmen door translokatie van de kern/nucleus naar de cytosol te initiëren, waarmee ze de transcriptiefactor fysiek van het DNA verwijderen. Groeifactoren remden inderdaad FoxO6 activiteit maar de remming kwam niet door translokatie. Een FoxO6-FoxO3 chimeer eiwit (hoofdstuk 5) had dezelfde transcriptionale activiteit als een wild-type FoxO6 en werd in dezelfde mate als een wild-type FoxO6 onderdrukt door groeifactoren (hoofdstuk 6). Mutatie-analyse onthulde dat Thr26 waarschijnlijk noodzakelijk is om remming van transcriptionale activiteit door stimulatie van de groeifactor te bemiddelen, daar waar Ser184 baseline niveaus van transcriptionale activiteit controleert (figuur 4). Naast groeifactoren reduceert ook stress de activiteit van FoxO6 op reporter constructs. De door stress veroorzaakte afname van activiteit was onafhankelijk van de fosforilatie staat van Ser184, hetgeen aangeeft dat stress aanvullende PI3K-onafhankelijke paden gebruikt bij het reguleren van FoxO6. Verdere analyse van FoxO6-bemiddelde activiteit op de glucose-6-fosfatase promotor onthulde dat FoxO6-activiteit afhangt van intacte FoxO binding plaatsen. Samengevat wordt transcriptionale activiteit van FoxO6 gereguleerd door fosforilatie van Thr26 en Ser184, onafhankelijk van translocatie naar de cytosol. Strakke controle op transcriptie door FoxO factoren is klaarblijkelijk mogelijk zonder het vereiste van cytoplasmische pendeling.

In zijn algemeenheid reguleert insuline intracellulaire cross-talk, faciliteert insuline synaptische plasticiteit en controleert insuline de activiteit van FoxO transcriptiefactoren. Alledrie de besproken aspecten hebben mogelijkerwijs implicaties voor de juiste ontwikkeling en onderhoud van het centrale zenuwstelsel.

Een rol voor insuline in neuro-ontwikkeling en neurogenese?

In hoofdstuk 2 beschreven we door insuline opgewekte crosstalk tussen PI3K en ERK1/2 signalering. Hoewel we niet onderzochten wat de uitwerking van deze crosstalk voor cellulaire stofwisseling is, bestaan er onderzoeken naar non-neuronale systemen die deze crosstalk beschrijven en die erop duiden dat insuline een belangrijke rol kan spelen in het reguleren van de balans tussen proliferatie en differentiatie. Een onderzoek naar MCF-7 borstkankercellen liet zien dat IGF-I ook aanzette tot crosstalk tussen PI3K en ERK1/2. In betreffend onderzoek werd aangetoond dat PKB/Akt de stroomopwaartse ERK1/2 activator Raf fosforileert en inactiveert. Remming van Raf door PKB verschuift de cellulaire reactie interessant genoeg van cell-cycle stilstand naar proliferatie. Zij postuleren dat de PI3K route proliferatie en overleving reguleert, daar waar de ERK1/2 route groeistilstand en differentiatie reguleert. Aangezien het systeem in reactie op verlengde hoge concentraties van insuline desensibiliseert zijn de dosis en aanwezigheid van insuline voorzichtig geïntegreerd in een cellulaire reactie. Als dat systeem functioneel zou zijn in ontwikkelende neuronen zou insuline de ruimtelijke en tijdelijke patronen van neuronale proliferatie en differentiatie kunnen controleren. Een variant van dit systeem zou ook neuronale axon guidance kunnen controleren. Insuline receptors op

