Analysis of FOXO transcriptional networks

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Analysis of FOXO transcriptional networks

Analyse van FOXO-geïnduceerde transcriptionele netwerken

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Kristan Elina van der Vos
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Promotor: Prof. dr. P.J. Coffer

Als je doel Ithaka is en je vertrekt daarheen,
dan hoop ik dat je tocht lang zal zijn,
en vol nieuwe kennis, vol avontuur.

Vrees geen Laistrigonen en Kyclopen,
of een woedende Poseidon;
je zult ze niet tegenkomen op je weg, als
je gedachten verheven zijn, en emotie
je lichaam en geest niet verlaat.
Laistrigonen en Kyclopen, en de razende Poseidon
zul je niet tegenkomen op je weg,
als je ze al niet meedroeg in je ziel, en
je ziel ze niet voor je voeten werpt.

Ik hoop dat je tocht lang mag zijn,
de zomerochtenden talrijk zijn, en
dat het zien van de eerste havens
je een ongekende vreugde geeft.
Ga naar de warenhuizen van Fenicië,
neem er het beste uit mee.
Ga naar de steden van Egypte, en
leer van een volk dat ons zoveel te leren heeft.

Verlies Ithaka niet uit het oog;
daar aankomen was je doel.
Maar haast je stappen niet;
het is beter dat je tocht duurt en duurt
en je schip pas ankert bij Ithaka,
wanneer je rijk geworden bent
van wat je op je weg hebt geleerd.

Verwacht niet dat Ithaka je meer rijkdom geeft.
Ithaka gaf je een prachtige reis;
zonder Ithaka zou je nooit vertrokken zijn.
Het gaf je alles al, meer geven kan het niet.

En mocht je vinden dat Ithaka arm is,
denk dan niet dat het je bedroog.
Want je bent een wijze geworden, hebt intens geleefd,
en dat is de betekenis van Ithaka.

Konstantinos Kavafis

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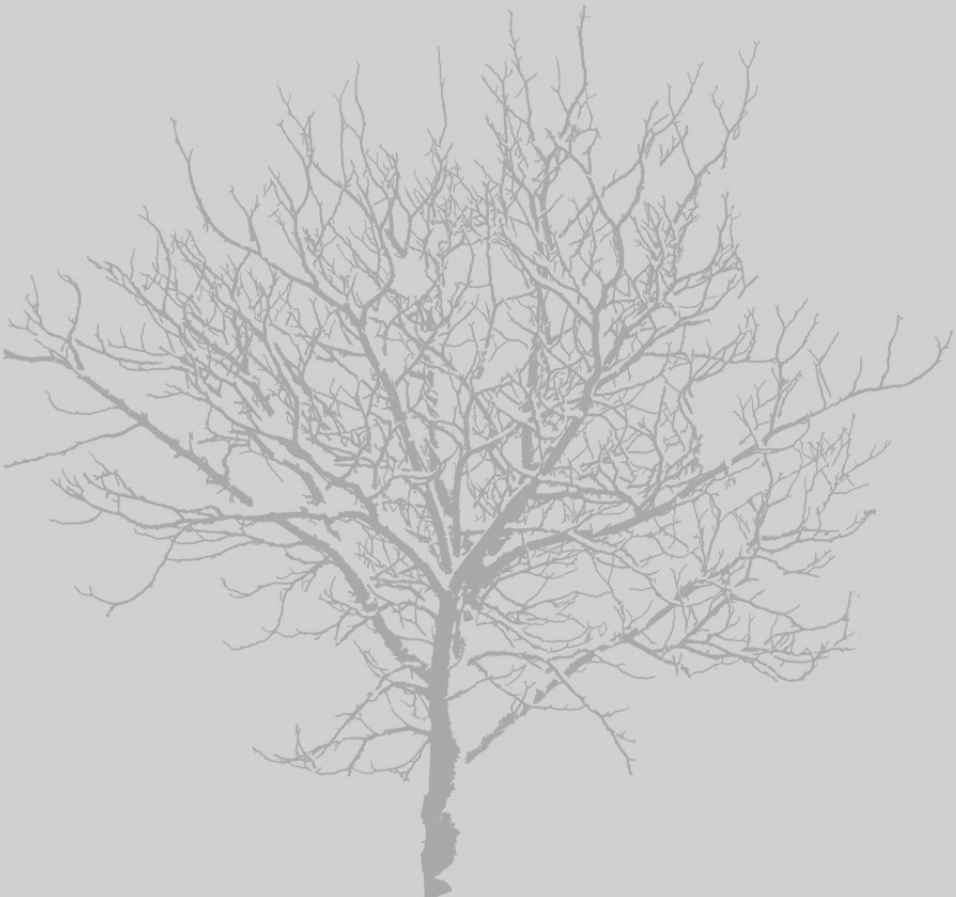
CHAPTER 1

General Introduction



Proliferation, differentiation and apoptosis are tightly regulated by extracellular stimuli including hormones, growth factors and cytokines. A balance between these processes is a critical requirement for tissue homeostasis and loss of this balance can result in development of disease. The binding of such mediators to their cognate receptors induces activation of intracellular signal transduction cascades that, through regulation of transcription, eventually results in altered gene expression and changes in cell fate decisions. Constitutive activation of oncogenic signalling pathways by loss of tumour suppressors and activation of oncogenes causes malignant transformation, which is characterised by a differentiation block and the ability of the cancer cells to proliferate and survive independent of growth factor signals. Understanding the molecular mechanisms underlying the development of neoplasia is crucial for the identification of novel targets for the development of novel cancer therapies.

The PI3K-PKB pathway

The PI3K-PKB pathway plays a critical role in the regulation of proliferation and survival of most cell types and inappropriate activation of this signalling module is frequently observed in human cancer. Phosphoinositide-3-kinase (PI3K) is activated in response to a diverse array of stimuli including hormones, cytokines and growth factors. When activated, PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP_2) lipids at the 3' position of the inositol ring resulting in the formation of phosphoinositol-3,4,5-triphosphate (PIP_3). PIP_3 in the plasma membrane then acts as a "docking site" for proteins containing a pleckstrin homology (PH) domain (Engelman, 2009). Importantly, phosphatase and tensin homolog (PTEN) counteracts PI3K signalling by dephosphorylating PIP_3(Maehama and Dixon, 1998) and is this protein is frequently mutated in cancer, resulting in a constitutive PI3K activation (Fig. 1) (Li *et al.*, 1997; Steck *et al.*, 1997). The relocalisation and "clustering" of PH-containing proteins at the plasma membrane results in activation of multiple downstream

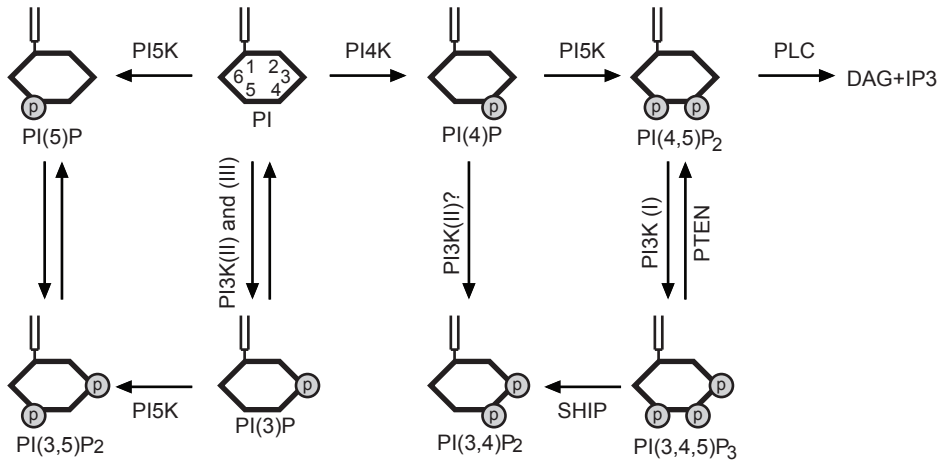


Figure 1. Overview of the phosphoinositide lipid cycle

A series of phosphoinositide kinases phosphorylate phosphoinositides on different residues resulting in the formation of different phospholipids that can act as important signal mediators. PI3K phosphorylates PI(4,5)P2 at the second position resulting in formation of PI(3,4,5)P3 in response to growth factor signalling. Once produced, PI(3,4,5)P3 is hydrolysed by the activation of either PTEN or SHIP to give PI(4,5)P2 or PI(3,4)P2 respectively.

signalling proteins (Engelman, 2009). One of these, is the serine threonine kinase protein kinase B (PKB, also called Akt), which upon activation mediates the proliferative and pro-survival activities of PI3K (Burgering and Coffer, 1995). Relocalisation of PKB to the plasma membrane brings the kinase in close proximity with phosphoinositide dependent kinase 1 (PDK1), which then activates PKB by phosphorylating threonine (Thr) 308 within its kinase domain (Fig. 2) (Stephens *et al.*, 1998). It has been suggested that phosporylation of Thr 308 might induce a conformational change, which abrogates the binding of the PH domain to PIP_3 and releases PKB from the plasma membrane (Ananthanarayanan *et al.*, 2007). In addition, PKB is phosphorylated on serine (Ser) 473. The identity of the kinase that phosphorylates Ser 473 was long unknown and multiple candidates have been proposed, including PDK1, integrin linked kinase and PKB itself (Manning and Cantley, 2007). However, recently it has been demonstrated that the mTORC2 complex is responsible for phosphorylating this residue (Sarbassov *et al.*, 2005). While phosphorylation of Thr 308 is essential for PKB activation, it has been proposed that phosphorylation of Ser 473 might determine substrate specificity. Upon stimulation of mouse embryonic fibroblasts lacking the mTORC2 component SIN1, PKB was phosphorylated on Thr 308, but phosphorylation of Ser 473 was undetectable (Jacinto *et al.*, 2006). However, although with slightly lower levels, PKB activity was still measurable, but the kinase was unable to phosphorylate a number of its substrates, suggesting that phosphorylation of Ser 473 can in some way influence substrate specificity (Jacinto *et al.*, 2006). The precise molecular mechanism

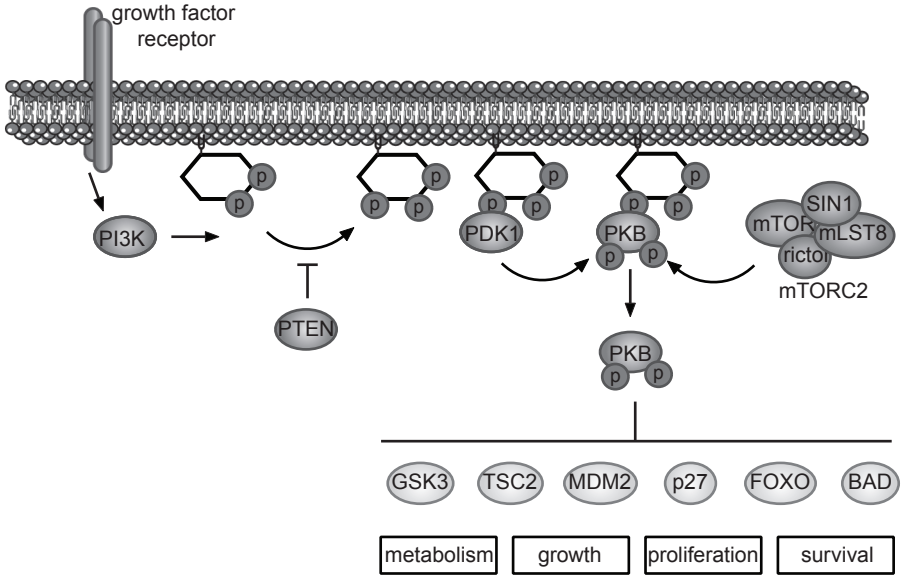


Figure 2. Activation of PI3K and PKB by growth factors
Activation of phosphoinositol-3-kinase (PI3K) by growth factors and cytokines increases survival and proliferation. After its activation, PI3K phosphorylates PIP2 in the cell membrane resulting in formation of PIP3. The formation of PIP3 results in recruitment of proteins containing a pleckstrin homology (PH) domain, through which they bind to PIP3. Among those are the serine-threonine kinase protein kinase B (also known as c-akt) and its upstream kinases PDK1 and PDK2, which phosphorylate PKB on two residues resulting in activation of PKB and release from the plasma membrane. The activation of PKB causes an increase in proliferation and survival by regulation of proteins involved in cell cycle regulation, apoptosis, growth and metabolism.

underlying this observation remains unclear, but one explanation is that the extent of PKB activation may play an important role. Through phosphorylation, PKB affects the activity of many substrates involved in metabolism, glycolysis, insulin signalling, growth and vascularisation (Fig. 2). PKB recognises and phosphorylates serine and threonine residues within the consensus motif RxRxxS/T, which for many targets causes functional inhibition (Manning and Cantley, 2007). One example of these are the FOXO transcription

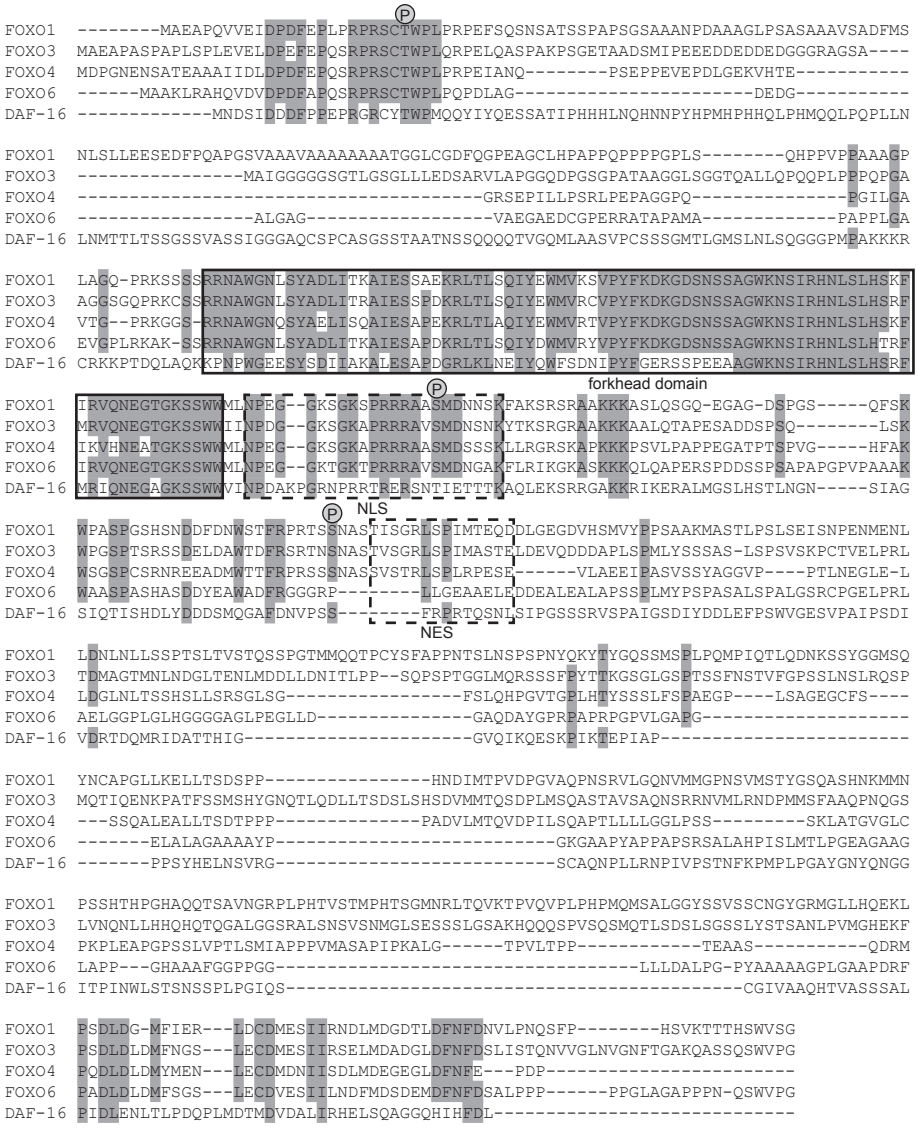


Figure 3. Alignment of FOXO1, FOXO3, FOXO4, FOXO6 and DAF-16
Alignment of the amino acid sequences of FOXO1, FOXO3, FOXO4, FOXO6 and DAF-16. Indicated are the PKB phosphorylation sites, the DNA binding domain and the NLS and NES.

factors, whose inactivation by PKB-mediated phosphorylation for the pro-survival effect of PKB (Brunet *et al.*, 1999; Kops *et al.*, 2002).

FOXO transcription factors

Forkhead box O transcription factors (FOXOs) are characterised by a winged-helix domain through which they bind DNA (Obsil and Obsilova, 2008). The first member of this family was identified in *Drosophila*; the *forkhead* gene which is important for terminal development in the Drosophila embryo (Weigel *et al.*, 1989). Mutations in this gene cause abnormalities in the development of the digestive system and foregut and hindgut are replaced by ectopic head structures, hence the name forkhead. Currently over 100 members have been identified based on sequence homology with roles in a wide variety of processes. In mammals FOXO transcription factors consists of four members: FOXO1, FOXO3, FOXO4 and FOXO6. FOXOs contain four domains: a highly conserved DNA binding domain, a nuclear localisation sequence (NLS), a nuclear export sequence (NES) and a C-terminal transactivation domain. Analysis of sequence alignment shows that several of these regions are highly conserved between the various FOXO isoforms (Fig. 3). Regions that show the highest homology include the region containing the first PKB phosphorylation site, the DNA binding domain and the region containing the NLS. Due to the high conservation within the DBD FOXOs share similar binding specificity to the DNA binding consensus sequence: TTGTTTAC (Furuyama *et al.*, 2000).

FOXOs are ubiquitously expressed but with varying expression levels. FOXO1 expression is high in adipose tissue, heart, spleen, and brain. FOXO3 is mainly expressed in muscle, heart, spleen and ovaries, while FOXO4 shows the highest expression in heart, brain, spleen and lung (Anderson *et al.*, 1998; Furuyama *et al.*, 2000; Greer and Brunet, 2005). FOXO6 has recently been identified in the murine brain, but whether it is expressed in other tissues remains unclear (Jacobs *et al.*, 2003).

FOXO transcription factors in aging

In the nematode worm *C elegans*, activation of an orthologue of the insulin receptor (DAF-2) results in activation of a PI3K orthologue (AGE-1), which induces activation of PDK1

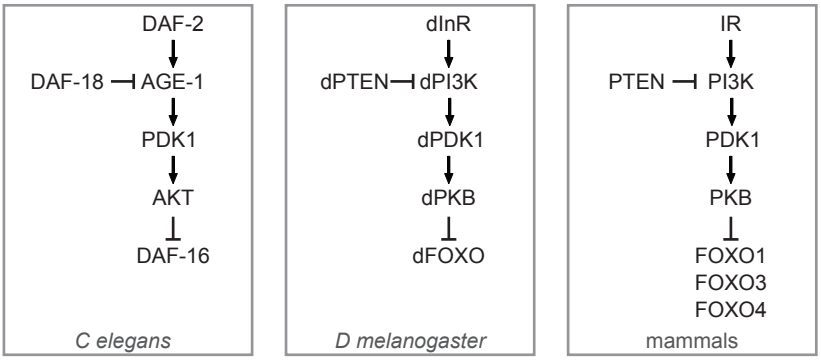


Figure 4. The insulin pathway is conserved between C elegans, D melanogaster and mammals

Activation of the insulin receptor (DAF-2) activates PI3K (AGE-1) resulting in the formation of PIP3. These phosphorylated lipids form docking sites for PDK1 and PKB (AKT) resulting in their activation. PKB phosphorylates and inhibits FOXO transcription factors. While C elegans and D melanogaster have a single FOXO isoform, mammals have three distinct FOXOs

and subsequently activates a PKB (AKT) orthologue (Kimura *et al.*, 1997; Paradis *et al.*, 1999; Dorman *et al.*, 1995; Paradis and Ruvkun, 1998). In addition, expression of the PTEN orthologue DAF-18 limits AKT activity by decreasing PIP_3 levels (Fig. 4)(Ogg *et al.*, 1997). This pathway plays an important role in regulation of so-called dauer formation. Normally the lifespan of *C elegans* is 2-3 weeks, however in the absence of nutrients the nematode worm can enter a stress-resistant stage in which the worms can survive up to 2-3 months (Mukhopadhyay *et al.*, 2006). The DAF-2 pathway negatively regulates the induction of dauer formation and worms that have loss of function mutations in the *daf-2* gene live twice as long as wild-type worms (Kenyon *et al.*, 1993). *Daf-2* mutant-induced dauer formation is dependent on the FOXO transcription factor DAF-16 since the long-lived phenotype is reverted in *daf-16* deficient organisms (Lin *et al.*, 1997; Ogg *et al.*, 1997). The ability of DAF-16 to induce dauer formation depends on regulation of transcriptional targets involved in stress resistance and metabolism, including superoxide dismutatase, catalase, apolipoprotein genes and small heat shock protein genes (Mukhopadhyay *et al.*, 2006). Similar to *C elegans, Drosophila melanogaster* has only a single FOXO isoform: dFOXO (Giannakou and Partridge, 2007). Overexpression of dFOXO or inhibition of insulin signalling by mutating the insulin receptor again extends lifespan, indicating that the role of insulin signalling in regulating lifespan is evolutionary conserved (Fig. 4) (Giannakou *et al.*, 2004; Giannakou and Partridge, 2007; Hwangbo *et al.*, 2004). The insulin pathway also regulates lifespan of the mosquito *Culex pipiens* in a FOXO-dependent manner. This fly enters a overwintering diapause in response to shortening of the day length (Emerson *et al.*, 2009). Knockdown of FOXO, inhibits diapause, reduces fat storage and decreases the mosquito's lifespan demonstrating that FOXO activity mediates the overwintering diapause response (Sim and Denlinger, 2008).
Accumulating evidence is emerging that the FOXO transcription factors also play an import role in human aging. SNP analyses have shown that specific *FOXO3* genotypes are associated with longevity (Flachsbart *et al.*, 2009; Willcox *et al.*, 2008). In studies among centenarians, genetic variation within the *FOXO3* gene was associated with the ability to attain exceptionally long age. Furthermore, one specific *FOXO3* genotype was also associated with increased insulin sensitivity, stressing the importance of this hormone in regulating human lifespan (Flachsbart *et al.*, 2009; Willcox *et al.*, 2008). However, further research is required to determine what the consequences are of these polymorphisms for the function of FOXO3.

FOXO mouse models

Deletion of specific FOXO genes in mice has revealed both redundant and non-redundant effects (Table 1). *Foxo1*-/- mice die during embryonic development due to incomplete vascular development. Foxo1 expression was found in a variety of embryonic vessels, suggesting a role in vascular development (Hosaka *et al.*, 2004). Foxo3-/- females showed an age-dependent infertility with an abnormal ovarian follicular development (Hosaka *et al.*, 2004). The *Foxo3*-/- female mice exhibit global follicular activation leading to oocyte death, resulting in early depletion of functional ovarian follicles and subsequently infertility. This *Foxo3*-/- phenotype resembles human premature ovararian failure, a common cause of infertility and aging in woman and indicates that Foxo3 normally suppresses follicular activation and helps to maintain a resting follicle pool (Castrillon *et al.*, 2003). Foxo3 is highly expressed in peripheral lymphoid tissue and examination of the lymphoid compartment revealed that *Foxo3* deficiency leads to spontaneous lymphoproliferation and wide-spread organ inflammation (Lin *et al.*, 2004). Helper T cells from *Foxo3*-/- mice are hyperactivated and show increased rates of proliferation and cytokine production compared to wild-type T

Table 1. Phenotypes of FOXO knockout mice

Hosaka *et al.*(2004)	*Foxo1* -/-	Lethal due to incomplete vascular development
Nakae *et al.*(2002)	*Foxo1* -/+	Restored insulin sensitivity and rescued diabetic phenotype in InsR mutant mice
Kitamura *et al.*(2002)	*Foxo1* -/+	Reversed β-cell failure in mice lacking Insulin receptor substrate 2 (Irs2 -/-)
Castrillon *et al.*(2003) Hosaka *et al.*(2004)	*Foxo3* -/-	Age-dependent infertility due to global ovarian follicle activation resulting in early oocyte depletion
Lin *et al.*(2004)	*Foxo3* -/-	Lymphoproliferation and widespread organ inflammation due to hyperactivated helper T cells
Hosaka *et al.*(2004)	*Foxo4* -/-	No phenotype detected yet
Paik *et al.*(2007)	*Foxo1* -/-, *Foxo3* -/-, *Foxo4* -/-	Uterine hemangiomas appear at 6 to 8 weeks of age, which progress to massive fatal hemaningiomas affecting numerous tissues. Lymphoblastic thymic lymphomas appear at 19 to 30 weeks of age
Tothova *et al.*(2007)	*Foxo1* -/-, *Foxo3* -/-, *Foxo4* -/-	Decrease in long-term hematopoietic stem cell population due to increased entry into cell cycle, decreased renewal capacity, increased apoptosis, which are caused by an increase in reactive oxygen species

cells (Lin *et al.*, 2004). In contrast to *Foxo1*- and *Foxo3*-deficient mice, *Foxo4*-/- mice are normal in appearance and did not show any abnormalities (Hosaka *et al.*, 2004).
The individual disruption of each of the Foxo genes resulted in distinct phenotypes suggesting that there are functional differences between FOXO1, FOXO3 and FOXO4. However, since the expression pattern of these genes partially overlaps, there is still the possibility of functional redundancy. The development of an inducible *Foxo1/3/4*-/- mouse model has highlighted this (Paik *et al.*, 2007; Tothova *et al.*, 2007). After conditional deletion of *Foxo1*, *Foxo3* and *Foxo4,* mice developed lymphoblastic thymic lymphomas and hemangiomas (Paik *et al.*, 2007). Disruption of only two *Foxo* genes resulted in less severe hemangiomas indicated by longer survival of the mice, and no sign of lymphomas. These results demonstrate that FOXOs are functional redundant tumour supressors (Paik *et al.*, 2007). Since FOXOs show a wide-spread tissue distribution, the restricted tumour phenotype was somewhat unexpected. Chromosomal translocations that disrupt the human *FOXO* gene have been associated with leukemia and alveolar rhadomyosarcoma (Anderson *et al.*, 2001; Barr, 2001; Borkhardt *et al.*, 1997; Galili *et al.*, 1993; Hillion *et al.*, 1997; Parry *et al.*, 1994). Furthermore, deregulation of FOXOs by constitutive activation of the PI3K pathway has been observed in a variety of tumours, including those arising from prostate, stomach, brain and breast. Thus it is surprising that after deletion of *Foxo* genes only tumours arose from endothelial and thymic origin (Arden, 2008).
Analysis of the haematopoietic system using the same inducible *Foxo1/3/4*-/- mouse model has demonstrated the role of FOXOs in oxidative stress resistance *in vivo* (Tothova *et al.*, 2007). Loss of *Foxo1*, *Foxo3* and *Foxo4* resulted in increased numbers of myeloid progenitors in peripheral blood, while in the bone marrow the number of haematopoietic stem cells (HSCs) was reduced. Transplantion experiments showed a decreased repopulating ability of bone marrow cells from triple *Foxo*-deficient mice, indicating that

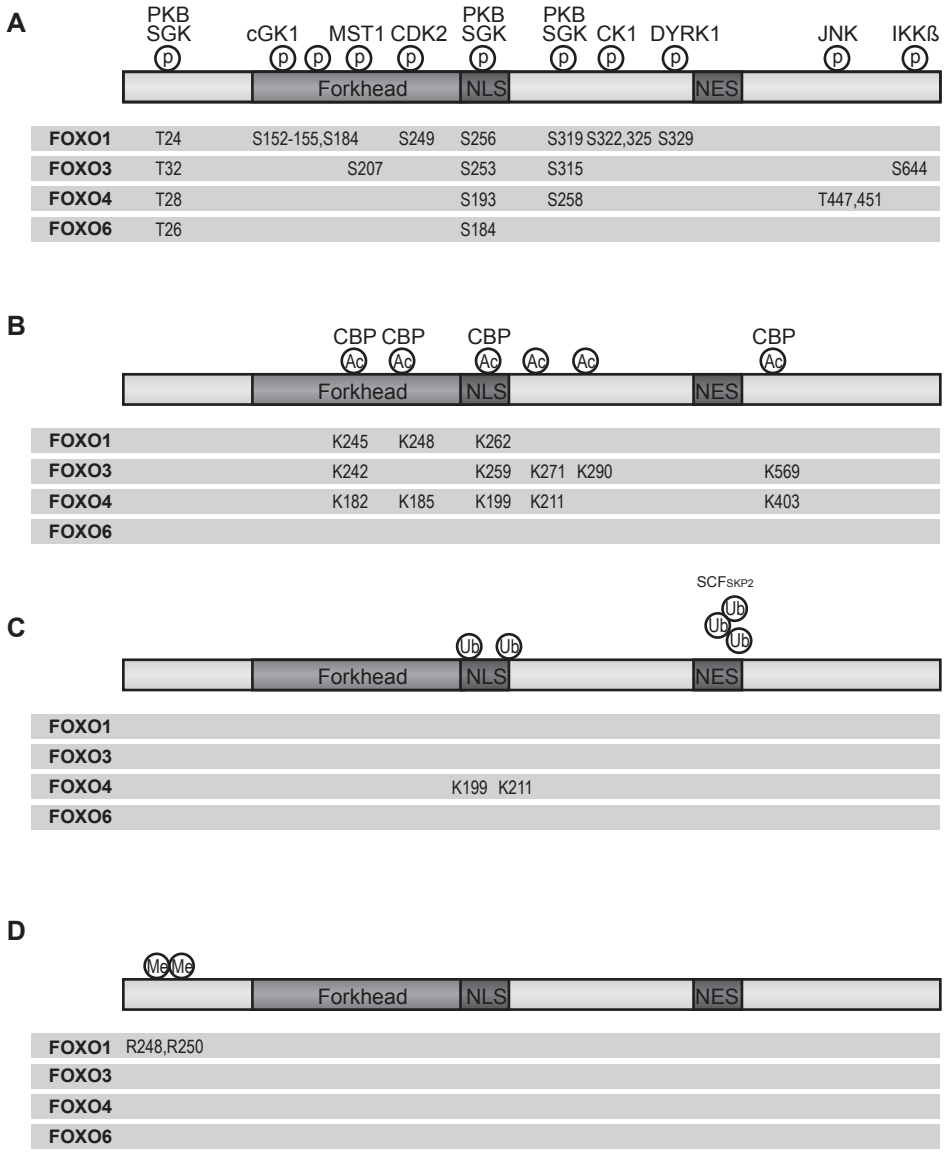


Figure 5. Schematic representation of FOXO post-translational modifications
Depicted are the localisation of multiple post-translational modifications on FOXOs including phosphorylation, acetylation, ubiquitination and methylation sites. Ac, acetylation;AKT/PKB, AKT/protein kinase B; CDK2, cyclin-dependent kinase-2; cGK1, cGMP-dependent protein kinase-1; CK1, casein kinase-1; DYRK1, dual-specificity tyrosine (Y)-phosphorylation-regulated kinase-1; IKKß, IκB kinase; JNK, c-Jun N-terminal kinase; Me, methylation; MST1, mammalian sterile 20 kinase-1; P, phosphorylation; PRMT1, protein arginine N-methyltransferase 1; SCFSkp2, the SKP1/cullin-1/F-box protein complex that contains the specific substrate-targeting F-box protein SKP2; SGK, serum- and glucocorticoid-inducible kinase; Ub, ubiquitylation. (adapted from Van der Horst and Burgering, Nature Cell Biology, 2007)

FOXOs are required for HSC renewal. Analysis of *Foxo*-deficient HSCs revealed that apoptosis and reactive oxygen species levels were increased in *Foxo*-deficient cells. Surprisingly, treatment of mice with antioxidant N-acetyl-cysteine resulted in reversion of the *Foxo*-deficient HSC phenotype. This demonstrates that FOXOs play an essential role in the response to oxidative stress and thereby mediate quiescence and survival in the haematopoietic system (Tothova *et al.*, 2007). Recently, it has been also been reported that deletion of Foxo1, Foxo3 and Foxo4 has similar effect on neural stem cells. Foxo-deficient mice showed a decline in the neural stem cell pool, due to increased proliferation and loss of self renewal, indicating that FOXOs play a critical role in stem cell homeostasis (Paik *et al.*, 2009). Remarkably, deletion of Foxo3 gave a similar phenotype, suggesting that Foxo1, Foxo4 and Foxo6 cannot compensate for the loss of Foxo3 in this specific stem cell population (Renault *et al.*, 2009).

Post-translational modifications of FOXOs

The transcriptional activity of FOXOs is tightly regulated by multiple post-translational modifications including phosphorylation, acetylation, ubiquitination and methylation (Fig. 5) (Calnan and Brunet, 2008). In the absence of growth factor signalling FOXOs are continuously cycling between nucleus and cytoplasm, however, the rate of import of unphosphorylated FOXO exceeds the export resulting in a predominantly nuclear localisation (Brownawell *et al.*, 2001). Phosphorylation of FOXOs by PKB on three conserved residues inhibits FOXO transcriptional activity by blocking DNA binding and relocalising FOXOs to the nucleus. Phosphorylation induces binding of 14-3-3 proteins to the first two PKB phosphorylation sites thereby blocking DNA binding (Boura *et al.*, 2007; Brunet *et al.*, 1999). In addition, phosphorylation and 14-3-3 binding blocks nuclear import, likely by interfering with the NLS, thereby shifting the localisation of FOXOs from the nucleus to the cytoplasm (Fig. 6) (Obsilova *et al.*, 2005; Brownawell *et al.*, 2001). In contrast to FOXO1, FOXO3 and FOXO4, FOXO6 expression is predominantly nuclear and although two PKB phosphorylation sites are conserved FOXO6 is not regulated by nucleo-cytoplasmic shuttling (Jacobs *et al.*, 2003). However, serum stimulation induced phosphorylation of the first PKB phosphorylation site (Thr 26) and inhibited FOXO6 activity in a reporter assay (van der Heide *et al.*, 2005). This indicates that FOXO6 can be regulated by growth factor-induced phosphorylation, but whether this is mediated by PKB remains unclear.

In the last decade, multiple kinases have been identified that regulate the activity of FOXO in both a positive an a negative manner, including SGK, CDK2, IKK, JNK and MST1 (Calnan and Brunet, 2008) The serum and glucocorticoid-inducible kinase (SGK) is able to phosphorylate FOXOs on the same residues as PKB. Similar to PKB, SGK is activated upon growth factor signalling by PI3K and promotes survival by inhibiting FOXO activity (Brunet *et al.*, 2001). CDK2 phosphorylates FOXO, resulting in cytoplasmic relocalisation and inhibition of transcriptional activity. CDK2 itself is inhibited during DNA damage-induced cell cycle arrest, thereby releasing the inhibition of FOXO1 (Huang *et al.*, 2006). Phosphorylation of FOXO3 by IKK links the NFκB signalling pathway to FOXOs (Hu *et al.*, 2004).

Oxidative stress can result in phosphorylation of FOXO4 by the stress kinase JNK, which can be activated by the small GTP-ase Ral (de Ruiter *et al.*, 2001). This phosphorylation induces nuclear translocation of FOXO4 and increases its activity (de Ruiter *et al.*, 2001; Essers *et al.*, 2004). However this phosphorylation site is not conserved in FOXO1, FOXO3 or FOXO6. In addition, oxidative stress can induce FOXO1 and FOXO3 phosphorylation through MST signalling (Lehtinen *et al.*, 2006). MST1 phosphorylates FOXOs on a

conserved site within the DNA binding domain. Phosphorylation disrupts the interaction with 14-3-3, promoting nuclear translocation and resulting in induction of apoptosis in neurons (Lehtinen *et al.*, 2006).

Phosphorylation of FOXOs in response to growth factors can also induce its poly-ubiquitination and subsequently proteosomal degradation (Matsuzaki *et al.*, 2003). The SKP2 poly-ubiquitination complex has been demonstrated to induce poly-ubiquitination and degradation of FOXO1 in a prostate cancer cell line (Huang *et al.*, 2005). However, it is unclear whether SKP2-mediated FOXO degradation is important for regulation of stability of FOXO1 and FOXO3 in other cell types, since in many reports FOXO expression remains stable after growth factor signalling. In response to oxidative stress FOXO4 can become mono-ubiquitinated resulting in nuclear relocalisation and increasing its transcriptional activity (van der Horst *et al.*, 2006). While the ubiquitin ligase responsible for this effect has not yet been identified, de-ubiquitination is carried out by the de-ubiquitinating enzyme USP7/HAUSP thereby negatively regulating FOXO activity (van der Horst *et al.*, 2006).

A recent paper demonstrates that in response to oxidative stress FOXO1 can be methylated by the methyl transferase PRMT1 (Yamagata *et al.*, 2008). PRMT1 methylated FOXO1 at R248 and R250 which are located within the PKB phosphorylation motif. This methylation blocked PKB-mediated phosphorylation at S253 and increased FOXO1 activity, indicating that arginine methylation can serve as an activating modification by decreasing PKB-

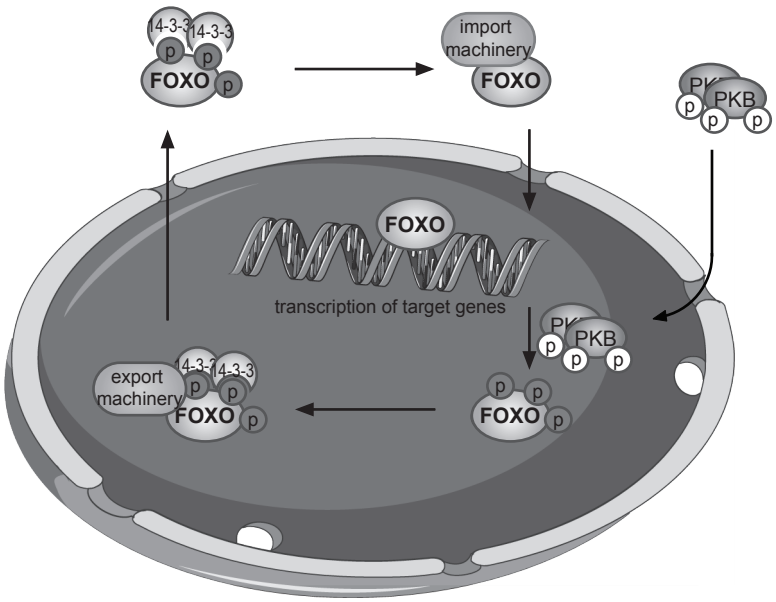


Figure 6. Translocation FOXO transcription factors upon growth factor signalling
Addition of growth/survival signals results in activation of PKB, which then translocates into the nucleus. Phosphorylation of FOXO by PKB results in release from DNA, and binding to 14-3-3 proteins. This complex is then transported out of the nucleus, where it remains inactive in the cytoplasm. Upon removal of growth/survival signals, FOXO is dephosphorylated, 14-3-3 is released, and FOXO is transported back into the nucleus where it is transcriptionally active.

mediated phosphorylation (Yamagata *et al.*, 2008).
Finally, FOXOs are acetylated by the acetyl transferases p300 and the cyclic-AMP responsive element binding (CREB)-binding protein, while the deacetylase SIRT1 can deacetylate FOXOs (Calnan and Brunet, 2008). Oxidative stress increases the interaction of FOXOs with acetyl transferases, which correlates with increased acetylation (Dansen *et al.*, 2009; Motta *et al.*, 2004). Deacetylation of FOXOs by SIRT is evolutionary conserved and the *C elegans* orthologue Sir2 increases lifespan in a *daf-16*-dependent manner, suggesting that DAF-16 is positively regulated by acetylation (Tissenbaum and Guarente, 2001). The effect of acetylation on FOXO activity in mammalian cells is still under debate and conflicting studies have reported both inhibitory and stimulatory effects (Brunet *et al.*, 2004; Dansen *et al.*, 2009; Motta *et al.*, 2004). It has been proposed that acetylation can influence the functional outcome of FOXO activation by stimulating the transcription of only a subset of FOXO targets (Brunet *et al.*, 2004). For example, expression of SIRT1 increases FOXO3-induced cell cycle arrest and resistance to oxidative stress, but inhibits FOXO3-induced cell death (Brunet *et al.*, 2004). However, the molecular mechanism underlying this model is unknown, and since acetylation of histones also affects transcription, complicating interpretation of these data, further research is required to elucidate the role of acetylation on FOXO function.

OUTLINE OF THIS THESIS

The PI3K-PKB-FOXO signalling module plays an important role in regulation of essential biological processes including proliferation, survival, differentiation and metabolism. A disturbed balance in the regulation of this processes might result in the development of disease. The focus of this thesis is the identification and characterisation of novel targets of the PI3K-PKB-FOXO signal transduction pathway that contribute to tumorigenesis. In **chapter 2** we give an overview of the functional consequences of FOXO activation by focussing on transcriptional targets, while **chapter 3** focusses on a detailed description of FOXO binding partners. In **chapter 4** we describe the identification of eIF4B as a PKB substrate, revealing a novel mechanism by which PKB can regulate translation. In response to stimulation with IL-3 or insulin, PKB was found to phosphorylate eIF4B on Ser 422. In addition, Ser 406 was found to be phosphorylated in response to insulin, which was dependent on MEK and mTOR signalling. The phosphorylation of these residues was found to be required for optimal translational activity of eIF4B.
To characterise functional differences between FOXO3 and FOXO4 and to identify novel FOXO targets we performed microarray analyses after activation of FOXO3 or FOXO4. We analysed this dataset by pathway analysis and confirmed JAK2 as a transcriptional target of FOXO activation in **chapter 5**. Furthermore, we performed an additional comparative analysis between the FOXO data set and transcripts regulated by PI3K and PKB activation. The results of this 'pathway analysis' are described in the **appendix** to this chapter. The identification and functional characterisation of Id1 as a novel FOXO3 target is the focus of **chapter 6**. FOXO3-induced downregulation of Id1 resulted in erythroid differentiation of a chronic myeloid leukemia cell line, indicating that inhibition of FOXO3 is critical for maintenance of the leukemic phenotype. Finally, in **chapter 7** we identified glutamine synthetase (GS) as a novel transcriptional target of the PI3K-PKB-FOXO pathway. The upregulation of GS expression and generation of increased levels of glutamine by FOXO3 induces autophagy and results in increased survival after FOXO activation. The regulation of GS by FOXO activation is conserved in *C elegans*, and might provide clues on the link between aging and autophagy. The consequences of these findings are discussed in **chapter 8**. Overall, these studies give more insights in the PI3K-PKB-FOXO network providing novel targets for anti-cancer therapy.

REFERENCES

Ananthanarayanan,B., Fosbrink,M., Rahdar,M., and Zhang,J. (2007). Live-cell molecular analysis of Akt activation reveals roles for activation loop phosphorylation. J. Biol. Chem. *282*, 36634-36641.

Anderson,M.J., Shelton,G.D., Cavenee,W.K., and Arden,K.C. (2001). Embryonic expression of the tumor-associated PAX3-FKHR fusion protein interferes with the developmental functions of Pax3. Proc. Natl. Acad. Sci. U. S. A *98*, 1589-1594.

Anderson,M.J., Viars,C.S., Czekay,S., Cavenee,W.K., and Arden,K.C. (1998). Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. Genomics *47*, 187-199.

Arden,K.C. (2008). FOXO animal models reveal a variety of diverse roles for FOXO transcription factors. Oncogene *27*, 2345-2350.

Barr,F.G. (2001). Gene fusions involving PAX and FOX family members in alveolar rhabdomyosarcoma. Oncogene *20*, 5736-5746.

Borkhardt,A., Repp,R., Haas,O.A., Leis,T., Harbott,J., Kreuder,J., Hammermann,J., Henn,T., and Lampert,F. (1997). Cloning and characterization of AFX, the gene that fuses to MLL in acute leukemias with a t(X;11)(q13;q23). Oncogene *14*, 195-202.

Boura,E., Silhan,J., Herman,P., Vecer,J., Sulc,M., Teisinger,J., Obsilova,V., and Obsil,T. (2007). Both the N-terminal loop and wing W2 of the forkhead domain of transcription factor Foxo4 are important for DNA binding. J. Biol. Chem. *282*, 8265-8275.

Brownawell,A.M., Kops,G.J., Macara,I.G., and Burgering,B.M. (2001). Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. Mol. Cell Biol. *21*, 3534-3546.

Brunet,A., Bonni,A., Zigmond,M.J., Lin,M.Z., Juo,P., Hu,L.S., Anderson,M.J., Arden,K.C., Blenis,J., and Greenberg,M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.

Brunet,A., Park,J., Tran,H., Hu,L.S., Hemmings,B.A., and Greenberg,M.E. (2001). Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). Mol. Cell Biol. *21*, 952-965.

Brunet,A., Sweeney,L.B., Sturgill,J.F., Chua,K.F., Greer,P.L., Lin,Y., Tran,H., Ross,S.E., Mostoslavsky,R., Cohen,H.Y., Hu,L.S., Cheng,H.L., Jedrychowski,M.P., Gygi,S.P., Sinclair,D.A., Alt,F.W., and Greenberg,M.E. (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.

Burgering,B.M. and Coffer,P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature *376*, 599-602.

Calnan,D.R. and Brunet,A. (2008). The FoxO code. Oncogene *27*, 2276-2288.

Castrillon,D.H., Miao,L., Kollipara,R., Horner,J.W., and DePinho,R.A. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science *301*, 215-218.

Dansen,T.B., Smits,L.M., van Triest,M.H., de Keizer,P.L., van,L.D., Koerkamp,M.G., Szypowska,A., Meppelink,A., Brenkman,A.B., Yodoi,J., Holstege,F.C., and Burgering,B.M. (2009). Redox-sensitive cysteines bridge p300/CBP-mediated acetylation and FoxO4 activity. Nat. Chem. Biol. *5*, 664-672.

de Ruiter,N.D., Burgering,B.M., and Bos,J.L. (2001). Regulation of the Forkhead transcription factor AFX by Ral-dependent phosphorylation of threonines 447 and 451. Mol. Cell Biol. *21*, 8225-8235.

Dorman,J.B., Albinder,B., Shroyer,T., and Kenyon,C. (1995). The age-1 and daf-2 genes function in a common pathway to control the lifespan of Caenorhabditis elegans. Genetics *141*, 1399-1406.

Emerson,K.J., Bradshaw,W.E., and Holzapfel,C.M. (2009). Complications of complexity: integrating

environmental, genetic and hormonal control of insect diapause. Trends Genet. *25*, 217-225.

Engelman,J.A. (2009). Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat. Rev. Cancer *9*, 550-562.

Essers,M.A., Weijzen,S., de Vries-Smits,A.M., Saarloos,I., de Ruiter,N.D., Bos,J.L., and Burgering,B. M. (2004). FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J. *23*, 4802-4812.

Flachsbart,F., Caliebe,A., Kleindorp,R., Blanche,H., von Eller-Eberstein,H., Nikolaus,S., Schreiber,S., and Nebel,A. (2009). Association of FOXO3A variation with human longevity confirmed in German centenarians. Proc. Natl. Acad. Sci. U. S. A *106*, 2700-2705.

Furuyama,T., Nakazawa,T., Nakano,I., and Mori,N. (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem. J. *349*, 629-634.

Galili,N., Davis,R.J., Fredericks,W.J., Mukhopadhyay,S., Rauscher,F.J., III, Emanuel,B.S., Rovera,G., and Barr,F.G. (1993). Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. Nat. Genet. *5*, 230-235.

Giannakou,M.E., Goss,M., Junger,M.A., Hafen,E., Leevers,S.J., and Partridge,L. (2004). Long-lived Drosophila with overexpressed dFOXO in adult fat body. Science *305*, 361.