de groeikoon van migrerende neuronen zouden een stijging van insuline kunnen waarnemen en zouden kunnen reageren door verder te prolifereren/migreren of door te differentiëren tot een functioneel neuron wanneer de context daartoe geschikt is. Wegnemen van de insuline receptor in drosophila/fruitvliegen zorgt interessant genoeg voor abnormale axon guidance in het visuele systeem van de fruitvlieg (Song. J., 2004), hetgeen de suggestie wekt dat insuline signalering in vivo belangrijk is voor normale neuronale groei, uitgroei en differentiatie. De juiste afzetting van een extracellulaire insuline gradiënt zou een eerste vereiste moeten zijn indien insuline neuronale migratie controleert. Het is echter niet bekend of zo'n insuline gradiënt aanwezig is in het centrale zenuwstelsel of hoe zo'n gradiënt zou kunnen worden gecreëerd. Insuline mRNA wordt interessant genoeg lokaal tot expressie gebracht in de hippocampale CA1 en CA3 regio's en zou een belangrijke factor kunnen zijn in neuronale ontwikkeling en onderhoud.
IGF-1 administratie direct naar de hersenen of periferale bloedstromen doet de hoeveelheid hippocampale neurogenese toenemen, zoals gemeten door 5-bromo-2-deoxyuridine incorporatie. Aangezien is aangetoond dat IGF-1 op dezelfde intracellulaire signaleringscomponenten werkt als insuline zou het best kunnen zijn insuline ook effect heeft op de hippocampale neurogenese, helemaal omdat zijn receptor ook tot expressie komt in de hippocampus (fig5.). Aangezien neurogenese onder strakke controle moet staan wil men proliferatie voorkomen, kan crosstalk tussen PI3K en ERK1/2 mogelijkerwijs de mate van celproliferatie controleren.

Een rol voor insuline in neuronale overleving en synaptische plasticiteit?

In dit proefschrift werd aangetoond dat de PI3K route NMDA receptor afhankelijke veranderingen in synaptische plasticiteit faciliteert. Als de effecten van insuline inderdaad voornamelijk worden bemiddeld door PI3K, zouden andere op deze route actieve compounds allemaal hetzelfde effect op synaptische plasticiteit moeten hebben (fig 5). Twee voorbeelden daarbij zijn leptine en BDNF: beiden werken via PI3K signalering en moduleren synaptische plasticiteit.
Leptine is een verzadiging hormoon dat betrokken is bij voeder-gedrag en dat – net als insuline - op een PI3K-afhankelijke wijze NMDA receptor-gemedieerde signalering faciliteert. BDNF werkt ook op PI3K signalering en is betrokken bij het consolideren van spatieel geheugen. Insuline, leptine en BDNF zijn naast hun vermogen tot beïnvloeden van synaptische plasticiteit, zeer effectief in neuroprotectie op celniveau, vermoedelijk door de PI3K-PKB route te activeren. Deze data gaf aanleiding tot de hypothese dat synaptische plasticiteit en -overleving aan elkaar gekoppeld zijn (hoofdstuk 3). Als de hypothese juist is, zouden signalen die zowel werken op neuronale overleving als op de PI3K route synaptische plasticiteit moeten veranderen. En synaptische activiteit zou moeten aanzetten tot overleving aangezien beiden intracellulair convergeren op het PI3K-PKB pad. Aangetoond werd dan ook dat LTP inductie, oftewel activering van de NMDA en mGluR receptor, in een activering van de PI3K-PKB route resulteerde en dat een en ander bijdraagt aan neuronale overleving.Belangrijk is dat dit suggereert dat de afwezigheid van synaptische activiteit grotendeels gecompenseerd kan worden door groeifactoren die werken op de PI3K-PKB route, hetgeen implicaties heeft voor ontwikkeling en ziekte(behandeling). Men heeft ontdekt dat bij Alzheimerpatienten die worden behandeld met insuline voor een korte periode een verbetering in hun geheugen