Giannakou,M.E. and Partridge,L. (2007). Role of insulin-like signalling in Drosophila lifespan. Trends Biochem. Sci. *32*, 180-188.

Greer,E.L. and Brunet,A. (2005). FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene *24*, 7410-7425.

Hillion,J., Le,C.M., Jonveaux,P., Berger,R., and Bernard,O.A. (1997). AF6q21, a novel partner of the MLL gene in t(6;11)(q21;q23), defines a forkhead transcriptional factor subfamily. Blood *90*, 3714-3719.

Hosaka,T., Biggs,W.H., III, Tieu,D., Boyer,A.D., Varki,N.M., Cavenee,W.K., and Arden,K.C. (2004). Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc. Natl. Acad. Sci. U. S. A *101*, 2975-2980.

Hu,M.C., Lee,D.F., Xia,W., Golfman,L.S., Ou-Yang,F., Yang,J.Y., Zou,Y., Bao,S., Hanada,N., Saso,H., Kobayashi,R., and Hung,M.C. (2004). IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell *117*, 225-237.

Huang,H., Regan,K.M., Lou,Z., Chen,J., and Tindall,D.J. (2006). CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. Science *314*, 294-297.

Huang,H., Regan,K.M., Wang,F., Wang,D., Smith,D.I., van Deursen,J.M., and Tindall,D.J. (2005). Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. Proc. Natl. Acad. Sci. U. S. A *102*, 1649-1654.

Hwangbo,D.S., Gershman,B., Tu,M.P., Palmer,M., and Tatar,M. (2004). Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature *429*, 562-566.

Jacinto,E., Facchinetti,V., Liu,D., Soto,N., Wei,S., Jung,S.Y., Huang,Q., Qin,J., and Su,B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell *127*, 125-137.

Jacobs,F.M., van der Heide,L.P., Wijchers,P.J., Burbach,J.P., Hoekman,M.F., and Smidt,M.P. (2003). FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. J. Biol. Chem. *278*, 35959-35967.

Kenyon,C., Chang,J., Gensch,E., Rudner,A., and Tabtiang,R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461-464.

Kimura,K.D., Tissenbaum,H.A., Liu,Y., and Ruvkun,G. (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science *277*, 942-946.

Kops,G.J., Medema,R.H., Glassford,J., Essers,M.A., Dijkers,P.F., Coffer,P.J., Lam,E.W., and Burgering,B.M. (2002). Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol. Cell Biol. *22*, 2025-2036.

Lehtinen,M.K., Yuan,Z., Boag,P.R., Yang,Y., Villen,J., Becker,E.B., DiBacco,S., de,l., I, Gygi,S., Blackwell,T.K., and Bonni,A. (2006). A conserved MST-FOXO signaling pathway mediates oxidative-stress responses and extends life span. Cell *125*, 987-1001.

Li,J., Yen,C., Liaw,D., Podsypanina,K., Bose,S., Wang,S.I., Puc,J., Miliaresis,C., Rodgers,L., McCombie,R., Bigner,S.H., Giovanella,B.C., Ittmann,M., Tycko,B., Hibshoosh,H., Wigler,M.H., and Parsons,R. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science *275*, 1943-1947.

Lin,K., Dorman,J.B., Rodan,A., and Kenyon,C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science *278*, 1319-1322.

Lin,L., Hron,J.D., and Peng,S.L. (2004). Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. Immunity. *21*, 203-213.

Maehama,T. and Dixon,J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. *273*, 13375-13378.

Manning,B.D. and Cantley,L.C. (2007). AKT/PKB signaling: navigating downstream. Cell *129*, 1261-1274.

Matsuzaki,H., Daitoku,H., Hatta,M., Tanaka,K., and Fukamizu,A. (2003). Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. Proc. Natl. Acad. Sci. U. S. A *100*, 11285-11290.

Motta,M.C., Divecha,N., Lemieux,M., Kamel,C., Chen,D., Gu,W., Bultsma,Y., McBurney,M., and Guarente,L. (2004). Mammalian SIRT1 represses forkhead transcription factors. Cell *116*, 551-563.

Mukhopadhyay,A., Oh,S.W., and Tissenbaum,H.A. (2006). Worming pathways to and from DAF-16/FOXO. Exp. Gerontol. *41*, 928-934.

Obsil,T. and Obsilova,V. (2008). Structure/function relationships underlying regulation of FOXO transcription factors. Oncogene *27*, 2263-2275.

Obsilova,V., Vecer,J., Herman,P., Pabianova,A., Sulc,M., Teisinger,J., Boura,E., and Obsil,T. (2005). 14-3-3 Protein interacts with nuclear localization sequence of forkhead transcription factor FoxO4. Biochemistry *44*, 11608-11617.

Ogg,S., Paradis,S., Gottlieb,S., Patterson,G.I., Lee,L., Tissenbaum,H.A., and Ruvkun,G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature *389*, 994-999.

Paik,J.H., Ding,Z., Narurkar,R., Ramkissoon,S., Muller,F., Kamoun,W.S., Chae,S.S., Zheng,H., Ying,H., Mahoney,J., Hiller,D., Jiang,S., Protopopov,A., Wong,W.H., Chin,L., Ligon,K.L., and DePinho,R.A. (2009). FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis. Cell Stem Cell *5*, 540-553.

Paik,J.H., Kollipara,R., Chu,G., Ji,H., Xiao,Y., Ding,Z., Miao,L., Tothova,Z., Horner,J.W., Carrasco,D. R., Jiang,S., Gilliland,D.G., Chin,L., Wong,W.H., Castrillon,D.H., and DePinho,R.A. (2007). FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell *128*, 309-323.

Paradis,S., Ailion,M., Toker,A., Thomas,J.H., and Ruvkun,G. (1999). A PDK1 homolog is necessary and sufficient to transduce AGE-1 PI3 kinase signals that regulate diapause in Caenorhabditis elegans. Genes Dev. *13*, 1438-1452.

Paradis,S. and Ruvkun,G. (1998). Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes Dev. *12*, 2488-2498.

Parry,P., Wei,Y., and Evans,G. (1994). Cloning and characterization of the t(X;11) breakpoint from a leukemic cell line identify a new member of the forkhead gene family. Genes Chromosomes. Cancer *11*, 79-84.

Renault,V.M., Rafalski,V.A., Morgan,A.A., Salih,D.A., Brett,J.O., Webb,A.E., Villeda,S.A., Thekkat,P. U., Guillerey,C., Denko,N.C., Palmer,T.D., Butte,A.J., and Brunet,A. (2009). FoxO3 regulates neural stem cell homeostasis. Cell Stem Cell *5*, 527-539.

Sarbassov,D.D., Guertin,D.A., Ali,S.M., and Sabatini,D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098-1101.

Sim,C. and Denlinger,D.L. (2008). Insulin signaling and FOXO regulate the overwintering diapause of the mosquito Culex pipiens. Proc. Natl. Acad. Sci. U. S. A *105*, 6777-6781.

Steck,P.A., Pershouse,M.A., Jasser,S.A., Yung,W.K., Lin,H., Ligon,A.H., Langford,L.A., Baumgard,M. L., Hattier,T., Davis,T., Frye,C., Hu,R., Swedlund,B., Teng,D.H., and Tavtigian,S.V. (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat. Genet. *15*, 356-362.

Stephens,L., Anderson,K., Stokoe,D., Erdjument-Bromage,H., Painter,G.F., Holmes,A.B., Gaffney,P. R., Reese,C.B., McCormick,F., Tempst,P., Coadwell,J., and Hawkins,P.T. (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science *279*, 710-714.

Tissenbaum,H.A. and Guarente,L. (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature *410*, 227-230.

Tothova,Z., Kollipara,R., Huntly,B.J., Lee,B.H., Castrillon,D.H., Cullen,D.E., McDowell,E.P., Lazo-Kallanian,S., Williams,I.R., Sears,C., Armstrong,S.A., Passegue,E., DePinho,R.A., and Gilliland,D. G. (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell *128*, 325-339.

van der Heide,L.P., Jacobs,F.M., Burbach,J.P., Hoekman,M.F., and Smidt,M.P. (2005). FoxO6 transcriptional activity is regulated by Thr26 and Ser184, independent of nucleo-cytoplasmic shuttling. Biochem. J. *391*, 623-629.

van der Horst,A., de Vries-Smits,A.M., Brenkman,A.B., van Triest,M.H., van den,B.N., Colland,F., Maurice,M.M., and Burgering,B.M. (2006). FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. Nat. Cell Biol. *8*, 1064-1073.

Weigel,D., Jurgens,G., Kuttner,F., Seifert,E., and Jackle,H. (1989). The homeotic gene fork head encodes a nuclear protein and is expressed in the terminal regions of the Drosophila embryo. Cell *57*, 645-658.

Willcox,B.J., Donlon,T.A., He,Q., Chen,R., Grove,J.S., Yano,K., Masaki,K.H., Willcox,D.C., Rodriguez,B., and Curb,J.D. (2008). FOXO3A genotype is strongly associated with human longevity. Proc. Natl. Acad. Sci. U. S. A *105*, 13987-13992.

Yamagata,K., Daitoku,H., Takahashi,Y., Namiki,K., Hisatake,K., Kako,K., Mukai,H., Kasuya,Y., and Fukamizu,A. (2008). Arginine methylation of FOXO transcription factors inhibits their phosphorylation by Akt. Mol. Cell *32*, 221-231.

TWO

CHAPTER 2

The extending network of FOXO transcriptional target genes

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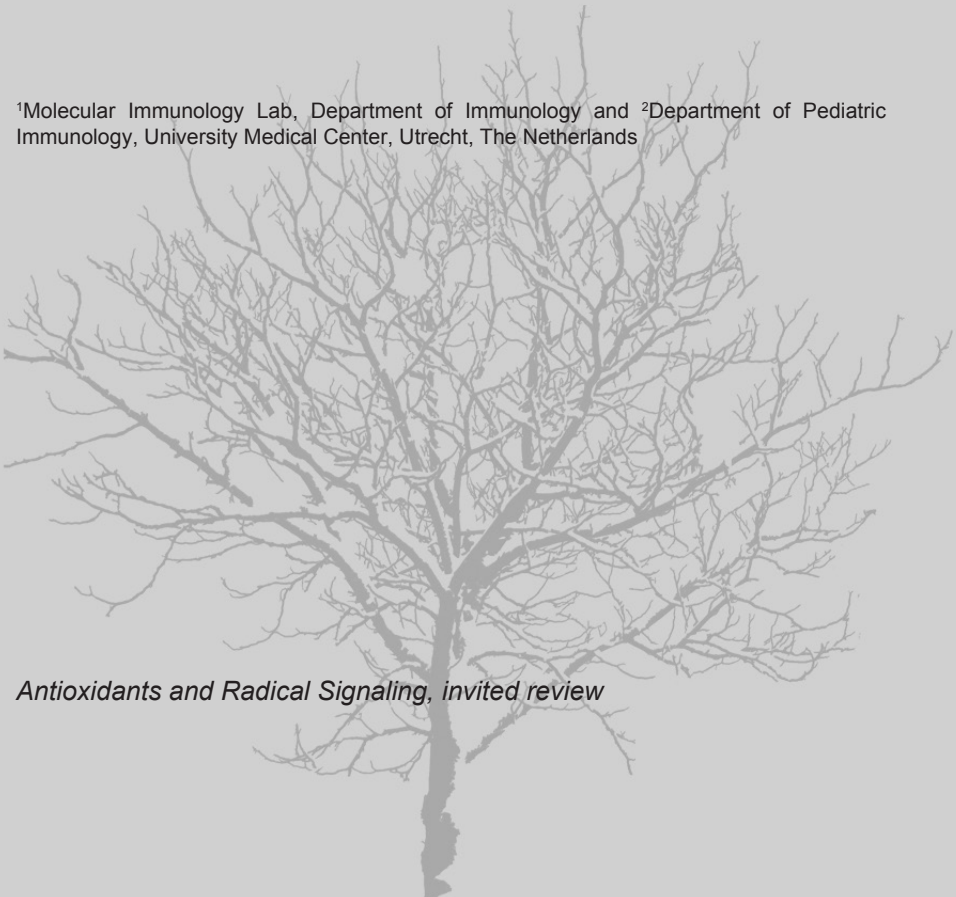

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INTRODUCTION

The forkhead transcription factor family is characterised by a winged-helix DNA binding motif, the forkhead domain (reviewed in Obsil and Obsilova, 2008) and the forkhead box O (FOXO) subfamily is based on sequence homology within this domain. A single FOXO transcription factor has been identified in both the nematode worm *C. elegans*, termed DAF-16, and in *D. melanogaster;* termed dFOXO, while four FOXO orthologues have been identified in mammalian cells. FOXO1, FOXO3, FOXO4 and FOXO6. FOXO1, FOXO3 and FOXO4 are ubiquitously expressed but with varying expression levels, FOXO1 expression being the highest in adipose tissue, FOXO3 is predominantly expressed in heart, brain, kidneys and ovaries and FOXO4 shows the highest expression in muscle cells and heart tissue (Anderson *et al.*, 1998; Biggs *et al.*, 2001; Furuyama *et al.*, 2003). FOXO6 is expressed in brain, but whether it is also expressed in other tissues remains unclear (Jacobs *et al.*, 2003).

Alignment of amino acid sequence of FOXO transcription factors with other forkhead transcription factors reveals that the DNA binding domain (DBD) is highly conserved (Fig. 1). Due to this FOXOs share a similar DNA binding specificity, with the core binding motif being defined as TTGTTTAC (Furuyama *et al.*, 2000). The DNA binding domain of FOXOs consists of three α-helices (H1, H2 and H3), three β-strands (S1, S2 and S3) and two wing-like loops (W1 and W2). Crystallisation of the FOXO4 DBD bound to DNA has shown that FOXOs bind DNA through multiple interactions within the N-terminal region, the second wing and the third helix (Boura *et al.*, 2007). FOXOs can function both as transcriptional activators and repressors, probably depending on the range of associated co-factors that they recruit upon DNA binding. Growth factors, cytokines and hormones negatively regulate FOXOs through inhibitory phosphorylation by protein kinase B (PKB/c-Akt) (Brunet *et al.*, 1999; Kops *et al.*, 2002b). This phosphorylation inactivates FOXOs by recruiting 14-3-3 proteins preventing DNA-binding and inhibiting nuclear import (reviewed in Obsil and Obsilova, 2008). In addition to PKB, several other protein kinases have been identified that upon phosphorylation induce the cytoplasmic relocalisation of FOXOs,

Figure 1. The DNA binding domain is highly conserved within the FOXO transcription factor family

Alignment of the amino acid sequence of the DNA binding domains of FOXO1, FOXO3, FOXO4, FOXO6 and DAF-16. The localisation of the three α-helices (H1, H2 and H3), three β-strands (S1, S2 and S3) and two wing-like loops (W1 and W2) are indicated at the top.

including serum- and glucocorticoid-inducible kinase (SGK), cyclin-dependent kinase-2 (CDK2), and IκB kinase (IKK) (Brunet *et al.*, 2001; Hu *et al.*, 2004; Huang *et al.*, 2006) . Deletion of the *Foxo* alleles in mice has revealed both redundant as well as isoform specific functions of FOXO1, FOXO3 and FOXO4. Deletion of *Foxo1* is lethal due to incomplete vascular development (Hosaka *et al.*, 2004). However, *Foxo3*$^{-/-}$ mice were found to be viable, but showed lymphoproliferation and widespread organ inflammation due to hyperactivated helper T cells (Lin *et al.*, 2004). Further examination in female mice revealed an age-dependent infertility due to global ovarian follicle activation resulting in early oocyte depletion (Castrillon *et al.*, 2003; Hosaka *et al.*, 2004). In contrast, *Foxo4*$^{-/-}$ mice no phenotype has been detected yet (Hosaka *et al.*, 2004). The development of a inducible *Foxo1*$^{-/-}$, *Foxo3*$^{-/-}$ and *Foxo4*$^{-/-}$ mouse model has revealed a redundant role for FOXOs in oncogenesis and stem cell homeostasis. Conditional deletion of *Foxo1*, *Foxo3* and *Foxo4* results in the development of lymphoblastic thymic lymphomas and hemangiomas, demonstrating that FOXOs act as functional tumour suppressors (Paik *et al.*, 2007). Analysis of the haematopoietic system after loss of *Foxo1*, *Foxo3* and *Foxo4* demonstrated increased numbers of myeloid progenitors in peripheral blood, while in the bone marrow the number of haematopoietic stem cells (HSCs) was reduced. Further analysis revealed that FOXOs are required for haematopoietic stem cell renewal by decreasing levels of reactive oxygen species (Tothova *et al.*, 2007). Recently, it has been demonstrated that deletion of *Foxo1*, *Foxo3* and *Foxo4* has a similar effect on neural stem cells (NSCs) (Paik *et al.*, 2009). *Foxo*-deficient mice showed a decline in the NSC pool, due to increased proliferation and los of self renewal, indicating that FOXOs may play a critical general role in stem cell homeostasis.

Over the last decade a plethora of studies have demonstrated that FOXOs play critical roles in a wide variety of cellular processes, including proliferation, apoptosis, autophagy, metabolism, inflammation, differentiation and stress resistance (Table 1). This review will focus on the functional consequences of FOXO activation based on our current knowledge of regulation of transcriptional targets.

Induction of cell cycle arrest

One of the functions initially attributed to FOXO activation was regulation of cell cycle progression. FOXOs have been shown to modulate both the G1-S transition and the G2-M phase by coordinating the expression of multiple important cell cycle regulators (Fig. 2) (reviewed in Ho *et al.*, 2008). Ectopic expression of constitutively active FOXO4, in which the inhibitory phosphorylation sites are mutated, induces a G1 cell cycle arrest in A14, U2OS and Jurkat cells, which is dependent on expression of the cell cycle inhibitor p27 (Medema *et al.*, 2000). The cell cycle is regulated by the coordinated activation of multiple cyclin/cyclin dependent kinases, which phosphorylate and regulate multiple substrates that are essential for cell cycle progression. p27 is a member of the Cip/Kip family of CDK inhibitors together with p21 and p57 and binds to both cyclin and CDK subunits inhibiting the activities of cyclin D-, E- and A-CDK complexes (reviewed in Besson *et al.*, 2008). Use of promoter luciferase reporter assays demonstrated that p27 expression was regulated through direct FOXO4-mediated transcription (Medema *et al.*, 2000). Cytokines are regulators of proliferation and survival of haematopoietic cells and cytokine deprivation will often result in arrest in the G1 phase of the cell cycle. Several reports have shown that cytokine deprivation induces the activation of FOXO3 resulting in an subsequent increase in p27 expression and a cell cycle arrest (Hideshima *et al.*, 2001; Stahl *et al.*, 2002; Dijkers *et al.*, 2000b). Activation of an inducible active FOXO3 mutant in bone marrow-derived Ba/F3 cells was found to result in increased transcription of the gene encoding for p27, which

Table 1. FOXO transcriptional target genes
Listed are transcriptional targets, which are directly regulated by FOXO1, FOXO3 or FOXO4. Listed are the FOXO targets grouped by cellular function. The effect of FOXO activation on the expression level; up-regulation or down-regulation, is indicated by - and +

Target	Up- or down-regulation	FOXO	Pathway	References
Cyclin D	-	FOXO3, FOXO4	Cell cycle	Schmidt *et al.*, 2002
Cyclin G2	+	FOXO1, FOXO3, FOXO4	Cell cycle	Chen *et al.*, 2006; Martinez-Gac *et al.*, 2004
P130	+	FOXO1, FOXO3, FOXO4	Cell cycle	Chen *et al.*, 2006; Kops *et al.*, 2002b
P15	+	FOXO1, FOXO3	Cell cycle	Katayama *et al.*, 2008
P19	+	FOXO1, FOXO3	Cell cycle	Katayama *et al.*, 2008
P21	+	FOXO1, FOXO3, FOXO4	Cell cycle	Nakae *et al.*, 2003; Seoane *et al.*, 2004
P27	+	FOXO1, FOXO3, FOXO4	Cell cycle	Dijkers *et al.*, 2000; Medema *et al.*, 2000; Stahl *et al.*, 2002
Plk	+	FOXO1	Cell cycle	Yuan *et al.* 2008
MnSOD	+	FOXO3	Stress resistance	Kops *et al.*, 2002a
catalase	+	FOXO3	Stress resistance	Sandri *et al.* 2004
Peroxiredoxin III	+	FOXO3	Stress resistance	Chiribau *et al.*, 2008
Sterol carrier protein	+	FOXO3	Stress resistance	Dansen *et al.*, 2004
Gadd45	+	FOXO3, FOXO4	DNA repair	Furukawa-Hibi *et al.*, 2002; Tran *et al.*, 2002
Fasl	+	FOXO1, FOXO3	Apoptosis	Brunet *et al.*, 1999; Ciechomska *et al.*, 2003
TRADD	+	FOXO1	Apoptosis	Rokudai *et al.*, 2002
TRAIL	+	FOXO1, FOXO3	Apoptosis	Modur *et al.*, 2002
Puma	+	FOXO3	Apoptosis	You *et al.*, 2006
Bcl 6	+	FOXO3, FOXO4	Apoptosis	Fernandez de *et al.*, 2004; Tang *et al.*, 2002
Pink1	+	FOXO3	Apoptosis	Mei *et al.*, 2009
G6Pase	+	FOXO1, FOXO3	Metabolism	Puigserver *et al.*, 2003; Onuma *et al.*, 2006
PEPCK	+	FOXO1	Metabolism	Sekine *et al.*, 2007
PGC1	+	FOXO1	Metabolism	Daitoku *et al.*, 2003
adiponectin	+	FOXO1	Metabolism	Qiao and Shao, 2006

Target	Up- or down-regulation	FOXO	Pathway	References
proopiomelano-cortin	-	FOXO1	Metabolism	Kim *et al.*, 2006; Kitamura *et al.*, 2006
neuropeptide Y	+	FOXO1	Metabolism	Kim *et al.*, 2006
apoC-III	+	FOXO1	Metabolism	Altomonte *et al.*, 2004
Pdx1	-	FOXO1	Metabolism	Kitamura *et al.*, 2002
BTG1	+	FOXO3	Differentiation	Bakker *et al.*, 2004
Id1	-	FOXO3	Differentiation	Birkenkamp *et al.*, 2007
Atrogin-1	+	FOXO3	Muscle atrophy	Sandri *et al.*, 2004
Bnip3	+	FOXO3	Muscle atrophy	Mammucari *et al.*, 2007; Zhao *et al.*, 2007
LC3	+	FOXO3	Muscle atrophy	Mammucari *et al.*, 2007; Zhao *et al.*, 2007
Garabl12	+	FOXO3	Muscle atrophy	Zhao *et al.*, 2007
IL7R	+	FOXO1	Inflammation	Ouyang *et al.*, 2009
C/EBPβ	+	FOXO1	Inflammation	Ito *et al.*, 2009
IL-1β	+	FOXO1	Inflammation	Su *et al.*, 2009
4EBP1	+	FOXO1, FOXO3	Insulin signalling	Puig *et al.*, 2003
InsR	+	FOXO1	Insulin signalling	Puig and Tjian, 2005
trible 3	-	FOXO1	Signalling	Matsumoto *et al.*, 2006
Caveolin-1	+	FOXO1, FOXO3, FOXO4	Signalling	Roy *et al.*, 2008; van den Heuvel *et al.*, 2005
PP2A	-	FOXO1	signalling	Ni *et al.*, 2007
FOXO1	+	FOXO1, FOXO3	Signalling	Essaghir *et al.*, 2009
FOXO3	+	FOXO1, FOXO3	Signalling	Essaghir *et al.*, 2009
P110α	+	FOXO3	Signalling	Hui *et al.*, 2008
collagenase	+	FOXO3	Extracellular matrix degradation	Mawal-Dewan *et al.*, 2002
MMP9	+	FOXO4	Extracellular matrix degradation	Li *et al.*, 2007
Mxi1	+	FOXO3	Tumour suppression	Delpuech *et al.*, 2007
Estrogen receptorα	+	FOXO3	Tumour suppression	Guo and Sonenshein, 2004
Myostatin	+	FOXO1	Differentiation	Allen and Unterman, 2007
eNOS	-	FOXO1, FOXO3	Vessel formation	Potente *et al.*, 2005
MDR1	+	FOXO1	Drug resistance	Han *et al.*, 2008
Cited2	+	FOXO3	Angiogenesis	Hui *et al.*, 2008

was associated with a cell cycle arrest, indicating that FOXO3 activation is sufficient for p27 upregulation and inhibition of proliferation (Dijkers *et al.*, 2000b). In addition to regulation of p27 expression levels, FOXOs have been shown to regulate the transcription of another Cip/Kip family member, namely p21. TGFβ can block proliferation of epithelial, neuronal and immune cells by activating Smad transcription factors that regulate expression of multiple cell cycle regulators including p21 (Seoane *et al.*, 2004). Immunoprecipitation experiments have demonstrated that Smad3 and Smad4 can bind to the DNA binding domain of FOXO1, FOXO3 and FOXO4 (Seoane *et al.*, 2004). Furthermore, in epithelial cells it has been demonstrated that Smads increase p21 expression by forming a complex with FOXOs (Seoane *et al.*, 2004). The p21 promoter contains both FOXO and Smad enhancer elements, which are both required for the induction of p21 expression by TGFβ. In addition, it has been shown that activation of the PI3K pathway and consequently inactivation of FOXOs blocks p21 transcription. (Seoane *et al.*, 2004). Nakae *et al.* have shown that the regulation of p21 expression by FOXO1 also plays an important role in the proliferation of adipocytes. In these cells insulin signalling repressed the upregulation of p21 expression resulting in increased proliferation while in the absence of insulin, FOXO1 activation results in increased p21 expression and a cell cycle arrest (Nakae *et al.*, 2003).

Besides regulation of p21 and p27, FOXOs have been described to regulate the expression of p15 and p19, CDK inhibitors of the INK4 family. These cell cycle inhibitors inhibit the cyclin D/CDK complex by binding to CDK4 and CDK6, thereby blocking the binding of

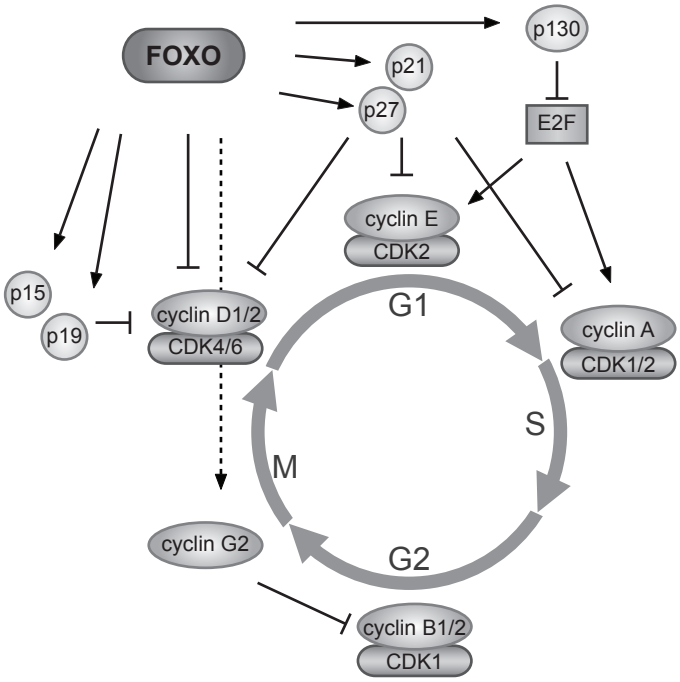

Figure 2 . Regulation of cell cycle progression by FOXOs
FOXOs can inhibit proliferation during distinct phases of the cell cycle. FOXOs block S phase entry and cause a G1 cell cycle arrest by upregulation of the cell cycle inhibitors p15, p19, p21 and p27, downregulation of cyclin D and upregulation of p130.

cyclin D (Besson *et al.*, 2008). FOXO1 and FOXO3 have been found to upregulate the expression of p15 and p19 by directly binding to FOXO enhancer elements present in their promoter (Katayama *et al.*, 2008). Moreover mouse embryonic fibroblasts from p15 or p19-null mouse failed to arrest in G1 after incubation with the PI3K inhibitor LY294002, indicating that the expression of both p15 and p19 is required for cell cycle arrest (Katayama *et al.*, 2008). These results are perhaps surprising since previous reports have shown that the increased p27 expression by FOXOs is also itself sufficient for cell cycle arrest (Dijkers *et al.*, 2000a). A possible explanation is that p27 requires a low level of p15 or p19 expression to block the cyclin-D/CDK complex.
Besides regulation of CDK inhibitors FOXOs have been described to block cell cycle progression by directly regulating the expression of cyclin D, cyclin G and the Rb family member p130. Overexpression of cyclin D1 partially rescues FOXO4-induced cell cycle arrest, suggesting that the effect of FOXO4 on proliferation depends on the repression of cyclin D expression and a decrease in cyclin D/CDK activity (Schmidt *et al.*, 2002). However the overexpression of cyclin D1 may also act to titrate away CDK inhibitors such as p21 and p27 thereby affecting cylin-CDK activity indirectly. p130 is a member of the Rb protein family, which repress the activity of E2F transcription factors and thereby regulates the expression of genes required for S phase entry, such as cyclin A and cyclin E (Sun *et al.*, 2007). It has been shown that FOXO4 can upregulate p130, however the functional consequences of this regulation remain unclear (Kops *et al.*, 2002b). Cyclin G2 and p130 levels are high in resting B cells, while mitogen stimulation induces a rapid decrease in their expression (Chen *et al.*, 2006). Activation of FOXO3 has been reported to induce cell cycle arrest in murine B cells and increase the expression of cyclin G2. In addition, overexpression of cyclin G2 results in a block in cell cycle progression, showing that FOXOs regulate lymphocyte quiescence through regulation of multiple cell cycle regulators (Chen *et al.*, 2006).

Regulation of genes involved in stress resistance
In the nematode worm *C. elegans* inactivation of the insulin pathway by a lack of nutrients induces dauer formation, a stress-resistant-state in which the worms lowers it metabolism and has an increased lifespan (Kimura *et al.*, 1997). Genetic analysis has revealed that the *C elegans* forkhead transcription factor DAF-16 is inhibited by insulin signalling and that its activity is required for lifespan extension in insulin receptor mutants (Kenyon *et al.*, 1993). DAF-16 exerts its effect on lifespan through regulation of genes involved in microbial defense, cellular stress response and metabolism (reviewed in Partridge and Bruning, 2008).
In humans accumulation of cellular damage by oxidative stress has been implicated in oncogenesis and aging (Finkel *et al.*, 2007). FOXOs have been described to protect cells from oxidative damage by increasing transcription of multiple genes involved in scavenging reactive oxygen species. Activation of FOXO3 either by treatment with the PI3K inhibitor LY294002, or by activating an inducible active FOXO3 mutant, increases expression of manganese superoxide dismutase (MnSOD) in a colon carcinoma cell line (Kops *et al.*, 2002a). MnSOD protects against oxidative damage through conversion of superoxide, which is formed as a by-product during generation of ATP in mithochondria into hydrogen peroxide. FOXO3 was found to upregulate MnSOD transcription through direct binding to its promoter (Kops *et al.*, 2002a). Activation of FOXO3 was subsequently found to rescue glucose-deprived cells from mitochondrial damage in wild-type mouse embryonic fibroblasts (MEFs) but not in MnSOD deficient MEFs, demonstrating that the FOXO3-induced MnSOD expression is required for survival after nutrient deprivation (Kops *et al.*, 2002a). In addition to regulation of MnSOD, FOXO3 also regulates catalase expression,

another antioxidant enzyme catalysing the conversion of hydrogen peroxide to water and oxygen. In the neuronal cell line PC12, increased catalase expression by FOXO3 was found to decrease oxidative stress resulting in increased survival (Nemoto and Finkel, 2002). Through coordinated regulation of MnSOD and catalase expression FOXOs are able to decrease oxidative damage and thereby increase cellular survival.
Experiments in human cardiac fibroblasts have revealed that FOXOs can also modulate the cellular response to hydrogen peroxide by expression of peroxiredoxin III, an antioxidant enzyme which can inactivate hydrogen peroxide (Chiribau *et al.*, 2008). Knockdown of FOXO3 in these cells revealed that expression of peroxiredoxin III was dependent on FOXO3. In addition, FOXO3 was shown to bind to the peroxiredoxin III promoter, demonstrating that peroxiredoxin III is a direct transcriptional target. While the accumulation of hydrogen peroxide and the percentage of apoptotic cells in response to serum starvation was increased after peroxiredoxin III knockdown, concomitant FOXO3 knockdown resulted in even higher levels of hydrogen peroxide suggesting that multiple FOXO targets are important for resistance to oxidative stress (Chiribau *et al.*, 2008).
Conditional deletion of *Foxo1*, *Foxo3* and *Foxo4* in the haematopoietic system of mice has further highlighted the physiological importance of regulation of ROS *in vivo* (Tothova *et al.*, 2007). Analysis of haematopoietic cells in the bone marrow showed that *Foxo* deficient mice had reduced numbers of haematopoietic stem cells (HSCs), while the number of myeloid progenitors in peripheral blood was increased, suggesting that FOXOs are important for maintaining HSCs in a quiesencent state. Repopulation experiments showed a decreased repopulating ability of bone marrow cells from *Foxo* deficient mice, indicating that FOXOs are required for stem cell self-renewal. In HSCs from triple $Foxo^{-/-}$ mice levels of reactive oxygen species and apoptosis were increased. Surprisingly, treatment of mice with the antioxidant N-acetyl-cysteine was sufficient to rescue the $Foxo^{-/-}$ HSC phenotype, indicating that FOXO-mediated resistance to oxidative stress is critical for homeostasis of the HSC compartment *in vivo* (Tothova *et al.*, 2007).

In addition to regulating oxidative damage by decreasing the availability of ROS, FOXOs also protect cells from DNA damage by increasing DNA repair. Rat1 fibroblasts showed a G_2-M delay after release from a chemically induced S-phase block when a constitutively active FOXO3 mutant was ectopically expressed (Tran *et al.*, 2002). The G_2-M checkpoint is activated after DNA damage, which pauses the cell cycle and gives the cell time to repair the damage before continuing to divide. The FOXO3-induced G_2-M arrest suggested a role for FOXO in DNA damage repair. It was shown that ectopic expression of a constitutive active FOXO3 mutant increased expression of an UV-damaged luciferase construct, suggesting that FOXO3 can indeed modulate DNA damage repair mechanisms (Tran *et al.*, 2002). Microarray analysis subsequently identified Growth arrest and DNA damage response gene GADDD45 as a novel FOXO transcriptional target. Gadd45 has been shown to participate in cell cycle arrest, DNA repair and survival in response to stress. Activation of FOXO3 increased the expression of Gadd45 on both the mRNA and protein level. In addition, FOXO3 activation restored expression of an UV-damaged luciferase construct, indicating that FOXO3 upregulates DNA damage repair mechanisms. The FOXO3-induced DNA damage repair was compromised in *Gadd45*-/- cells, suggesting that Gadd45 expression is required for FOXO3-mediated DNA repair (Tran *et al.*, 2002).
While high levels of ROS are detrimental to cellular survival, low levels of ROS are often required for intracellular signalling by acting as secondary messengers (reviewed in Stone and Yang, 2006). Stimulation of cultured neonatal rat cardiomyocytes with insulin increases the intracellular concentration of ROS and results in an increase in cell size (Tan *et al.*, 2008). This insulin-induced hypertrophy can be inhibited by the antioxidant

N-acetyl-cysteine, suggesting that insulin can regulate cell size by increasing ROS levels. The increase of ROS levels observed after insulin stimulation correlate with a decrease in phosphorylated FOXO3 and a decreased expression of the antioxidant enzyme catalase. Furthermore, knockdown of FOXO3 is sufficient to induce hypertrophy and can be abrogated by ectopic expression of catalase, suggesting that insulin signalling induces ROS-mediated hypertrophy by inhibiting FOXO3 function (Tan *et al.*, 2008). In patients with heart failure, high insulin levels in plasma are associated with cardiac hypertrophy (Paolisso *et al.*, 1995). The repression of cell size in cardiomyocytes by FOXO3 suggests that insulin-mediated inhibition of FOXO3 might thus play a role in heart failure *in vivo*.

Life and death decisions

Programmed cell death, also known as apoptosis, can be induced by activation of either intrinsic or extrinsic pathways. In the extrinsic pathway binding of death receptor ligands to their receptors triggers the formation of a death inducing signalling complex and consequently activation of caspases (reviewed in Guicciardi and Gores, 2009). In contrast, intracellular stress can induce apoptosis by activating pro-apoptotic Bcl-2 proteins, which modulate release of cytochrome c from mitochondria resulting in caspase-9 activation and subsequently activation of downstream effector caspases, which execute the apoptotic program (reviewed in Brunelle and Letai, 2009).

FOXOs have been reported to be required for the induction of apoptosis after growth factor removal in haematopoietic and neuronal cells. FOXOs can activate the intrinsic apoptotic pathway through upregulation of multiple Bcl-2 family members, while upregulation of

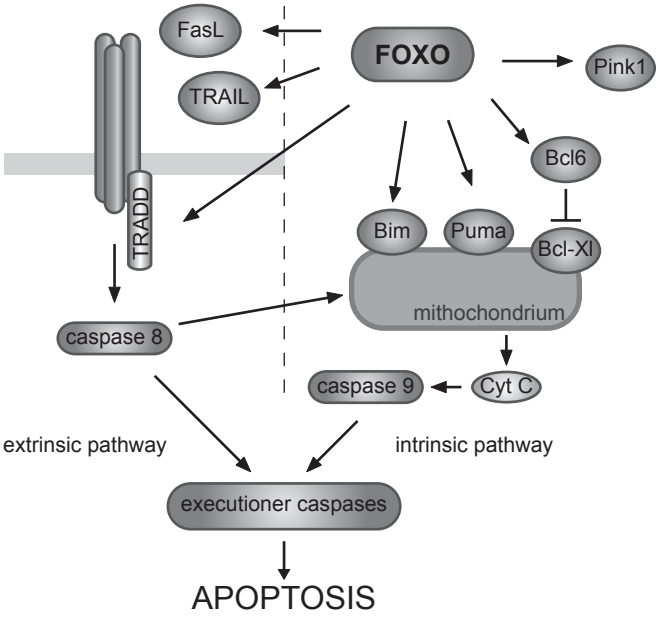

Figure 3. Regulation of apoptosis by FOXOs

FOXOs can induce apotosis by regulating the expression of multiple proteins involved in the apoptotic pathway. Upregulation of the death receptor ligands FasL and TRAIL will induce apoptosis through activation of the extrinsic pathway. While regulation of the expression of Bim, Puma, BclXl and Pink1 by FOXOs can induce apoptosis via the intrinsic pathway.

death receptor ligands can activate the extrinsic pathway (Fig. 3). Cytokine deprivation of bone marrow-derived Ba/F3 cells results in activation of FOXO3 and cytochrome c release, DNA laddering and subsequently induction of apoptosis (Dijkers *et al.*, 2000a). Similar effects on apoptosis were observed after specific activation of FOXO3, demonstrating that FOXO3 activation is itself sufficient to induce apoptosis. FOXO3 was found to directly upregulate the expression of the pro-apoptotic Bcl-2 family member Bim and overexpression of Bcl-2 rescued cells from FOXO3-induced apoptosis (Dijkers *et al.*, 2000a). Stahl *et al.* demonstrated that the regulation of Bim expression by FOXO3 also plays an important role in the survival of activated T cells (Stahl *et al.*, 2002). T cells require IL-2 for proliferation and survival, and cytokine deprivation results in activation of FOXO3 and upregulation of Bim protein levels. A recent report showed that FOXO3 also upregulates the pro-apoptotic Bcl-2 family member Puma after IL-2 withdrawal (You *et al.*, 2006). T cells derived from *Bim*$^{-/-}$ and *Puma*$^{-/-}$ mice were resistant to apoptosis after IL-2 deprivation demonstrating that regulation of both Bcl-2 proteins by FOXO3 is important for the induction of apoptosis in the absence of cytokines (You *et al.*, 2006). The induction of Bim expression by FOXO also plays a role in the induction of neuronal apoptosis during embryogenesis. Using sympathetic neurons, which depend on nerve growth factor (NGF) as a model, inhibition of FOXO activity was found to delay NGF withdrawal-induced death (Gilley *et al.*, 2003). Ectopic expression of FOXO3 induced apoptosis which was itself dependent on Bim expression (Gilley *et al.*, 2003). Additionally, it has been shown that FOXO4 can induce apoptosis through upregulation of the transcriptional repressor Bcl-6 (Tang *et al.*, 2002). Promoter reporter analysis has demonstrated that Bcl-6 itself can subsequently down-regulate the expression of the anti-apoptotic protein Bcl-X_L (Tang *et al.*, 2002). Taken together these studies show that FOXO3 can induce apoptosis by activation of the intrinsic apoptotic pathway through modulation of expression of several Bcl-2 family members.

Pink1 was originally identified as a PTEN induced transcript and mutations in this gene are linked with autosomal recessive Parkinson's disease (Valente *et al.*, 2004). Pink1 has been linked with survival of neuronal cells and loss of PINK1 expression is associated with dysregulated mitochondrial function, however the precise mechanisms how Pink1 exerts its functions remain unclear (Bueler, 2009). Recently, it was reported that in T cells Pink1 mRNA expression is increased by either cytokine starvation or FOXO3 overexpression (Mei *et al.*, 2009). Promoter reporter assays and ChIP analysis revealed that FOXO3 can directly regulate PINK1 expression through binding to its promoter. Furthermore, depletion of PINK1 by siRNA-mediated knockdown sensitized cells to IL-2 withdrawal-induced cell death, suggesting that in lymphocytes regulation of Pink1 expression by FOXOs is important in cellular survival after growth factor deprivation (Mei *et al.*, 2009).

One of the first reported transcriptional targets for FOXO was Fas-ligand (FasL), which can induce cell death in neuronal and lymphoid cells (Brunet *et al.*, 1999). Ectopic expression of a constitutively active FOXO3 mutant was found to increase FasL promoter activity in reporter assays. Furthermore Jurkat cells that were deficient in components of the Fas signalling cascade failed to undergo apoptosis after expression of FOXO3, indicating that the Fas-mediated signalling is required for induction of apoptosis by FOXO3 (Brunet *et al.*, 1999). Overexpression of FOXO1 and FOXO3 in prostate carcinoma cells also induces apoptosis and this correlates with upregulation of Tumour Necrosis Factor-related Apoptosis Inducing Ligand (TRAIL) (Modur *et al.*, 2002). Utilising promoter reporter assays it was also shown that TRAIL is a direct transcriptional target of FOXOs (Modur *et al.*, 2002).

Rodukai *et al.* have demonstrated that treatment of lung cancer cells with a PDK1 inhibitor

sensitized the cells to chemotherapeutic drug-induced apoptosis (Rokudai *et al.*, 2002). Further experiments revealed that the PDK1 inhibitor resulted in activation of FOXO1 and increased expression of tumour necrosis factor receptor-associated death domain (TRADD). FOXO1 was shown to directly regulate the expression of TRADD through binding to a conserved FOXO enhancer element in the promoter of the TRADD gene. Ectopic expression of a TRADD mutant lacking the death domain attenuated chemotherapeutic drug-induced cell death, demonstrating the importance of this FOXO target gene in regulating apoptosis (Rokudai *et al.*, 2002).

While most studies report that activation of FOXOs induces cell cycle arrest and induction of apoptosis, a study by Jonsson *et al.* suggests that FOXOs may also increase cellular survival through repression of FasL expression (Jonsson *et al.*, 2005). In a murine model for rheumatoid arthritis, loss of Foxo3 expression protected against immune complex-mediated inflammation. Administration of serum from arthritic mice to healthy littermates caused a severe inflammatory arthritis, while Foxo3 deficient mice were resistant to this. Adoptive transfer of wild type neutrophils in *Foxo3*$^{-/-}$ mice restored their susceptibility to arthritis indicating that the resistance to induction of arthritis is caused by an intrinsic neutrophil defect. Neutrophils isolated from FOXO3 deficient mice showed higher levels of apoptosis compared to wildtype cells. After stimulation with inflammatory cytokines, Foxo3 deficient neutrophils also showed high levels of FasL expression, suggesting that Foxo3 represses FasL expression in neutrophils. Furthermore, transfection of a FasL reporter in neutrophils demonstrated that Foxo3 can indeed down-regulate FasL promoter activity (Jonsson *et al.*, 2005). This data is in contrast to previous studies in which FOXO3 induced apoptosis through upregulation of FasL expression in cerebellar granule cells and Jurkat cells (Brunet *et al.*, 1999). It is possible that FOXOs may interact with alternative cofactors in primary neutrophils, resulting in suppression of FasL promoter activity.

The role of FOXO1 in glucose metabolism and diabetes

Insulin signalling results in PKB-mediated inactivation of FOXOs, a pathway which is conserved between *C elegans, D melanogaster* and mammals. In mammals, insulin signalling ensures glucose homeostasis by adjusting endogenous glucose production as well as glucose uptake by peripheral tissue. FOXO1 is highly expressed in insulin responsive tissues and has been shown to play an important role in metabolic changes during adaptation to fasting (Altomonte *et al.*, 2003; Altomonte *et al.*, 2004; Puigserver *et al.*, 2003) *Foxo1*$^{-/+}$ in mice have highlighted the importance of FOXO1 in the development of type 2 diabetes. Deletion of one *Foxo1* allele restored insulin sensitivity and rescued diabetic phenotype in insulin receptor mutant mice (Nakae *et al.*, 2002).

Activation of FOXO1 in the liver after decreased insulin signalling increases gluconeogenesis, while in the pancreas FOXO1 is an important regulator of proliferation and beta cell function (Fig. 4) (Kitamura *et al.*, 2002). During fasting the upregulation of gluconeogenic genes in the liver, such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) ensures a stable blood glucose level (Barthel and Schmoll, 2003). Insulin blocks gluconeogenesis in the liver through inhibition of the transcription of these enzymes. Puigserver *et al.* have shown that FOXO1 increases G6Pase expression in mouse hepatocytes, which can be inhibited by insulin. (Puigserver *et al.*, 2003). This regulation of G6Pase expression requires the liver specific transcription factor PGC1. Both FOXO1 and PGC1 associate with the G6Pase promoter and expression of both factors showed a synergistic effect on G6Pase mRNA expression. This PGC1-FOXO1 complex is disrupted after PKB-mediated phosphorylation of FOXO1 in response to insulin, resulting in decreased G6Pase expression. Furthermore, injection

of mice with an adenoviral vector expressing a dominant negative FOXO1 mutant inhibited the induction of G6Pase by PGC1 in the liver (Puigserver *et al.*, 2003). In addition, it has been demonstrated that FOXO1 can also regulate the expression of PGC1 itself, thereby regulating PEPCK and G6Pase expression levels indirectly (Daitoku *et al.*, 2003). It has been suggested that the regulation of G6Pase and PEPCK by FOXO1 plays an important role in the development of type 2 diabetes. FOXO1 expression in the liver was increased in diabetic mice, and this was associated with increased expression of PEPCK and G6Pase (Altomonte *et al.*, 2003). Inhibition of Foxo1 activity by expression of a FOXO1 dominant negative mutant decreased PEPCK and G6Pase expression and returned blood glucose levels to normal levels (Altomonte *et al.*, 2003). Furthermore, expression of FOXO1 was found to be increased during differentiation of fetal liver cells, correlating with increased G6Pase and PEPCK mRNA levels (Sekine *et al.*, 2007). These data suggest that FOXO1-mediated expression of G6Pase and PEPCK is critical for gluconeogenesis in the liver during fasting and deregulation of its expression is involved in diabetes.