optreedt, hetgeen wellicht kan worden verklaard door het effect dat insuline heeft op synaptische plasticiteit. Aan de andere kant kan de waargenomen toename van neuronale degeneratie getuigen van een verlies aan insuline signalering door de afname van de hoeveelheid insuline receptoren. Insuline of insulineachtige factoren kunnen mogelijkerwijs worden aangepast/gemodificeerd in een behandelingsparadigma teneinde de symptomen van Alzheimer te vertragen. Men kan zeggen dat diabetespatiënten de nadelen ondervinden van grote hoeveelheden insuline aangezien patiënten met hoge insulineniveaus het grootse risico hebben dement te worden (hoofdstuk 1). Langdurige hoge insulineniveaus bevorderen synaptische plasticiteit en neuronale overleving wellicht niet omdat een algemene ongevoeligheid voor insuline en insulineachtige compounds wordt opgewekt. Aangezien we in vitro hebben waargenomen dat insuline op selectieve wijze de PI3K route ongevoelig maakt, zou een en ander ten grondslag kunnen liggen aan het risico dementie te ontwikkelen. Het stimuleren van synaptische activiteit door het stimuleren van PI3K zou kunnen compenseren voor de door de insuline veroorzaakte ongevoeligheid. Hoewel speculatief zou een en ander kunnen verklaren waarom geheugenoefening voor ouderen effectief is in het vertragen van de eerste symptomen van dementie en in het versterken van de cognitieve functies.

FoxO6 is een transcriptioneel eindpunt van insuline signalering. FoxO6 komt tot hoge expressie in de hippocampus, hetgeen doet vermoeden dat het op negatieve wijze door herseninsuline wordt gereguleerd. Aangezien FoxO6 factoren vermeende targets zouden kunnen hebben, zou FoxO6 verschillende cellulaire functies kunnen hebben en zou FoxO6 betrekking kunnen hebben op alle hier eerder beschreven effecten die aan insuline toegeschreven worden (hoofdstuk 4 en dit hoofdstuk).
Onderzoek naar modelsystemen heeft aangetoond dat naast cel-autonome functies insulineachtige signalering non-autonoom van een beperkte set van cellen of een specifiek weefsel kan opereren. Een insulineachtig signaleringspad controleert de veroudering, stofwisseling en ontwikkeling van *Caenorhabditis elegans*. Mutaties in het insulineachtige daf-2 receptor gen of het stroomafwaartse age-1 PI3K gen, verlengen de volwassen levensduur twee of drie maal. Tot neuronen, spieren of ingewanden beperkt herstel van insulineachtige signalering, liet zien dat insulineachtige signalering in neuronen voldoende was om wild-type levensduur te specificeren, maar signalering in spier of ingewanden niet. Geactiveerde dFOXO in het hoofd vet lichaam van *Drosophila* is voldoende om de mannelijke en vrouwelijk levensduur te verlengen, de weerstand tegen oxidatieve prikkels te vergroten en de lipide stofwisseling van het hele dier te veranderen. Deze bevindingen stellen vast dat het centrale zenuwstelsel een centrale regulator is van de hoge ouderdom van *C.elegans* en *Drosophila* en duiden op een belangrijke rol voor FoxO factoren in het reguleren van de stofwisseling in muis en mens.

Curriculum vitae

Lars Philip van der Heide werd geboren op 8 maart 1977 te Leerdam. Hij behaalde het VWO diploma in juni 1995 aan het Wagenings Lyceum. In september van datzelfde jaar begon hij aan de studie Medische Biologie aan de Universiteit Utrecht. In 2000 werd het doctoraal examen behaald, met bijvak aan de vakgroep Moleculaire Cel Biologie van de Universiteit Utrecht (o.l.v. Dr. Jan Andries Post). De expressie van cell adhesie moleculen en de gevolgen van oxidatieve stress werden in het bijvak onderzocht. Het hoofdvak werd gevolgd op het Rudolf Magnus Instituut voor Neurowetenschappen (o.l.v. Dr. Loes Schrama). De functie van het eiwit B-50 op neuronale uitgroei werd in het hoofdvak onderzocht. Van september 2000 tot sepetember 2004 werkte hij als assistent in opleiding (o.l.v. Dr. Marco Hoekman, Dr. Marten Smidt, Prof. Peter Burbach en Prof. Willem Hendrik Gispen) bij het Rudolf Magnus Instituut voor neurowetenschappen aan het onderzoek beschreven in dit proefschrift.

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LP