FOXO1 also plays an important role in development of the pancreas in response to insulin during embryonic development. To investigate the role of FoxO1 in beta cell function, the effect of loss of FoxO1 expression in *IRS2*-/- mice was investigated in mice (Kitamura *et al.*, 2002). Inactivation of insulin signalling by IRS2 deletion impaires beta cell proliferation and function. FoxO1 haplo-insufficiency restores proliferation in beta cells from *IRS2*-/- mice, which correlates with expression of the pancreatic transcription factor Pdx1. This protein plays an important role in the development of the pancreas as well as maintenance of beta cell function. It was shown that FoxO1 acts as transcriptional repressor of Pdx1 expression in the pancreas (Kitamura *et al.*, 2002) and this suggests that FOXO1 blocks beta cell proliferation and function through repression of Pdx1 expression.

In addition to the regulation of glucose metabolism, FOXO1 is also an important regulator of lipid metabolism through modulation of apoliprotein(apo) C-III expression levels. ApoC-III is an inhibitor of lipoprotein lipase (LPL) and its synthesis in the liver is blocked by insulin (Altomonte *et al.*, 2004). Elevated apoC-III levels have been associated with the development of hypertriglyceridemia in diabetic patients (Shachter, 2001). It has been shown that ectopic expression FOXO1 in rat primary hepatocytes increases apocIII mRNA, which can be blocked by insulin stimulation (Altomonte *et al.*, 2004). Infection of mice with

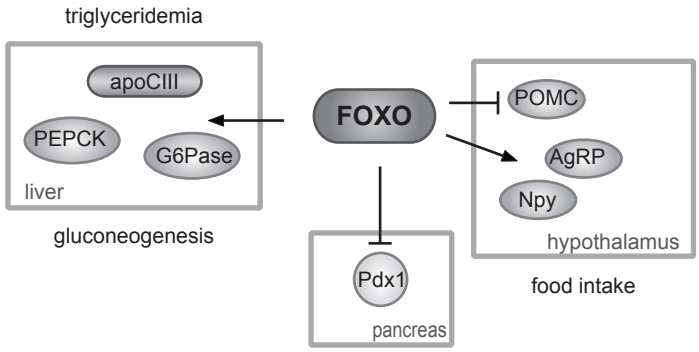

Figure 4. Role of FOXO1 in metabolism
FOXO1 plays an important role in metabolism by upregulating genes in the liver involved in gluconeogensis including PEPCK and G6Pase and lipid metabolism such as apoCIII. FOXO1 is also involved in β-cell function in the pancreas by suppressing Pdx1, while regulation of POMC, Agrp and Npy in the hypothalamus regulates food intake.

an adenovirus expressing FOXO1 increased apoC-III and triglyceride levels in plasma and additionally high Foxo1 expression in diabetic mice correlated with high apoC-III plasma levels (Altomonte *et al.*, 2004). Taken together, activation of FOXO1 activity contributes to the development of diabetes through transcriptional regulation of G6Pase, PEPCK and apoC-III in the liver and repressing Pdx1 in beta cells, resulting in decreased insulin sensitivity, decreased beta cell numbers and increased triglyceride levels in the blood.

In addition to the effects on genes involved in metabolism in the liver, pancreas and adipose tissue, FOXO1 is also involved in hormonal regulation of food intake in the hypothalamus (Kim *et al.*, 2006; Kitamura *et al.*, 2006). The anorexigenic hormone leptin decreases food intake through direct actions in hypothalamus through binding to the leptin receptor and activation of PI3K signalling (reviewed in Schwartz and Porte, Jr., 2005). It has been shown that Foxo1 is expressed in the hypothalamus in mice and its expression is decreased upon stimulation with leptin (Kim *et al.*, 2006). In mice, microinjection of an adenovirus encoding a constitutively active Foxo1 mutant in the hypothalamus inhibited leptin-induced reduction in food intake and decreased body weight, indicating that leptin-mediated inhibition of Foxo1 is required for the anorexigenic actions of this hormone. Previous research has indicated that leptin decreases in food intake by down-regulation of the hormones Agouti-related protein (Agrp) and neuropeptide Y (NpY), while enhancing the expression of pro-opiomelanocortin (POMC) (reviewed in Schwartz and Porte, Jr., 2005). In the hypothalamus expression of constitutively active Foxo1 inhibited the regulation of these genes by leptin (Kitamura *et al.*, 2006). In addition, ectopic expression of Foxo1 directly increases the expression of Agrp and NpY, while decreasing POMC expression by direct association with their promoters (Kim *et al.*, 2006; Kitamura *et al.*, 2006). These results demonstrate that Foxo1 is both necessary and sufficient for regulating Agrp, NpY and POMC expression in response to leptin. The differential effect of Foxo1 on these genes might result from association of distinct coactivator-corepressor complexes to the promoters. While active Foxo1 increased binding of the nuclear coactivator p300 to the Agrp promoter, inhibiton of Foxo1 expression resulted in binding of the corepressor NCoR. In contrast, the POMC promoter showed an opposite pattern, suggesting that the FOXO1-induced recruitment of either repressors or coactivators to the promoter is responsible for the differential effect on Agrp and POMC expression (Kitamura *et al.*, 2006).

FOXOs in the immune system

It has been suggested that low-grade inflammation of adipose tissue can contribute to insulin resistance in type 2 diabetes (Shoelson *et al.*, 2006). Stimulating adipocytes with TNFα blocked insulin-induced phosphorylation of FOXO1 suggesting that pro-inflammatory cytokines can modulate FOXO activity. In addition, FOXO1 activity was found to increase the expression of the transcription factor C/EBPβ (Ito *et al.*, 2009). Knockdown of FOXO1 in adipocytes decreased C/EBPβ expression and reduced expression of the pro-inflammmatory cytokines MCP-1 and IL-6. These results suggest that local inflammation might increase FOXO1 activity in adipose tissue, thereby providing a link between inflammation and insulin resistance (Ito *et al.*, 2009).

FOXO1 itself might also increase inflammation since it has recently been shown that FOXO1 can increase production of the inflammatory cytokine IL-1β (Su *et al.*, 2009). Ectopic exression of Foxo1 in a macrophage cell line was found to increase the level of IL-β and IL-2 production after stimulation with LPS (Su *et al.*, 2009). Furthermore, in macrophages isolated from LPS-treated mice, higher mRNA levels of Foxo1 and IL-1β were observed, correlating with increased plasma concentrations of IL-1β. ChIP analysis confirmed that IL-1β is a direct transcriptional target of Foxo1 and it was shown that

activation of NFκB could increase Foxo1 binding to the IL-β promoter. In macrophages from diabetic mice the expression of Foxo1 and IL-1β were both increased, suggesting that FOXO1 also regulates IL-1β expression *in vivo* (Su *et al.*, 2009). Taken together, these results suggest that FOXO1 might form a link between inflammation and diabetes.

The role of FOXO1 in T cell function has been investigated in mice with T cell specific deletion of *Foxo1* (Ouyang *et al.*, 2009). Analysis of T cells isolated from the spleen of these mice revealed that the percentage of activated CD4 and CD8 T cells was increased, while the percentage of naive CD4 and CD8 T cells was decreased. Phenotypic analysis of the marker expression profile of *Foxo1* deficient T cells demonstrated a decreased expression of the IL-7 receptor (IL-7R) on mature T cells. IL-7 is required for survival and homeostatic proliferation of peripheral T cells and stimulation of *Foxo1* deficient T cells with IL-7 *in vitro* could not rescue cells from starvation-induced cell death. IL7-R was found to be a direct transcriptional target of Foxo1. These data suggest that FOXO1 plays an important role in T cell homeostasis by increasing IL7-R, which is important for the maintenance of naieve T cells (Ouyang *et al.*, 2009).

Regulation of proteosomal and lysomal proteolysis in muscle atrophy

During fasting and in a variety of diseases including diabetes, cancer and sepsis, muscle size decreases in a process termed atrophy, a state which is characterised by accelerated proteolysis (Zhao *et al.*, 2008). It has been reported that proteolysis is caused through increased protein turnover through the ubiquitin-proteosome pathway as well as increased lysosomal proteolysis as a consequence of autophagy (Zhao *et al.*, 2007). Recently, FOXOs have been shown to play an important role in both these processes.

During starvation of murine muscle cells the expression of the muscle-specific ubiquitin

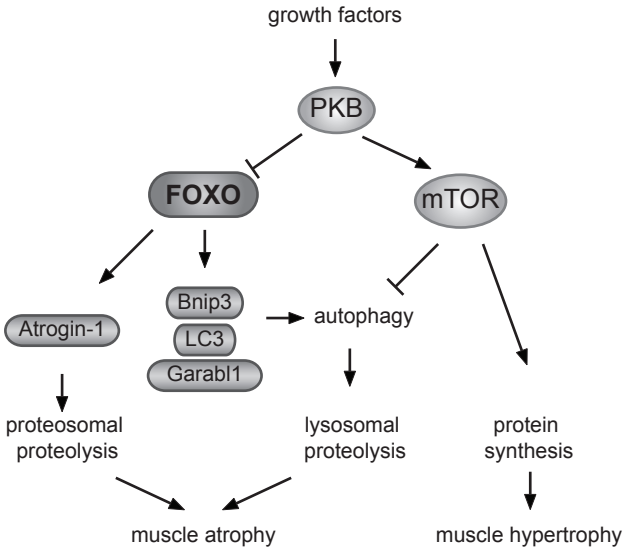

Figure 5. Role of FOXOs in muscle atrophy

FOXOs can induce atrophy in muscle cells by stimulating both lysosomal and proteosomal protein breakdown. Upregulation of Bnip3, LC3 and garabl12 are associated with FOXO-induced autophagy and increased lysosomal proteolysis, while the upregulation of the ubiquitin ligase atrogin3 increases proteolysis via the proteosome.

ligase atrogin-1 increases, which can be blocked by activation of PKB (Sandri *et al.*, 2004). Further experiments revealed that FOXO3 can directly regulate the expression of atrogin-1. Ectopic expression of an active FOXO3 mutant not only caused increased atrogin-1 expression but resulted in reduction in muscle fiber size (Sandri *et al.*, 2004). Furthermore Skurk *et al.* demonstrated that FOXO3 can also regulate cell size in cardiac muscle *in vivo*. Injection of viral vectors expressing FOXO3 directly in the heart of mice increased atrogin-1 expression and reduced the cell size, indicating that *in vivo* FOXO3 can induce cardiac hypertrophy (Skurk *et al.*, 2005).

Two recent studies have demonstrated that in addition to proteosomal degradation, FOXO3 can also induce lysosomal degradation through induction of autophagy. Using specific inhibitors for either proteosomal or lysosomal proteolysis it was shown that in muscle cells both pathways contribute to FOXO3-induced protein degradation (Zhao *et al.*, 2007). Furthermore it was shown that ectopic expression of an active FOXO3 mutant in adult muscle fibers from mice induced the formation of autophagosomes, resulting in an increase in lysosomal proteolysis. In contrast, knockdown of Foxo in these muscles fibers blocked autophagosome formation after starvation, demonstrating that FOXO3 activity is both required and sufficient for induction of autophagy in muscle cells. Microarray and ChIP analysis revealed the upregulation of a number of mRNA transcripts involved in protein breakdown including proteins involved in degradation through the proteosome including atrogin-1 and autophagy-related genes including Gabarapl1, atg12l and Beclin1 (Zhao *et al.*, 2007). However, it is unclear whether upregulation of these autophagy-related genes directly drives autophagic flux or whether they are upregulated to replace the components which are consumed during this process. A second report by Mammucari *et al.* demonstrated that in skeletal muscle mRNA levels of multiple proteins involved in protein degradation increased after fasting, including LC3, GabarapL1, Bnip3 and atrogin-1 (Mammucari *et al.*, 2007). Furthermore analysis of LC3 expression by fluorescent microscopy revealed the formation of autophagosomes in muscles from fasted animals and these effects could be blocked by knockdown of Foxo3 expression. Expression of a constitutively active FOXO3 mutant in muscles was sufficient to induce autophagosomes *in vivo*, which was in turn dependent on transcriptional upregulation of Bnip3, a Bcl-2-related protein that is involved in regulation of autophagy. Bnip3 overexpression was also sufficient to induce autophagosomes, indicating that *in vivo* FOXO3 controls autophagy through regulation of Bnip3 expression (Mammucari *et al.*, 2007). However, these results are in contrast to a report by Zhao *et al.* in which no upregulation of Bnip3 expression was observed after FOXO3 activation *in vitro* (Zhao *et al.*, 2007). How FOXO3 induces autophagy in the absence of Bnip3 expression remains unclear and awaits further research.

Besides regulation of atrophy in skeletal muscle FOXOs have also been shown to play a role in autophagy in cardiomyocytes during fasting (Sengupta *et al.*, 2009). Ectopic expression of a dominant negative FOXO1 mutant blocked the starvation-induced reduction in cell size of cultured cardiomyocytes. In contrast, ectopic expression of FOXO1 or FOXO3 increased autophagosome formation, reduced cell size and induced the expression of the autophagy-related genes LC3, Gabarapl1 and Atg12. (Sengupta *et al.*, 2009). These results suggest that FOXOs can directly regulate cardiomyocyte cell size through modulation of autophagy.

Besides regulating atrophy by upregulating targets involved in protein degradation, FOXOs have also been described to induce atrophy in skeletal muscle by upregulation of myostatin; a secreted factor which potently induces atrophy by inhibiting protein synthesis (Allen and Unterman, 2007). Activation of FOXO1 in myoblasts increased myostatin mRNA and increased activity of a myostatin promoter reporter (Allen and Unterman, 2007). However the importance of the upregulation of myostatin expression in FOXO-induced atrophy

remains unclear.
Taken together, FOXO3 is an important regulator of muscle atrophy by regulating both proteosomal as well as lysomal proteolysis resulting in decreased muscle function (Fig. 5).

FOXOs act as tumour suppressors
Although they are regulated by distinct intracellular events, both FOXOs and p53 regulate a similar set of target genes, including proteins involved in cell cycle arrest and apoptosis, suggesting that similar to p53, FOXOs might also act as true tumour suppressors. This is supported by the finding that the PI3K-PKB pathway is frequently overactivated in cancer resulting in FOXO inactivation (Engelman, 2009). In a number of studies it has been shown that reactivation of FOXOs either by ectopic expression or by inhibition of PI3K in a variety of cancer cells resulted in induction of apoptosis. FOXO3-mediated upregulation of the pro-apoptotic Bcl-2 family member Bim has been shown to induce apoptosis in breast cancer cells, chronic leukaemia cells and gastric cancer cells (Essafi *et al.*, 2005; Sunters *et al.*, 2003; Yamamura *et al.*, 2006). Furthermore, treatment of glioma cells with the chemotherapeutic drug cyclosporin A induced apoptosis through FOXO1-mediated FasL expression (Ciechomska *et al.*, 2003). These results suggest that the inactivation of FOXOs plays an important role in survival of tumour cells. The development of a inducible *Foxo1*-/-, *Foxo3*-/- and *Foxo4*-/- mouse model demonstrated the importance of FOXOs in oncogenesis (Paik *et al.*, 2007). After conditional deletion of *Foxo1*, *Foxo3* and *Foxo4* mice developed lymphoblastic thymic lymphomas and hemangiomas. Disruption of only two *Foxo* genes resulted in a more moderate phenotype demonstrating that FOXOs are functional redundant tumour suppressors (Paik *et al.*, 2007).
It has been demonstrated that FOXO3 can influence the transcription of a large subset of target genes by inhibiting the proto-oncogene c-myc (Delpuech *et al.*, 2007). C-myc is a positive regulator of proliferation and survival is found to be upregulated in a variety of cancers. In a colon carcinoma cell line microarrray analysis after FOXO3 activation identified Mxi1, a transcriptional inhibitor of c-myc, as a putative FOXO3 target. Comparative analysis of the FOXO3-regulated transcripts with a database of C-myc target genes revealed that an overlapping set of transcripts with FOXO3-down-regulated genes, suggesting that FOXO3 could directly inhibit c-myc signalling. Knockdown of Mxi1 expression increased the expression of the FOXO3-repressed c-myc targets, suggesting that the induction of Mxi expression contributes to the FOXO3-induced down-regulation of c-myc signalling. In addition, knockdown of Mxi1 reduced the block in cell cycle progression by FOXO3 activation, indicating that Mxi1 contributes to inhibition of proliferation by FOXO3 (Delpuech *et al.*, 2007).

In some cell types FOXO transcriptional activity not only prevents cells from proliferating but actively induces a differentiation program. Chromic myeloid leukemia (CML) is characterized by the expression of the oncogenic fusion protein Bcr-Abl, which results in constitutive activation of multiple signalling pathways including the PI3K-PKB pathway (Jagani *et al.*, 2008). Inhibition of the kinase activity of Bcr-Abl with the specific inhibitor imatinib induced activation of FOXO3 in the CML celline K562 and, utilising microarray analysis, the helix-loop-helix protein Id1 was identified as a novel FOXO target (Birkenkamp *et al.*, 2007). Inhibition of Bcr-Abl or overexpression of active FOXO3 induced differentiation of CML cells towards erythrocytes, which was blocked by Id1 specific knockdown (Birkenkamp *et al.*, 2007). This suggests that Bcr-Abl maintains the leukemic phenotype by repressing FOXO3-induced differentiation.

Besides the tumour-suppressor function of FOXOs it has also been suggested that FOXOs can actually contribute to enhanced survival of drug-resistant oncogenic cells. It was observed that in doxyrubicin-resistant K562 CML cells the levels of dephosphorylated FOXO3 are increased compared to the parental cell line, while phosphorylation and activity of PKB was also increased (Hui *et al.*, 2008). Furthermore, activation of FOXO3 in K562 cells increased PKB phosphorylation, suggesting that FOXO3 acts in a positive feedback loop to activate PKB. Activation of FOXO3 resulted in transcriptional upregulation of one of the catalytic subunits of PI3K; p110α, suggesting that the FOXO3-induced activation of PKB was mediated by increasing PI3K activity. However knockdown of p110α did not decrease FOXO3-induced phosphorylation of PKB, indicating that other mechanisms play a role in this feedback loop (Hui *et al.*, 2008).

CONCLUSION

Through regulation of multiple transcriptional targets FOXOs modulate various cellular functions including proliferation, apoptosis, stress resistance and metabolism. Since many of these cellular responses are deregulated in cancer, FOXOs are important regulators of tissue homeostasis. The outcome of FOXO activation depends largely on the cellular context. This is highlighted by fact that comparative analysis of FOXO-regulated transcripts in NSCs, HSCs and lymphomas from the *Foxo* triple knockout mice demonstrated very little overlap, indicating that FOXOs regulate their targets in a highly cell type-specific manner. FOXOs associate with a large variety of co-factors that influence their transcriptional program and detailed knowledge about the specific interactions in different cell types might provide clues on the cell type specific consequences of FOXO activation (reviewed in van der Vos and Coffer, 2008). n addition, although the different FOXOs isoforms show an overlapping expression pattern, deletion of the individual FOXO genes in mice gives distinct phenotypes indicating non-redundant roles for FOXOs *in vivo.* The identification of differential regulated transcriptional targets will give more insights in the complex biology of FOXO-mediated transcription.

REFERENCES

Allen,D.L. and Unterman,T.G. (2007). Regulation of myostatin expression and myoblast differentiation by FoxO and SMAD transcription factors. Am. J. Physiol Cell Physiol *292*, C188-C199.

Altomonte,J., Cong,L., Harbaran,S., Richter,A., Xu,J., Meseck,M., and Dong,H.H. (2004). Foxo1 mediates insulin action on apoC-III and triglyceride metabolism. J. Clin. Invest *114*, 1493-1503.

Altomonte,J., Richter,A., Harbaran,S., Suriawinata,J., Nakae,J., Thung,S.N., Meseck,M., Accili,D., and Dong,H. (2003). Inhibition of Foxo1 function is associated with improved fasting glycemia in diabetic mice. Am. J. Physiol Endocrinol. Metab *285*, E718-E728.

Anderson,M.J., Viars,C.S., Czekay,S., Cavenee,W.K., and Arden,K.C. (1998). Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. Genomics *47*, 187-199.

Barthel,A. and Schmoll,D. (2003). Novel concepts in insulin regulation of hepatic gluconeogenesis. Am. J. Physiol Endocrinol. Metab *285*, E685-E692.

Besson,A., Dowdy,S.F., and Roberts,J.M. (2008). CDK inhibitors: cell cycle regulators and beyond. Dev. Cell *14*, 159-169.

Biggs,W.H., Cavenee,W.K., and Arden,K.C. (2001). Identification and characterization of members of the FKHR (FOX O) subclass of winged-helix transcription factors in the mouse. Mamm. Genome *12*, 416-425.

Birkenkamp,K.U., Essafi,A., van,d., V, da,C.M., Hui,R.C., Holstege,F., Koenderman,L., Lam,E.W., and Coffer,P.J. (2007). FOXO3a induces differentiation of Bcr-Abl-transformed cells through transcriptional down-regulation of Id1. J. Biol. Chem. *282*, 2211-2220.

Boura,E., Silhan,J., Herman,P., Vecer,J., Sulc,M., Teisinger,J., Obsilova,V., and Obsil,T. (2007). Both the N-terminal loop and wing W2 of the forkhead domain of transcription factor Foxo4 are important for DNA binding. J. Biol. Chem. *282*, 8265-8275.

Brunelle,J.K. and Letai,A. (2009). Control of mitochondrial apoptosis by the Bcl-2 family. J. Cell Sci. *122*, 437-441.

Brunet,A., Bonni,A., Zigmond,M.J., Lin,M.Z., Juo,P., Hu,L.S., Anderson,M.J., Arden,K.C., Blenis,J., and Greenberg,M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.

Brunet,A., Park,J., Tran,H., Hu,L.S., Hemmings,B.A., and Greenberg,M.E. (2001). Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). Mol. Cell Biol. *21*, 952-965.

Bueler,H. (2009). Impaired mitochondrial dynamics and function in the pathogenesis of Parkinson's disease. Exp. Neurol. *218*, 235-246.

Castrillon,D.H., Miao,L., Kollipara,R., Horner,J.W., and DePinho,R.A. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science *301*, 215-218.

Chen,J., Yusuf,I., Andersen,H.M., and Fruman,D.A. (2006). FOXO transcription factors cooperate with delta EF1 to activate growth suppressive genes in B lymphocytes. J. Immunol. *176*, 2711-2721.

Chiribau,C.B., Cheng,L., Cucoranu,I.C., Yu,Y.S., Clempus,R.E., and Sorescu,D. (2008). FOXO3A regulates peroxiredoxin III expression in human cardiac fibroblasts. J. Biol. Chem. *283*, 8211-8217.

Ciechomska,I., Pyrzynska,B., Kazmierczak,P., and Kaminska,B. (2003). Inhibition of Akt kinase signalling and activation of Forkhead are indispensable for upregulation of FasL expression in apoptosis of glioma cells. Oncogene *22*, 7617-7627.

Daitoku,H., Yamagata,K., Matsuzaki,H., Hatta,M., and Fukamizu,A. (2003). Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. Diabetes *52*, 642-649.

Delpuech,O., Griffiths,B., East,P., Essafi,A., Lam,E.W., Burgering,B., Downward,J., and Schulze,A. (2007). Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression. Mol. Cell Biol. *27*, 4917-4930.

Dijkers,P.F., Medema,R.H., Lammers,J.W., Koenderman,L., and Coffer,P.J. (2000a). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol. *10*, 1201-1204.

Dijkers,P.F., Medema,R.H., Pals,C., Banerji,L., Thomas,N.S., Lam,E.W., Burgering,B. M., Raaijmakers,J.A., Lammers,J.W., Koenderman,L., and Coffer,P.J. (2000b). Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol. Cell Biol. *20*, 9138-9148.

Engelman,J.A. (2009). Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat. Rev. Cancer *9*, 550-562.

Essafi,A., Fernandez de,M.S., Hassen,Y.A., Soeiro,I., Mufti,G.J., Thomas,N.S., Medema,R. H., and Lam,E.W. (2005). Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. Oncogene *24*, 2317-2329.

Finkel,T., Serrano,M., and Blasco,M.A. (2007). The common biology of cancer and ageing. Nature *448*, 767-774.

Furuyama,T., Kitayama,K., Yamashita,H., and Mori,N. (2003). Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation. Biochem. J. *375*, 365-371.

Furuyama,T., Nakazawa,T., Nakano,I., and Mori,N. (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem. J. *349*, 629-634.

Gilley,J., Coffer,P.J., and Ham,J. (2003). FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. J. Cell Biol. *162*, 613-622.

Guicciardi,M.E. and Gores,G.J. (2009). Life and death by death receptors. FASEB J. *23*, 1625-1637.

Hideshima,T., Nakamura,N., Chauhan,D., and Anderson,K.C. (2001). Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. Oncogene *20*, 5991-6000.

Ho,K.K., Myatt,S.S., and Lam,E.W. (2008). Many forks in the path: cycling with FoxO. Oncogene *27*, 2300-2311.

Hosaka,T., Biggs,W.H., III, Tieu,D., Boyer,A.D., Varki,N.M., Cavenee,W.K., and Arden,K. C. (2004). Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc. Natl. Acad. Sci. U. S. A *101*, 2975-2980.

Hu,M.C., Lee,D.F., Xia,W., Golfman,L.S., Ou-Yang,F., Yang,J.Y., Zou,Y., Bao,S., Hanada,N., Saso,H., Kobayashi,R., and Hung,M.C. (2004). IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell *117*, 225-237.

Huang,H., Regan,K.M., Lou,Z., Chen,J., and Tindall,D.J. (2006). CDK2-dependent phosphorylation of FOXO1 as an apoptotic response to DNA damage. Science *314*, 294-297.

Hui,R.C., Gomes,A.R., Constantinidou,D., Costa,J.R., Karadedou,C.T., Fernandez de,M.S., Wymann,M.P., Brosens,J.J., Schulze,A., and Lam,E.W. (2008). The forkhead transcription factor FOXO3a increases phosphoinositide-3 kinase/Akt activity in drug-resistant leukemic cells through induction of PIK3CA expression. Mol. Cell Biol. *28*, 5886-5898.

Ito,Y., Daitoku,H., and Fukamizu,A. (2009). Foxo1 increases pro-inflammatory gene

expression by inducing C/EBPbeta in TNF-alpha-treated adipocytes. Biochem. Biophys. Res. Commun. *378*, 290-295.

Jacobs,F.M., van der Heide,L.P., Wijchers,P.J., Burbach,J.P., Hoekman,M.F., and Smidt,M.P. (2003). FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. J. Biol. Chem. *278*, 35959-35967.

Jagani,Z., Singh,A., and Khosravi-Far,R. (2008). FoxO tumor suppressors and BCR-ABL-induced leukemia: a matter of evasion of apoptosis. Biochim. Biophys. Acta *1785*, 63-84.

Jonsson,H., Allen,P., and Peng,S.L. (2005). Inflammatory arthritis requires Foxo3a to prevent Fas ligand-induced neutrophil apoptosis. Nat. Med. *11*, 666-671.

Katayama,K., Nakamura,A., Sugimoto,Y., Tsuruo,T., and Fujita,N. (2008). FOXO transcription factor-dependent p15(INK4b) and p19(INK4d) expression. Oncogene *27*, 1677-1686.

Kenyon,C., Chang,J., Gensch,E., Rudner,A., and Tabtiang,R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461-464.

Kim,M.S., Pak,Y.K., Jang,P.G., Namkoong,C., Choi,Y.S., Won,J.C., Kim,K.S., Kim,S.W., Kim,H.S., Park,J.Y., Kim,Y.B., and Lee,K.U. (2006). Role of hypothalamic Foxo1 in the regulation of food intake and energy homeostasis. Nat. Neurosci. *9*, 901-906.

Kimura,K.D., Tissenbaum,H.A., Liu,Y., and Ruvkun,G. (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science *277*, 942-946.

Kitamura,T., Feng,Y., Kitamura,Y.I., Chua,S.C., Jr., Xu,A.W., Barsh,G.S., Rossetti,L., and Accili,D. (2006). Forkhead protein FoxO1 mediates Agrp-dependent effects of leptin on food intake. Nat. Med. *12*, 534-540.

Kitamura,T., Nakae,J., Kitamura,Y., Kido,Y., Biggs,W.H., III, Wright,C.V., White,M.F., Arden,K.C., and Accili,D. (2002). The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth. J. Clin. Invest *110*, 1839-1847.

Kops,G.J., Dansen,T.B., Polderman,P.E., Saarloos,I., Wirtz,K.W., Coffer,P.J., Huang,T. T., Bos,J.L., Medema,R.H., and Burgering,B.M. (2002a). Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature *419*, 316-321.

Kops,G.J., Medema,R.H., Glassford,J., Essers,M.A., Dijkers,P.F., Coffer,P.J., Lam,E. W., and Burgering,B.M. (2002b). Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol. Cell Biol. *22*, 2025-2036.

Lin,L., Hron,J.D., and Peng,S.L. (2004). Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. Immunity. *21*, 203-213.

Mammucari,C., Milan,G., Romanello,V., Masiero,E., Rudolf,R., Del,P.P., Burden,S.J., Di,L.R., Sandri,C., Zhao,J., Goldberg,A.L., Schiaffino,S., and Sandri,M. (2007). FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab *6*, 458-471.

Medema,R.H., Kops,G.J., Bos,J.L., and Burgering,B.M. (2000). AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature *404*, 782-787.

Mei,Y., Zhang,Y., Yamamoto,K., Xie,W., Mak,T.W., and You,H. (2009). FOXO3a-

dependent regulation of Pink1 (Park6) mediates survival signaling in response to cytokine deprivation. Proc. Natl. Acad. Sci. U. S. A *106*, 5153-5158.

Modur,V., Nagarajan,R., Evers,B.M., and Milbrandt,J. (2002). FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. J. Biol. Chem. *277*, 47928-47937.

Nakae,J., Biggs,W.H., III, Kitamura,T., Cavenee,W.K., Wright,C.V., Arden,K.C., and Accili,D. (2002). Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. Nat. Genet. *32*, 245-253.

Nakae,J., Kitamura,T., Kitamura,Y., Biggs,W.H., III, Arden,K.C., and Accili,D. (2003). The forkhead transcription factor Foxo1 regulates adipocyte differentiation. Dev. Cell *4*, 119-129.

Nemoto,S. and Finkel,T. (2002). Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. Science *295*, 2450-2452.

Obsil,T. and Obsilova,V. (2008). Structure/function relationships underlying regulation of FOXO transcription factors. Oncogene *27*, 2263-2275.

Ouyang,W., Beckett,O., Flavell,R.A., and Li,M.O. (2009). An essential role of the Forkhead-box transcription factor Foxo1 in control of T cell homeostasis and tolerance. Immunity. *30*, 358-371.

Paik,J.H., Ding,Z., Narurkar,R., Ramkissoon,S., Muller,F., Kamoun,W.S., Chae,S.S., Zheng,H., Ying,H., Mahoney,J., Hiller,D., Jiang,S., Protopopov,A., Wong,W.H., Chin,L., Ligon,K.L., and DePinho,R.A. (2009). FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis. Cell Stem Cell *5*, 540-553.

Paik,J.H., Kollipara,R., Chu,G., Ji,H., Xiao,Y., Ding,Z., Miao,L., Tothova,Z., Horner,J. W., Carrasco,D.R., Jiang,S., Gilliland,D.G., Chin,L., Wong,W.H., Castrillon,D.H., and DePinho,R.A. (2007). FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell *128*, 309-323.

Paolisso,G., Galzerano,D., Gambardella,A., Varricchio,G., Saccomanno,F., D'Amore,A., Varricchio,M., and D'Onofrio,F. (1995). Left ventricular hypertrophy is associated with a stronger impairment of non-oxidative glucose metabolism in hypertensive patients. Eur. J. Clin. Invest *25*, 529-533.

Partridge,L. and Bruning,J.C. (2008). Forkhead transcription factors and ageing. Oncogene *27*, 2351-2363.

Puigserver,P., Rhee,J., Donovan,J., Walkey,C.J., Yoon,J.C., Oriente,F., Kitamura,Y., Altomonte,J., Dong,H., Accili,D., and Spiegelman,B.M. (2003). Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. Nature *423*, 550-555.

Rokudai,S., Fujita,N., Kitahara,O., Nakamura,Y., and Tsuruo,T. (2002). Involvement of FKHR-dependent TRADD expression in chemotherapeutic drug-induced apoptosis. Mol. Cell Biol. *22*, 8695-8708.

Sandri,M., Sandri,C., Gilbert,A., Skurk,C., Calabria,E., Picard,A., Walsh,K., Schiaffino,S., Lecker,S.H., and Goldberg,A.L. (2004). Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell *117*, 399-412.

Schmidt,M., Fernandez de,M.S., van der,H.A., Klompmaker,R., Kops,G.J., Lam,E. W., Burgering,B.M., and Medema,R.H. (2002). Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. Mol. Cell Biol. *22*, 7842-7852.

Schwartz,M.W. and Porte,D., Jr. (2005). Diabetes, obesity, and the brain. Science *307*, 375-379.

Sekine,K., Chen,Y.R., Kojima,N., Ogata,K., Fukamizu,A., and Miyajima,A. (2007). Foxo1 links insulin signaling to C/EBPalpha and regulates gluconeogenesis during liver development. EMBO J. *26*, 3607-3615.

Sengupta,A., Molkentin,J.D., and Yutzey,K.E. (2009). FoxO transcription factors promote autophagy in cardiomyocytes. J. Biol. Chem. *284*, 28319-28331.

Seoane,J., Le,H.V., Shen,L., Anderson,S.A., and Massague,J. (2004). Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. Cell *117*, 211-223.

Shachter,N.S. (2001). Apolipoproteins C-I and C-III as important modulators of lipoprotein metabolism. Curr. Opin. Lipidol. *12*, 297-304.

Shoelson,S.E., Lee,J., and Goldfine,A.B. (2006). Inflammation and insulin resistance. J. Clin. Invest *116*, 1793-1801.

Skurk,C., Izumiya,Y., Maatz,H., Razeghi,P., Shiojima,I., Sandri,M., Sato,K., Zeng,L., Schiekofer,S., Pimentel,D., Lecker,S., Taegtmeyer,H., Goldberg,A.L., and Walsh,K. (2005). The FOXO3a transcription factor regulates cardiac myocyte size downstream of AKT signaling. J. Biol. Chem. *280*, 20814-20823.

Stahl,M., Dijkers,P.F., Kops,G.J., Lens,S.M., Coffer,P.J., Burgering,B.M., and Medema,R. H. (2002). The forkhead transcription factor FoxO regulates transcription of p27Kip1 and Bim in response to IL-2. J. Immunol. *168*, 5024-5031.

Stone,J.R. and Yang,S. (2006). Hydrogen peroxide: a signaling messenger. Antioxid. Redox. Signal. *8*, 243-270.

Su,D., Coudriet,G.M., Kim,D.H., Lu,Y., Perdomo,G., Qu,S., Slusher,S., Tse,H.M., Piganelli,J., Giannoukakis,N., Zhang,J., and Dong,H.H. (2009). FoxO1 Links Insulin Resistance to Proinflammatory Cytokine IL-1beta Production in Macrophages. Diabetes.

Sun,A., Bagella,L., Tutton,S., Romano,G., and Giordano,A. (2007). From G0 to S phase: a view of the roles played by the retinoblastoma (Rb) family members in the Rb-E2F pathway. J. Cell Biochem. *102*, 1400-1404.

Sunters,A., Fernandez de,M.S., Stahl,M., Brosens,J.J., Zoumpoulidou,G., Saunders,C.A., Coffer,P.J., Medema,R.H., Coombes,R.C., and Lam,E.W. (2003). FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. J. Biol. Chem. *278*, 49795-49805.

Tan,W.Q., Wang,K., Lv,D.Y., and Li,P.F. (2008). Foxo3a inhibits cardiomyocyte hypertrophy through transactivating catalase. J. Biol. Chem. *283*, 29730-29739.

Tang,T.T., Dowbenko,D., Jackson,A., Toney,L., Lewin,D.A., Dent,A.L., and Lasky,L.A. (2002). The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. J. Biol. Chem. *277*, 14255-14265.

Tothova,Z., Kollipara,R., Huntly,B.J., Lee,B.H., Castrillon,D.H., Cullen,D.E., McDowell,E.

P., Lazo-Kallanian,S., Williams,I.R., Sears,C., Armstrong,S.A., Passegue,E., DePinho,R. A., and Gilliland,D.G. (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell *128*, 325-339.

Tran,H., Brunet,A., Grenier,J.M., Datta,S.R., Fornace,A.J., Jr., DiStefano,P.S., Chiang,L. W., and Greenberg,M.E. (2002). DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science *296*, 530-534.

Valente,E.M., bou-Sleiman,P.M., Caputo,V., Muqit,M.M., Harvey,K., Gispert,S., Ali,Z., Del,T.D., Bentivoglio,A.R., Healy,D.G., Albanese,A., Nussbaum,R., Gonzalez-Maldonado,R., Deller,T., Salvi,S., Cortelli,P., Gilks,W.P., Latchman,D.S., Harvey,R.J., Dallapiccola,B., Auburger,G., and Wood,N.W. (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science *304*, 1158-1160.

van der Vos and Coffer,P.J. (2008). FOXO-binding partners: it takes two to tango. Oncogene *27*, 2289-2299.

Yamamura,Y., Lee,W.L., Inoue,K., Ida,H., and Ito,Y. (2006). RUNX3 cooperates with FoxO3a to induce apoptosis in gastric cancer cells. J. Biol. Chem. *281*, 5267-5276.

You,H., Pellegrini,M., Tsuchihara,K., Yamamoto,K., Hacker,G., Erlacher,M., Villunger,A., and Mak,T.W. (2006). FOXO3a-dependent regulation of Puma in response to cytokine/ growth factor withdrawal. J. Exp. Med. *203*, 1657-1663.

Zhao,J., Brault,J.J., Schild,A., Cao,P., Sandri,M., Schiaffino,S., Lecker,S.H., and Goldberg,A.L. (2007). FoxO3 coordinately activates protein degradation by the autophagic/ lysosomal and proteasomal pathways in atrophying muscle cells. Cell Metab *6*, 472-483.

Zhao,J., Brault,J.J., Schild,A., and Goldberg,A.L. (2008). Coordinate activation of autophagy and the proteasome pathway by FoxO transcription factor. Autophagy. *4*, 378-380.

CHAPTER 3

FOXO binding partners: it takes two to tango

THREE

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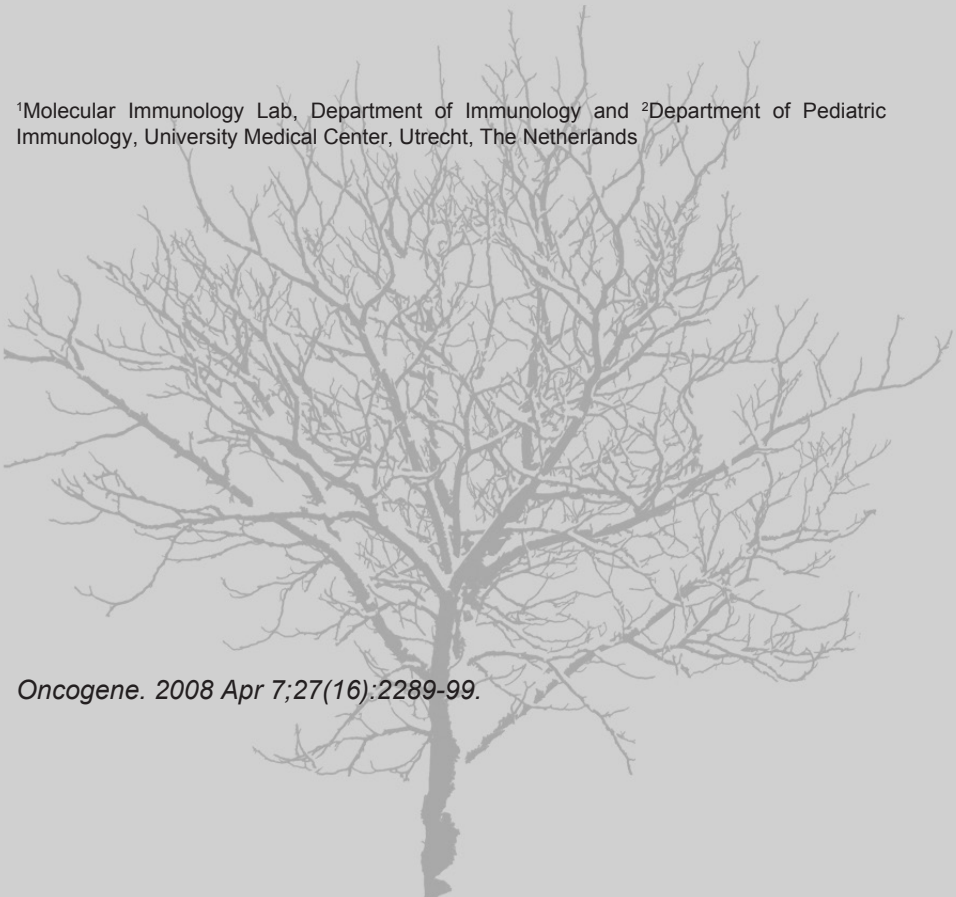

Oncogene. 2008 Apr 7;27(16):2289-99.

ABSTRACT

Modulation FOXO transcription factor activities can lead to a variety of cellular outputs resulting in changes in proliferation, apoptosis, differentiation and metabolic responses. Although FOXO proteins all contain an identical DNA-binding domain their cellular functions appear to be distinct, as exemplified by differences in the phenotype of Foxo1, Foxo3 and Foxo4 null mutant mice. While some of these differences may be attributable to the differential expression patterns of these transcription factors, many cells and tissues express several FOXO isoforms. Recently it has become clear that FOXO proteins can regulate transcriptional responses independently of direct DNA-binding. It has been demonstrated that FOXOs can associate with a variety of unrelated transcription factors, regulating activation or repression of diverse target genes. The complement of transcription factors expressed in a particular cell type is thus critical in determining the functional endpoint of FOXO activity. These interactions greatly expand the possibilities for FOXO-mediated regulation of transcriptional programs. This review details currently described FOXO-binding partners and examines the role of these interactions in regulating cell fate decisions.

INTRODUCTION

Over the last few years the mammalian DAF-16-like transcription factors FOXO1, FOXO3 and FOXO4 have been demonstrated to play crucial roles in a plethora of cellular processes including proliferation, apoptosis, differentiation, stress resistance and metabolic responses (Birkenkamp and Coffer 2003; van der Horst and Burgering 2007). To ensure that the correct, cell-type specific effect is initiated by these widely expressed factors, FOXOs utilise a wide range of binding partners allowing for a much broader transcriptional response. The consequences of such physical associations are perhaps best highlighted by oncogenic FOXO fusion proteins responsible for mixed lineage leukemia (MLL) and alveolar rhabdmyosarcoma (ARMS) (Barr 2001; So and Cleary 2003). A frequent target of chromosomal translocations in human leukemias, both FOXO3 and FOXO4 are MLL fusion partners. Fusion tends to occur in the middle of the Forkhead binding domain resulting in chimeric proteins harbouring the transcriptional activation domains of the respective forkhead proteins. Similarly PAX3-FOXO1 fusions found in ARMS exhibit a similar chimeric structure containing the FOXO transactivation domain and PAX3 DNA-binding domains. The FOXO1 transactivation domain has more robust transcriptional activation potential than that of PAX3 and can thus more strongly activate PAX3 mediated transcription. This has led to the hypothesis that fusion with FOXO1 drives oncogenesis through enhanced

Table 1. FOXO transcription factor binding partners

Androgen Receptor (AR)	(Fan *et al.,* 2007; Li *et al.,* 2003)
β-catenin	(Essers *et al.,* 2005; Almeida *et al.,* 2007)
Constitutive Androstane Receptor (CAR)	(Kodama *et al.,* 2004)
Cs1	(Kitamura *et al.,* 2007)
C/EBPα	(Sekine *et al.,* 2007; Qiao and Shao, 2006)
C/EBPβ	(Gomis *et al.,* 2006; Christian *et al.,* 2002)
Estrogen Receptor (ER)	(Schuur *et al.,* 2001; Schuur *et al.,* 2001)
FoxG1	(Seoane *et al.,* 2004)
Follicle Stimulating Hormone Receptor (FSHR)	(Nechamen *et al.,* 2007)
Hepatic nuclear factor-4 (HNF4)	(Hirota *et al.,* 2003)
HOXA5	(Foucher *et al.,* 2002)
HOXA10	(Kim *et al.,* 2003)
Myocardin	(Liu *et al.,* 2005)
PGC-1α	(Puigserver *et al.,* 2003)
PPARα	(Qu *et al.,* 2007)
PPARγ	(Dowell *et al.,* 2003)
Pregnane X receptor (PXR)	(Kodama *et al.,* 2004)
Progesterone Receptor (PR)	(Kim *et al.,* 2005; Rudd *et al.,* 2007)
Retinoic Acid Receptor (RAR)	(Zhao *et al.,* 2001)
RUNX3	(Yamamura *et al.,* 2006)
Smad3	(Seoane *et al.,* 2004)
Smad4	(Seoane *et al.,* 2004)
STAT3	(Kortylewski *et al.,* 2003)
Thyroid hormone receptor (TR)	(Zhao *et al.,* 2001)

transcriptional activation of PAX3 target genes. These “physical interactions” demonstrate that functional association of FOXO proteins with other transcription factors can have dramatic consequences on transcriptional programs which may even lead to cellular transformation.

The first real evidence that association of FOXOs with accessory proteins played a critical role in transcriptional regulation came from work by Sellers and co-workers (Ramaswamy *et al.,* 2002). Using transcriptional profiling, chromatin immunoprecipitation and functional experiments to identify FOXO target genes, Ramaswamy *et al.,* demonstrated that a FOXO mutant, in which DNA-binding was abolished, was still able to effectively regulate a specific subset of these genes. The authors proposed that DNA-binding was not required for FOXO-dependent tumour suppression and cell-cycle regulation. This surprising result could be explained by a possible “altered” DNA-binding specificity of the FOXO mutant utilised, but more likely is that FOXO regulates a subset of target genes through interaction with other transcription factors. Indeed it has become apparent that FOXO proteins are able to associate with a wide variety of diverse transcription factor partners resulting in a far broader spectrum of gene regulation (Table 1). Here we will discuss the various associating proteins and their implications for regulating cellular responses to FOXO activation.

Integration of PI3K/FOXO and TGFβ/Smad pathways

In the nematode worm *C. elegans* the FOXO transcription factor DAF-16 regulates metabolism, development and longevity (Gottlieb and Ruvkun 1994; Larsen *et al.,* 1995). Many of these effects are mediated through DAF-2, a homologue of the mammalian insulin receptor, and AGE-1, the nematode PI3-kinase. Null mutations in *daf-16* were found to suppress the effects of mutations in *daf-2* or *age-1*, while genetic ablation of *daf-16* bypasses the need for insulin receptor-like signalling (Ogg *et al.,* 1997). A parallel TGFβ/DAF-7 and Smad/DAF-3 pathway also regulates *C. elegans* metabolism and development (Patterson *et al.,* 1997; Ren *et al.,* 1996). In mammals, TGFβ activates a receptor serine/threonine kinase complex that phosphorylates Smad2 and Smad3 (Shi and Massague 2003). Once activated, Smads translocate to the nucleus where they form transcriptional complexes with Smad4 plus additional co-activators and repressors. In the nematode worm *daf-3* acts in the TGFβ pathway in an analogous manner to *daf-16* in the insulin-like pathway, and DAF-3 activity is negatively regulated by upstream TGFβ signalling. Importantly, the DAF-2 pathway was found to exhibit genetic synergy with the nematode DAF-7/DAF-3 pathway, suggesting that DAF-16 can cooperate with nematode SMAD proteins in regulating the transcription of key metabolic and developmental control genes (Ogg *et al.,* 1997).

On basis of these findings, Ruvkun and co-workers proposed that DAF-16 and DAF-3 might form heteromers that repress the expression of key genes regulating metabolism and reproductive development (Ogg *et al.,* 1997). This can be extended to mammals where it can be envisaged that FOXO proteins might functionally interact with SMAD transcription factors. This hypothesis was further investigated in studies analysing the regulation of neuroepothelial and glioblastoma cell proliferation by TGFβ (Seoane *et al.,* 2004). TGFβ delivers cytostatic signals to epithelial, neuronal and immune cells, and its subversion can contribute to tumour development. Transcriptional activation of *p21Cip1* and *p15Ink4b*, cell cycle inhibitors, and repression of growth promoting *Id1* and *c-myc* are critical for this TGFβ driven cytostatic program. In support of the genetic analysis performed in *C. elegans*, Massagué and colleagues identified FOXO proteins as key partners of Smad3 and Smad4 in TGFβ-dependent formation of a *p21Cip1* transactivation complex (Seoane *et al.,* 2004). TGFβ results in the reorganisation of a transcriptional complex

on the *p21Cip1* promoter with removal of c-Myc and binding of Smad2/3 and Smad 4. The binding of Smads to the *p21Cip1* promoter overlaps with a consensus Forkhead binding element and mutation of this site was found to abrogate the TGFβ response of this promoter. FOXO-mediated induction of *p21Cip1* promoter activity in TGFβ treated cells was also increased by Smad overexpression. The cooperation of these distinct transcription factor families suggested that they may form a TGFβ-regulated complex. Indeed co-immunoprecipitation experiments confirmed that FoxO1, FoxO3 and FoxO4 can all bind to Smad3 and Smad4 in a TGFβ-dependent manner. By generation of mutant proteins it was demonstrated that the FOXO transactivation domain was critical for TGFβ-mediated $p21^{Cip1}$ expression, and that this was not due to general sequestration of Smads, but formation of a specific complex at the *p21Cip1* promoter. As previously proposed by work in the nematode worm, these data suggest that insulin and TGFβ signalling could be integrated at the level of FOXOs. Indeed inhibition of PI3-kinase signalling potentiated the induction of *p21Cip1* by suboptimal concentrations of TGFβ. This model of integration of insulin and TGFβ signalling is further complicated by the identification of FoxG1 as an antagonist of FOXOs (Seoane *et al.,* 2004). FoxG1 is a transcriptional repressor whose function is to protect neuroepithelial progenitor cells from cytostatic signals (Hanashima *et al.,* 2004). FoxG1 overexpression inhibits *p21Cip* induction by TGFβ, and it could also be co-imunoprecipitated with FOXOs. An analysis of glioblastomas revealed high PI3K activity and FOXG1 expression with an inability of cells to increase *p21Cip1* levels after TGFβ treatment. Thus FOXOs can be considered a nodal point for the integration of Smad, PI3K and FoxG1 signalling modules. In support of this it has recently been demonstrated that in human keratinocytes, FOXOs are essential for 11 of 115 immediate gene activation responses to TGFβ (Gomis *et al.,* 2006). Taken together, these results suggest that formation of a FOXO and Smad transcription factor complex is critical in the control of cell growth and proliferation, and that perturbation of the association or function of this complex could contribute to neoplasia.

β-catenin and FOXOs fighting stress together

As already alluded to, the *C. elegans* FOXO transcription factor DAF-16 increases longevity in the nematode worm by inducing entry into the dauer diapause, an alternative larval stage. Korswagen and colleagues found that in animals lacking the β-catenin gene, *bar-1*, dauer development and nematode life-span are perturbed (Essers *et al.,* 2005). Further genetic analysis has revealed that BAR-1 is required for DAF-16 function (when DAF-16 activity is limiting), raising the intriguing possibility that β-catenin may also be required for FOXO function in mammalian cells. Indeed co-expression of these proteins resulted in increased transcription from several FOXO promoter reporters and they were also found to physically associate. In *C. elegans*, as well as in mammalian cells, oxidative stress activates FOXO transcriptional activity by stimulating nuclear relocalisation (Brunet *et al.,* 2004; Essers *et al.,* 2004). Increasing levels of oxidative stress by hydrogen peroxide treatment of cells resulted in increased association of β-catenin and FOXO4. Importantly, in the nematode worm DAF-16 induced transcriptional responses to oxidative stress require BAR-1.

The best characterised β-catenin binding partner is TCF, a transcription factor required for a variety of developmental processes (Arce *et al.,* 2006). One consequence of oxidative stress-induced association of FOXOs and β-catenin is that this would divert a limited pool of intracellular β-catenin away from TCF. β-catenin/TCF-mediated transcription is required for osteoblast differentiation, and hydrogen peroxide induces reciprocal changes in FOXO- and TCF-mediated transcription in osteoblastic cells (Almeida *et al.,* 2007; Glass and Karsenty 2007). Increased levels of reactive oxygen species are also able to suppress

osteoblast differentiation affecting skeletal homeostasis. Thus it appears that levels of oxidative stress, which are thought to increase during the age of an organism, will result in increased β-catenin/FOXO association at the expense of TCF. This could be an important pathogenic factor in the development of skeletal involution which is associated with old age.

CCAAT/enhancer-binding protein interactions

In a subsequent study, Massagué and colleagues characterised a subset of FOXO/Smad-dependent TGFβ gene responses which were found to additionally require the transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) (Gomis *et al.,* 2006). C/EBPβ was found to be essential for TGFβ induction of the cell cycle inhibitor *p15INK4b* by a FOXO-Smad complex and repression of *c-MYC* by an E2F4/5-Smad complex in human epithelial cells (Gomis *et al.,* 2006d). The molecular mechanisms underlying C/EBPβ mediated effects have not been completely resolved but diverse configurations of FOXO and Smad binding elements in the promoters of target genes were identified. C/EBP transcription factors exhibit a broad expression pattern with tissue specific transcriptional responses controlling diverse cellular processes including hematopoiesis, adipocyte differentiation and gluconeogenesis in the liver (Nerlov 2007). More recently, their position at the crossroads between proliferation and differentiation has made them strong candidate regulators of tumorigenesis, and C/EBPs have been described as both tumor promoters and tumor suppressors.

One such process where C/EBPs play a role is decidualisation of uterine endometrial stroma (ES). This is characterized by the morphological and biochemical transformation of the ES in which the stromal fibroblasts differentiate to become rounded, secretory decidual cells. It is regulated by ovarian estrodiol and progesterone and appears to require elevated cAMP levels and sustained activation of protein kinase A (PKA) (Brar *et al.,* 1997). C/EBPβ expression is upregulated during ES cell differentiation and this is cAMP-inducible (Pohnke *et al.,* 1999). Expression of decidual prolactin (dPRL) by ES cells is a widely used biochemical marker of decidual differentiation and C/EBPβ forms part of a transcriptional complex binding to the dPRL promoter upon PKA activation. Treatment of primary ES cell cultures with cAMP was found to induce the sustained expression of nuclear localised FOXO1 (Christian *et al.,* 2002). Ectopic expression of FOXO1 was found to regulate expression of several decidualization-specific genes such as dPRL and, in the absence of exogenous hormones, also results in a noticeable change in stromal cell shape (Buzzio *et al.,* 2006). FOXO1 was found to transcriptionally activate the dPRL promoter and mutation of C/EBP-binding sites in the dPRL promoter abolished this effect (Christian *et al.,* 2002). This suggests a physical interaction between C/EBPβ and FOXO1, and this has been subsequently confirmed by *in vitro* binding assays. Furthermore, attenuation of FOXO1 levels in hormone-treated ES cells by RNAi resulted in the dramatic inhibition of expression of marker genes associated with decidualisation (Grinius *et al.,* 2006). FOXO1 is therefore an important effector of the decidual response in part through interaction with C/EBPβ. These studies also reveal a novel functional interaction with the PKA/cAMP signal transduction module. The cooperative action of FOXO transcription factors in endometrial decidualisation is not only restricted to C/EBPβ. The homeobox (HOX) protein HOXA10 also follows similar patterns of expression to FOXO1 during different stages of the baboon menstrual cycle and pregnancy (Kim *et al.,* 2003). HOX proteins are developmentally regulated transcription factors that are important for spatial identity and differentiation of tissues in the developing embryo. HOXA10 null mutant mice exhibit infertility due to compromised endometrial decidualisation during blastocyst implantation (Benson *et al.,* 1996). Kim *et al.,* were able to demonstrate a direct *in vitro* association between HOXA10

and FOXO1 as well as cooperative transactivation of the insulin-like growth factor binding protein-1 (IGFBP-1) promoter (Kim *et al.,* 2003). During pregnancy IGFBP-1 is expressed in decidualised stromal cells where it is thought to play a role during blastocyst implantation (Giudice *et al.,* 1993). In a human fibroblast cell line (HuF) FOXO1 was found to cooperatively regulate IGFBP-1 expression together with another HOX protein, HOXA5 (Foucher *et al.,* 2002). However in the same study it was demonstrated that in the HepG2 cell line, HOXA5 actually represses FOXO1-induced IGFBP-1 transcription. Interestingly, these observations suggests that association between FOXO proteins and other transcription factors will have cell context dependent effects.

FOXO1 has also recently been demonstrated to link insulin signalling to another C/EBP family member, C/EBPα, and can thereby regulate gluconeogenesis in the liver (Sekine *et al.,* 2007). During mammalian development the liver progresses from a major site of hematopoiesis in the foetus to a central metabolic tissue in the adult. Newborns have to rapidly cope with the loss of maternal nutrient feeding after birth and adaptation in the liver is reflected by transcriptional changes. C/EBPα is critical for regulation of glucose metabolism during this adaptation phase and genetic ablation of this transcription factor leads to low blood glucose levels and subsequent neonatal death (Wang *et al.,* 1995). While glucose metabolism in the liver is precisely controlled by insulin which represses gluconeogenesis, it has remained unclear until recently how this was linked to C/EBPα function. C/EBPα is expressed in the fetal liver and this expression does not change dramatically after birth. In contrast, FOXO1 expression is low in early fetal liver, but increases dramatically during development (Sekine *et al.,* 2007). Functional interaction between FOXO1 and C/EBPα were observed when analysing their coordinated ability to regulate the phosphoenolpyruvate carboxykinase (PEPCK) promoter, a gluconeogenic gene. Co-immunoprecipitation experiments, utilising neonatal liver, demonstrated that C/EBPα and FOXO1 physically interact. Recruitment of C/EBPα-FOXO1 complexes to the PEPCK promoter *in vivo*, was confirmed using chromatin immunoprecipitation (ChIP) assays with neonatal liver extracts. Critically, using C/EBPα (-/-) cells it was shown that FOXO1 promoter association requires C/EBPα. Since insulin treatment results in PKB-mediated FOXO phosphorylation and nuclear exclusion, this suggests a simple model whereby insulin suppresses the expression of gluconeogenic genes. Indeed suppression of PEPCK expression is observed when cultured fetal liver cells are treated with insulin (Sekine *et al.,* 2007). The synergistic activity and physical interaction between FOXO1 and C/EBPα has been further confirmed in differentiated 3T3-L1 adipocytes (Qiao and Shao 2006). FoxO1 expression is induced early during adipocyte differentiation and FoxO1 haploinsufficiency leads to significant reduction of adiponectin gene expression in adipose tissue (Nakae *et al.,* 2003). Adiponectin enhances insulin sensitivity, improves fatty acid oxidation in skeletal muscle and suppresses hepatic gluconeogenesis (Berg *et al.,* 2001). FOXO-binding sites in the adiponectin promoter were found to bind a transcriptional complex containing FOXO1 and C/EBPα (Qiao and Shao 2006). Furthermore, the association of FOXO1 and C/EBPα was found to be regulated by SIRT1 activity, an NAD+-dependent protein deacetylase that is also involved in adipogenesis (Picard *et al.,* 2004). SIRT1 deacetylates three lysine residues in the FOXO1 forkhead domain, which is the region that interacts with C/EBPα (Brunet *et al.,* 2004). This suggests the interesting possibility that the post-translational modification status of FOXOs regulates their ability to interact with other co-factors.

FOXO partners regulating muscle homeostasis

Smooth muscle cells (SMCs) are unique in that they exhibit phenotypic plasticity and can transition between a quiescent contractile phenotype and a proliferative phenotype

(Owens *et al.,* 2004). This is critical in response to vascular injury where they are induced to dedifferentiate and proliferate. The PI3-kinase signalling pathway has been demonstrated to stimulate SMC differentiation (Hayashi *et al.,* 1999). Based on this observation, Olson and co-workers investigated the role of Foxo4 on phenotypic modulation of vascular smooth muscle cells (Liu *et al.,* 2005). Several critical observations were initially made: (i) IGF-1 promotes SMC differentiation in a PI3-kinase dependent manner, (ii) ectopically expressed Foxo4 was found to inhibit SMC differentiation, and (iii) Foxo4 siRNA promotes expression of SM contractile genes. Importantly the inhibitory effect of Foxo4 on SMC differentiation was independent of DNA-binding, however Foxo4 was found to associate with promoters of SMC marker genes *in vivo*. This suggests that Foxo4 must be associating with additional transcription factors or co-factors to regulate promoter activity of these genes. Expression of the transcription factor myocardin is sufficient activate a program of SM differentiation in fibroblasts, and was identified as a direct Foxo4 binding partner through co-immunoprecipitation and GST pull-down assays. Myocardin itself associates with, and is a potent co-factor for, serum response factor (SRF) (Wang *et al.,* 2004). SRF also associates with Foxo4, and the interaction of Foxo4 with myocardin is enhanced in the presence of SRF (Liu *et al.,* 2005). Taken together this suggests that Foxo4 forms a ternary complex with myocardin and SRF. While Foxo4 was found to repress the transcriptional activity of myocardin, and this was dependent on physical association, Foxo1 and Foxo3 were unable to recapitulate these effects. In reponse to insulin or IGF-1 stimulation, SMCs adopt a differentiated phenotype while mitogenic stimulation, or injury, results in dedifferentiation and enhanced proliferation of SMCs. Foxo4 therefore represents a link between these mitogenic effects and regulation of myocardin transcriptional activity. But how precisely does Foxo4 inhibit myocardin-dependent transcription? It doesn't appear to be due to displacement of SRF, since myocardin-SRF-FOXO4 were found associated in a ternary complex. The current mechanism is unknown but it could simply involve Foxo4-mediated recruitment of conventional corepressors, such as HDACs, to target promoters. Since Foxo4 activation has been reported to result in cell-cycle arrest (Medema *et al.,* 2000), it is perhaps rather surprising that nuclear Foxo4 is associated with SMC proliferation. A possible explanation is that the unique transcriptional program initiated by Foxo4-myocardin overrides any direct effects modulated by Foxo4 itself. In contrast, Foxo1 and Foxo3, which do not bind myocardin, may have an anti-proliferative role in SMCs. This is supported by the finding that overexpression of Foxo3a in SMCs of rat carotoid artery results in smooth muscle cell-cycle arrest (Park *et al.,* 2005).

It is not only in SMCs where FOXO transcription play a role in regulating myogenesis, it has recently been demonstrated that Foxo1 can also regulate myogenic differentiation in skeletal muscle (Kitamura *et al.,* 2007c). The Notch pathway plays a critical role in muscle differentiation during embryogenesis (Luo *et al.,* 2005). After ligand-induced cleavage, the intracellular domain of the Notch receptor translocates to the nucleus where it interacts with the DNA-binding protein Cs1 to generate an active transcriptional complex. Accili and colleagues made the connection that Foxo gain-of-function has similar effects on myoblast differentiation as Notch1 activation, while Foxo1 ablation in mice has a similar phenotype to Notch1 (-/-) animals (Hosaka *et al.,* 2004; Krebs *et al.,* 2000). In C2C12 cells growth factor withdrawal results in myogenic conversion, and ectopic expression of a constitutively active Foxo1 mutant blocked this effect in a DNA-binding independent manner (Kitamura *et al.,* 2007). Constitutively active Notch1 had identical effects in blocking myoblast differentiation, and Foxo siRNA rescued the inhibition. These data indicate that Foxo1 and Notch1 signalling are functionally connected. Demonstration that Foxo1 directly interacts with Cs1 using *in vitro* association assays, co-immunoprecipitation and ChIP provided a molecular mechanism by which Foxo1 could modulate Notch1 signalling. Interaction of

Foxo1 and Cs1 was required for Notch1-mediated induction of the transcriptional target *Hes1*, and this is was independently of Foxo1 transcriptional function. Instead, it appears that Foxo1 acts to aid displacement of Cs1-associated co-repressors (NcoR/Smrt) allowing association of co-activators (Maml1). These findings provide a molecular mechanism by which two distinct signalling modules, PI3K and Notch, can co-ordinately and synergistically regulate muscle differentiation. The ability of Notch/Foxo1 to functionally interact may allow the integration of diverse environmental cues (through Notch) and metabolic cues (through Foxo1) to regulate progenitor cell maintenance and differentiation in multiple cellular contexts. Acilli *et al* suggest that this might allow committed progenitor cells to avoid differentiation in response to developmental cues when Foxo1 is active, for example in the absence of growth factors.

FOXOs, steroid hormone receptors and cancer

FOXO proteins haven been shown to interact with multiple members of the nuclear hormone receptor (NHR) family, leading to changes in the transcriptional activity of both proteins (Fig. 1). NHRs have a modular structure with two domains that can act independently: a ligand binding domain, a central hinge region and a DNA binding domain. Binding of the cognate ligand induces conformational changes leading to dimerisation, recruitment of coactivator complexes and binding to hormone response elements located in target genes (Biggins and Koh 2007; Pardee *et al.,* 2004).

The association of FOXOs with steroid receptors has been shown to either inhibit or enhance their transcriptional activity. These interactions could potentially play a role in the development of steroid-dependent cancers, such as prostate cancer, breast cancer and ovarian cancer. The first hint of a functional link between FOXOs and steroid hormone receptors came from the observation that androgen protects prostate cancer cells from PTEN-induced apoptosis (Li *et al.,* 2001). The androgen receptor (AR) belongs to the subfamily of steroid receptors and its ligands include testosterone and 5α-dihydrotesterone (5α-DHT). AR-dependent gene expression in androgen target tissues, including prostate, skeletal muscle, liver and central nervous system is responsible for male sexual differentiation and male pubertal changes (Gao *et al.,* 2005). In addition, functional androgen receptor signalling is necessary for the development and maintenance of prostate cancer and antagonists are currently used for therapy (Gao *et al.,* 2005). The ability of androgens to inhibit apoptosis in both normal and malignant prostatic cells has been well documented. However, the underlying molecular mechanisms are understood poorly. Li *et al.,* observed that inhibition of PI3-kinase was able to inhibit the transcriptional activity of AR resulting in decreased androgen-induced proliferation (Li *et al.,* 2001). FOXO1 was found to directly associate with the androgen receptor, and thereby inhibit its transcriptional activity (Fan *et al.,* 2007; Li *et al.,* 2003). Utilising transcription reporter assays it was shown that AR, in a ligand-dependent manner, could also reciprocally inhibit both FOXO1 and FOXO3 activity. This effect is not due to altered FOXO phosphorylation status since a FOXO1 null phosphorylation mutant was still potently inhibited by AR (Li *et al.,* 2003). Interaction with AR decreased FOXO1 DNA binding and importantly rescued prostate cancer cells from FOXO1-induced cell death. Alternative mechanisms might also be relevant since it was also observed that addition of androgens can lead to proteolysis of FOXO1 by acidic cysteine proteases (Huang *et al.,* 2004). The importance of the FOXO1-AR interaction is strengthened by the observation that expression of FOXO1 in prostate cancer cells is reduced when compared to normal prostates (Dong *et al.,* 2006). Hemizygous deletion of *FOXO1A* was detected in 31% of primary prostate cancers, while ectopic expression of FOXO1 in two prostate cancer cell lines inhibited their proliferation. These results suggest that FOXO1 can be considered as a tumor suppressor in prostate

cancer. It is likely that in the prostate a tight balance between androgen receptor signalling and FOXO signalling ensures an equilibrium between cell proliferation and death. FOXO1 inactivation will lead to enhanced androgen receptor activities and the development and progression of prostate cancer. These effects are not unique to FOXO1 since FOXO3 is also expressed in prostate tissue and it has been reported that binding of the androgen receptor to FOXO3 can inhibit its transcriptional activity (Li *et al.,* 2003; Li *et al.,* 2007).
A second steroid receptor implicated in the development and maintenance of cancer cells is the estrogen receptor (ER). This receptor is mainly expressed in mammary gland tissue, ovarian tissue and the uterus. Binding of estrogen leads to homodimerisation and

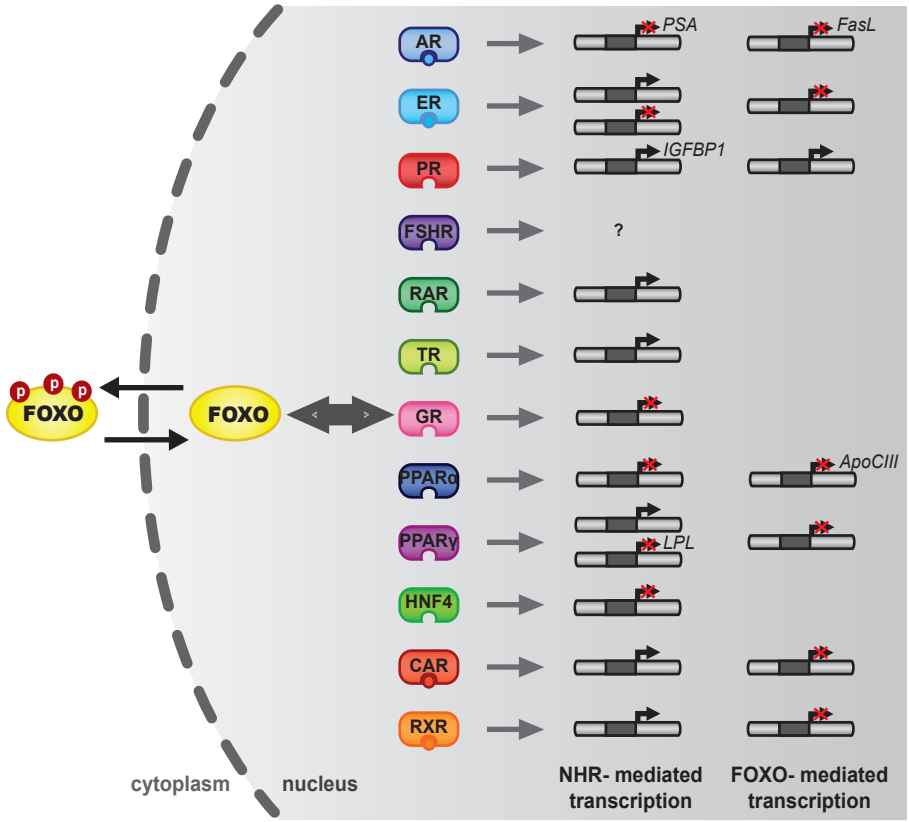

Figure 1. FOXO interaction partners: the Nuclear Hormone Receptors
FOXOs have been shown to interact with a large number of nuclear hormone receptors, resulting in changes in transcriptional activity of both proteins. FOXO interacts in a ligand-dependent manner with the androgen receptor (AR), the estrogen receptor (ER), constitutive androstane receptor (CAR) and pregnane X receptor (PXR). Interaction with the progesterone receptor (PR), the follicle stimulating hormone receptor (FSHR), the thyroid hormone receptor (TR), the retinoid acid receptor (RAR), the glucocorticoid receptor (GR), peroxisome proliferator-activated receptor α (PPARα), peroxisome proliferator-activated receptor γ (PPARγ) and hepatic nuclear factor-4 (HNF4) is independent of the presence of a NHR ligand. In most cases phosphorylation of FOXOs leads to a disruption of the complex. Increased transcriptional activity is indicated by an arrow, while a red cross indicates decreased activity of either FOXO or the associated NHR. The affected target genes which have been described are indicated in italic.

transcription of estrogen-responsive genes, which stimulate cell proliferation, invasion, metastasis and angiogenesis while they inhibit apoptosis (Deroo and Korach 2006). The receptor is frequently overexpressed in breast cancer cells and the cumulative exposure of breast epithelium to estrogen has been associated with the development of breast cancer. Ablation of the ERα gene delays the onset of tumour development in mouse models, indicating that estrogen receptor-mediated signalling indeed plays an important role breast cancer (Bocchinfuso and Korach 1997). FOXO1 was found to interact with ERα in a ligand-dependent manner, however there are conflicting reports as to the effect of this interaction on ER transcriptional activity (Schuur *et al.,* 2001; Zhao *et al.,* 2001). Schuur *et al.,* have also reported that ERα reciprocally repressed FOXO1-mediated promoter transactivation, while cell cycle arrest induced by FOXO1 in MCF7 cells was abrogated by estradiol (Schuur *et al.,* 2001). The physiological relevance of this interaction was tested in estrogen-dependent human breast cancer cells, where overexpression of FOXO1 inhibits proliferation (Zhao *et al.,* 2001). However, it remains unclear whether the FOXO1-mediated inhibition of proliferation is specifically dependent on its interaction with ERα.

It has been suggested that FOXOs may interact with NHRs through a LxxLL motif located C-terminal of the Forkhead DNA-binding domain (Zhao *et al.,* 2001). The LxxLL motif is present in critical co-activators and co-repressors that interact with NHRs, for example the histone acetyl transferase p300 (Plevin *et al.,* 2005). All four FOXO family members contain a LxxLL motif but it is not present in the *D. melangaster* dFOXO protein, or in the *C. elegans* homologue DAF16 (Table 2). Regions flanking the LxxLL motif differ between FOXOs and might play a role in NHR selectivity.

These findings provide an important link between cell surface signaling mechanisms that act through the PI3-kinase pathway and nuclear hormone receptors. Furthermore, they provide an alternative mechanism of steroid hormone action in responsive cells. It suggests that one of the oncogenic properties of steroid hormones might result from inhibition of FOXO activity, and supports a role for FOXO transcription factors as tumour suppressors.

Regulation of glucose and fatty acid metabolism by FOXO-interactions

Hepatic gluconeogenesis is a requirement for survival during prolonged fasting or starvation but is inappropriately activated in diabetes mellitus. Glucocorticoids and glucagon have potent gluconeogenic actions in the liver, while insulin suppresses this. FOXO1 is the most abundant FOXO isoform in insulin-responsive tissue, and negatively regulates insulin insensistivity in liver, adipose tissue and pancreas, by mediating insulin-induced changes in gluneogenic enzymes (Barthel *et al.,* 2005; Nakae *et al.,* 2001). It has been demonstrated that FOXO1 haploinsufficiency restores insulin insensitivity and rescues diabetic phenotype in insulin resistant mice by reducing expression of gluconeogenic enzymes in the liver (Nakae *et al.,* 2002). In contrast, targeting a gain-of function FOXO1 mutant to liver and pancreas results in promotion of diabetes. These effects of FOXO1 on insulin sensitivity can in part be explained by the observations that FOXOs can interact with peroxisome proliferator-activated receptors (PPARs). This group of nuclear receptors

FOXO1	APGL***LKELL***TSDS--PPHNDIMT
FOXO3	GNQT***LQDLL***TSDS-LSHSDVMMT
FOXO4	SSGA***LEALL***TSDTPPPPADVLMT
FOXO6	PPGA***LPALL***PPP-PPAP------
dFOXO	TTTMSPAYPNSEPSSDSLNTYSN
DAF16	QIKQESKPIKTEPIAPPPSYHEL
FOXA1	GPGALASVPPAS----------

Table 2. LxxLL motif in FOXOs
All mammalian FOXO isoforms contain an LxxLL motif that is postulated to interact with nuclear hormone receptors. This sequence is absent in non-vertebrate FOXOs and other Forkhead transcription factors (Zhao *et al,* 2001).

is involved in nutrient sensing and regulation of carbohydrate and lipid metabolism. For example, PPARγ can increase insulin sensitivity by regulating adipocytes hormones and cytokines (Fievet *et al.,* 2006).
Differentiated adipocytes secrete a variety of cytokines that affect adiposity and insulin resistance. It has been suggested that insulin resistance in adipocytes is the first metabolic manifestation leading to development of type 2 diabetes (Pilch and Bergenhem 2006). Foxo1 expression is induced in early stages of adipocyte differentiation, but activation is delayed until the end of the clonal expansion phase (Nakae *et al.,* 2003). Expressing an active Foxo1 mutant in preadipocytes inhibits differentiation, while an inhibitory Foxo1 mutant is able to restore differentiation in fibroblasts from insulin receptor deficient mice. PPARγ is an important regular of adipocyte differentiation and the observation that FOXO1 binding to PPARγ anatgonized PPARγ function could explain the FOXO1-mediated differentiation block. One proposed mechanism by which FOXO1 could inhibit PPARγ function is through disrupting formation of a PPARγ/RXR complex resulting in loss of DNA binding (Dowell *et al.,* 2003). Reducing transcription of the glucose reporter GLUT4 by PPARγ can lead to a decrease in insulin sensitivity in adipocytes (Armoni *et al.,* 2003). Thus in addition to inhibiting differentiation, FOXO1 activation leads to upregulated GLUT4 levels and a further increase in cellular insulin sensitivity (Armoni *et al.,* 2006; Armoni *et al.,* 2007). Evidence also suggests that FOXO1 might function as a co-activator of PPARα in myocytes. FOXO1 was found to enhance expression of LPL (lipoprotein lipase), a PPARα target gene, in a myocyte cell line (Kamei *et al.,* 2003). LPL plays a role in lipid usage in muscle cells by hydrolyzing plasma triglycerides into fatty acids, and is upregulated during fasting, exercise and diabetes. FOXO1-induced LPL levels increased even further in the presence of PPARα ligand, however whether this was due to a direct interaction between PPARα and FOXO1 needs to be determined (Kamei *et al.,* 2003). Adding further complexity, chromatin immunoprecipations have revealed that PPARα can inhibit FOXO1 transcriptional activity by decreasing the DNA binding capacity (Qu *et al.,* 2007).

PPARγ co-activaor-1 (PGC-1α) interacts with several transcription factors and plays important roles in regulation of mitochondrial biogenesis, respiration, thermogenesis and hepatic gluconeogenesis (Finck and Kelly 2006). Spiegelman *et al.,* have shown that the binding of PGC-1α results in co-activation of Foxo1 (Puigserver *et al.,* 2003). Furthermore expression of an inhibitory mutant of Foxo1 *in vivo* revealed that Foxo1 is required for the PGC-1α induced increase in glucose-6-phosphatase (G6Pase) levels in murine liver cells. Increased expression of G6pase contributes to the increasing production of glucose by the liver that occurs in individuals with diabetes. The authors propose a model in which the direct interaction of PGC-1α with Foxo1 leads to increased binding of Foxo1 to the promoter of G6Pase (Puigserver *et al.,* 2003). However a recent report has suggested that the synergism between PGC1α and Foxo1 is not the consequence of a direct Foxo1 PGC-1α interaction, but rather results from the presence of both Foxo1 and nuclear receptor binding sites in the G6Pase promoter (Schilling *et al.,* 2006). In this experimental setup, mutation of FOXO binding-sites did not decrease the ability of PGC1α to increase G6Pase expression, while mutating the nuclear receptor binding site did (Schilling *et al.,* 2006). However while these *in vitro* experiments show that the synergism between Foxo1 and PGC-1α can result from the presence of multiple binding sites in the promoter, they do not exclude effects of Foxo1 on PGC-1α activity. In conclusion the interplay between PGC-1α and Foxo1 plays an important role in regulating the transcription of genes involved in gluconeogenesis. Whether Foxo1 might function as a true PGC-1α co-activator, thereby explaining the negative effect on G6Pase expression in mice expressing an inhibitory Foxo1 mutant, requires further research.

In muscle, maintaining size and fiber composition requires contractile activity. This in turn stimulates the expression of PGC-1α which promotes fiber-switching from glycolytic toward more oxidative fibers. Upon fasting, as well as in many systemic diseases, muscles undergo atrophy and FOXO proteins have been implicated in this loss of muscle mass (Sandri *et al.,* 2004). In contrast to liver, it has been reported that in skeletal muscle that PGC-1α expression inhibits Foxo3-dependent transcription (Sandri *et al.,* 2006). Transgenic expression of PGC-1α alters the expression of key atrophy-specific genes as well as reducing the ability of Foxo3 to cause muscle atrophy. However it remains inconclusive whether this effect is due to a direct interaction between Foxo3 and PGC-1α.

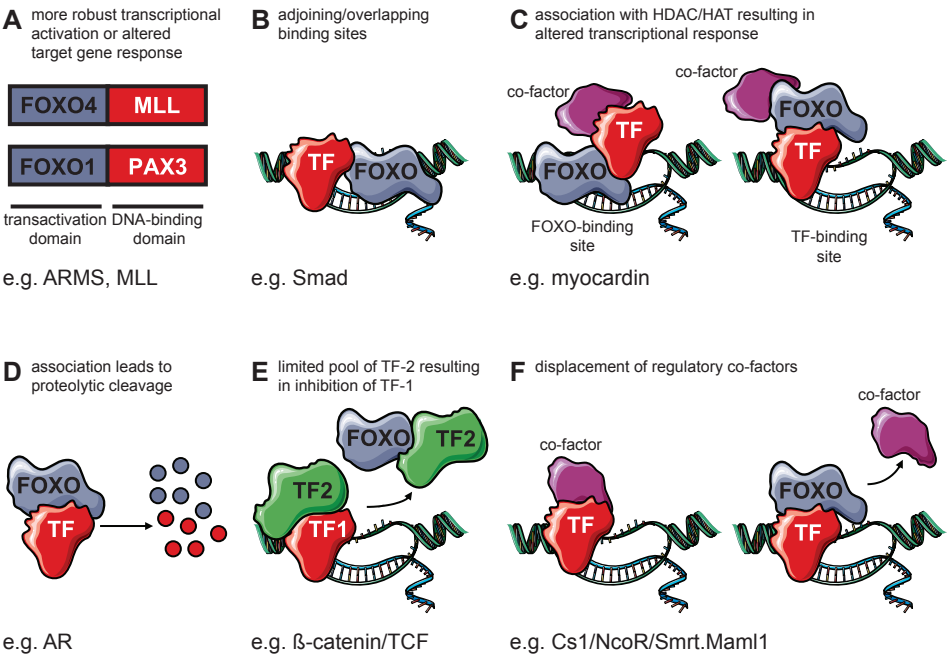

Figure 2. Mechanisms of altered transcriptional regulation through FOXO-interactions. Interaction of FOXO proteins with diverse transcription factor families or co-factors can lead to altered transcriptional responses through a variety of mechanisms. **(A)** *Fusion proteins.* Chromosomal translocations in mixed lineage leukaemia (MLL) or alveolar rhabdomyosarcoma result in the the generation of FOXO fusion proteins. These are thought to have both more robust and altered transcriptional responses resulting in oncogenesis. **(B)** *Transcriptional synergy.* Often FOXO binding elements are found adjoining or overlapping with other those of other transcription factors. Association between these proteins can often result in enhanced transcriptional responses. **(C)** *Recruitment of conventional co-factors.* The recruitment of histone acetylase transferases (HATs) or histone deacetylases (HDACs) to promoters through association with transcription factors can lead to activation or suppression of transcription. FOXO-transcription factor associations can result in altered co-factor distribution to target promoters. **(D)** *Proteolytic degradation.* Association of FOXO proteins may lead to increased proteolytic degradation of FOXOs or associating transcription factors. **(E)** *Transcription factor sequestration.* Transcription factors often form heterodimeric complexes when binding DNA. When one of these components is a limiting factor and also binds FOXOs, it may result in inhibition of transcription. **(F)** *Displacement of regulatory co-factors.* Association of transcription factors with co-activators or co-repressors modulates transcription. Displacement of these complexes by FOXO binding will result in altered transcriptional responses.

The regulatory role of FOXO1 in inhibiting insulin sensitivity in diabetic mice, makes it a promising target for therapeutic intervention. Indeed in support of this, targeted reduction of Foxo1 levels by antisense oligonucleotides decreased expression of G6Pase, lowered plasma glucose concentration and improved insulin sensitivity in diabetic mice (Samuel *et al.,* 2006).

CONCLUDING REMARKS

FOXO transcription factors have a similar, if not identical, DNA-binding domain, however ablation of Foxo1, Foxo3 and Foxo4 in mice has overlapping but distinct effects (Hosaka *et al.,* 2004). As previously discussed, studies by Sellers and colleagues demonstrated that FOXO proteins can induce transcriptional responses independently of DNA-binding (Ramaswamy *et al.,* 2002). The studies highlighted in this review demonstrate that direct association of FOXO proteins with diverse transcription factor families can mediate the regulation of a plethora of cellular processes independently of FOXO DNA-binding (Fig. 2). Furthermore, it suggests a mechanism by which specific FOXO isoforms can uniquely regulate transcriptional programs. For example, the ability of Foxo4 to repress myocardin-mediated transcription is not recapitulated by Foxo1 or Foxo3 (Liu *et al.,* 2005). Adding complexity to this, cell context-specific effects have also been observed. For example HOXA5 can represses FOXO-induced IGFBP-1 transcription in liver cells but cooperatively activates transcription in fibroblasts (Foucher *et al.,* 2002). Since FOXO proteins are exquisitely regulated by a variety of post-translational modifications, modulation of these events also allows a further level of control modulating FOXO transcriptional targets.

It is likely that we have only just started to uncover the full complement of FOXO transcriptional targets and the possibilities of therapeutically modulating FOXO function in disease has only recently been investigated. Total ablation of FOXO activity might have detrimental consequences since FOXOs can be considered to be tumour suppressors (Paik *et al.,* 2007). The ability to design pharmacological compounds that subtly manipulate FOXO-interactions with other transcription factors might prove to have beneficial therapeutic effects for treatment of a wide variety of diseases.

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REFERENCES

Almeida M, Han L, Martin-Millan M, O'Brien CA and Manolagas SC. (2007). *J Biol Chem,* 282, 27298-27305.

Arce L, Yokoyama NN and Waterman ML. (2006). *Oncogene,* 25, 7492-7504.

Armoni M, Harel C, Karni S, Chen H, Bar-Yoseph F, Ver MR, Quon MJ and Karnieli E. (2006). *J Biol Chem,* 281, 19881-19891.

Armoni M, Harel C and Karnieli E. (2007). *Trends Endocrinol Metab,* 18, 100-107.

Armoni M, Kritz N, Harel C, Bar-Yoseph F, Chen H, Quon MJ and Karnieli E. (2003). *J Biol Chem,* 278, 30614-30623.

Barr FG. (2001). *Oncogene,* 20, 5736-5746.

Barthel A, Schmoll D and Unterman TG. (2005). *Trends Endocrinol Metab,* 16, 183-189.

Benson GV, Lim H, Paria BC, Satokata I, Dey SK and Maas RL. (1996). *Development,* 122, 2687-2696.

Berg AH, Combs TP, Du X, Brownlee M and Scherer PE. (2001). *Nat Med,* 7, 947-953.

Biggins JB and Koh JT. (2007). *Curr Opin Chem Biol,* 11, 99-110.

Birkenkamp KU and Coffer PJ. (2003). *J Immunol,* 171, 1623-1629.

Bocchinfuso WP and Korach KS. (1997). *J Mammary Gland Biol Neoplasia,* 2, 323-334.

Brar AK, Frank GR, Kessler CA, Cedars MI and Handwerger S. (1997). *Endocrine,* 6, 301-307.

Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY, Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW and Greenberg ME. (2004). *Science,* 303, 2011-2015.

Buzzio OL, Lu Z, Miller CD, Unterman TG and Kim JJ. (2006). *Endocrinology,* 147, 3870-3876.

Christian M, Zhang X, Schneider-Merck T, Unterman TG, Gellersen B, White JO and Brosens JJ. (2002). *J Biol Chem,* 277, 20825-20832.

Deroo BJ and Korach KS. (2006). *J Clin Invest,* 116, 561-570.

Dong XY, Chen C, Sun X, Guo P, Vessella RL, Wang RX, Chung LW, Zhou W and Dong JT. (2006). *Cancer Res,* 66, 6998-7006.

Dowell P, Otto TC, Adi S and Lane MD. (2003). *J Biol Chem,* 278, 45485-45491.

Essers MA, de Vries-Smits LM, Barker N, Polderman PE, Burgering BM and Korswagen HC. (2005). *Science,* 308, 1181-1184.

Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruiter ND, Bos JL and Burgering BM. (2004). *EMBO J,* 23, 4802-4812.

Fan W, Yanase T, Morinaga H, Okabe T, Nomura M, Daitoku H, Fukamizu A, Kato S, Takayanagi R and Nawata H. (2007). *J Biol Chem,* 282, 7329-7338.

Fievet C, Fruchart JC and Staels B. (2006). *Curr Opin Pharmacol,* 6, 606-614.

Finck BN and Kelly DP. (2006). *J Clin Invest,* 116, 615-622.

Foucher I, Volovitch M, Frain M, Kim JJ, Souberbielle JC, Gan L, Unterman TG, Prochiantz A and Trembleau A. (2002). *Development,* 129, 4065-4074.

Gao W, Bohl CE and Dalton JT. (2005). *Chem Rev,* 105, 3352-3370.

Giudice LC, Dsupin BA, Jin IH, Vu TH and Hoffman AR. (1993). *J Clin Endocrinol Metab,* 76, 1115-1122.

Glass DA and Karsenty G. (2007). *Endocrinology,* 148, 2630-2634.

Gomis RR, Alarcon C, He W, Wang Q, Seoane J, Lash A and Massague J. (2006a). *Proc Natl Acad Sci U S A,* 103, 12747-12752.

Gomis RR, Alarcon C, Nadal C, Van PC and Massague J. (2006b). *Cancer Cell,* 10, 203-214.

Gottlieb S and Ruvkun G. (1994). *Genetics,* 137, 107-120.

Grinius L, Kessler C, Schroeder J and Handwerger S. (2006). *J Endocrinol,* 189, 179-187.

Hanashima C, Li SC, Shen L, Lai E and Fishell G. (2004). *Science,* 303, 56-59.

Hayashi K, Takahashi M, Kimura K, Nishida W, Saga H and Sobue K. (1999). *J Cell Biol,* 145, 727-740.

Hirota K, Daitoku H, Matsuzaki H, Araya N, Yamagata K, Asada S, Sugaya T and Fukamizu A. (2003). *J Biol Chem,* 278, 13056-13060.

Hosaka T, Biggs WH, III, Tieu D, Boyer AD, Varki NM, Cavenee WK and Arden KC. (2004). *Proc Natl Acad Sci U S A,* 101, 2975-2980.

Huang H, Muddiman DC and Tindall DJ. (2004). *J Biol Chem,* 279, 13866-13877.

Kamei Y, Mizukami J, Miura S, Suzuki M, Takahashi N, Kawada T, Taniguchi T and Ezaki O. (2003). *FEBS Lett,* 536, 232-236.

Kim JJ, Taylor HS, Akbas GE, Foucher I, Trembleau A, Jaffe RC, Fazleabas AT and Unterman TG. (2003). *Biol Reprod,* 68, 24-30.

Kim JJ, Buzzio OL, Li S and Lu Z. (2005). *Biol Reprod,* 73, 833-839.

Kitamura T, Kitamura YI, Funahashi Y, Shawber CJ, Castrillon DH, Kollipara R, DePinho RA, Kitajewski J and Accili D. (2007). *J Clin Invest,* 117, 2477-2485.

Kodama S, Koike C, Negishi M and Yamamoto Y. (2004). *Mol Cell Biol,* 24, 7931-7940.

Kortylewski M, Feld F, Kruger KD, Bahrenberg G, Roth RA, Joost HG, Heinrich PC, Behrmann I and Barthel A. (2003). *J Biol Chem,* 278, 5242-5249.

Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, Gallahan D, Closson V, Kitajewski J, Callahan R, Smith GH, Stark KL and Gridley T. (2000). *Genes Dev,* 14, 1343-1352.

Larsen PL, Albert PS and Riddle DL. (1995). *Genetics,* 139, 1567-1583.

Li P, Nicosia SV and Bai W. (2001). *J Biol Chem,* 276, 20444-20450.

Li P, Lee H, Guo S, Unterman TG, Jenster G and Bai W. (2003). *Mol Cell Biol,* 23, 104-118.

Li Y, Wang Z, Kong D, Murthy S, Dou QP, Sheng S, Reddy GP and Sarkar FH. (2007). *J Biol Chem,* 282, 21542-21550.

Liu ZP, Wang Z, Yanagisawa H and Olson EN. (2005). *Dev Cell,* 9, 261-270.

Luo D, Renault VM and Rando TA. (2005). *Semin Cell Dev Biol,* 16, 612-622.

Medema RH, Kops GJ, Bos JL and Burgering BM. (2000). *Nature,* 404, 782-787.

Nakae J, Kitamura T, Silver DL and Accili D. (2001). *J Clin Invest,* 108, 1359-1367.

Nakae J, Biggs WH, III, Kitamura T, Cavenee WK, Wright CV, Arden KC and Accili D. (2002). *Nat Genet,* 32, 245-253.

Nakae J, Kitamura T, Kitamura Y, Biggs WH, III, Arden KC and Accili D. (2003). *Dev Cell,* 4, 119-129.

Nechamen CA, Thomas RM and Dias JA. (2007). *Mol Cell Endocrinol,* 260-262, 93-99.

Nerlov C. (2007). *Trends Cell Biol,* 17, 318-324.

Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, Tissenbaum HA and Ruvkun G. (1997). *Nature,* 389, 994-999.

Owens GK, Kumar MS and Wamhoff BR. (2004). *Physiol Rev,* 84, 767-801.

Paik JH, Kollipara R, Chu G, Ji H, Xiao Y, Ding Z, Miao L, Tothova Z, Horner JW, Carrasco DR, Jiang S, Gilliland DG, Chin L, Wong WH, Castrillon DH and DePinho RA. (2007). *Cell,* 128, 309-323.

Pardee K, Reinking J and Krause H. (2004). *Sci Aging Knowledge Environ,* 2004, re8.

Park KW, Kim DH, You HJ, Sir JJ, Jeon SI, Youn SW, Yang HM, Skurk C, Park YB, Walsh K and Kim HS. (2005). *Arterioscler Thromb Vasc Biol,* 25, 742-747.

Patterson GI, Koweek A, Wong A, Liu Y and Ruvkun G. (1997). *Genes Dev,* 11, 2679-2690.

Picard F, Kurtev M, Chung N, Topark-Ngarm A, Senawong T, hado De OR, Leid M, McBurney MW and Guarente L. (2004). *Nature,* 429, 771-776.

Pilch PF and Bergenhem N. (2006). *Mol Pharmacol,* 70, 779-785.

Plevin MJ, Mills MM and Ikura M. (2005). *Trends Biochem Sci,* 30, 66-69.

Pohnke Y, Kempf R and Gellersen B. (1999). *J Biol Chem,* 274, 24808-24818.

Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D and Spiegelman BM. (2003). *Nature,* 423, 550-555.

Qiao L and Shao J. (2006). *J Biol Chem,* 281, 39915-39924.

Qu S, Su D, Altomonte J, Kamagate A, He J, Perdomo G, Tse T, Jiang Y and Dong HH. (2007). *Am J Physiol Endocrinol Metab,* 292, E421-E434.

Ramaswamy S, Nakamura N, Sansal I, Bergeron L and Sellers WR. (2002). *Cancer Cell,* 2, 81-91.

Ren P, Lim CS, Johnsen R, Albert PS, Pilgrim D and Riddle DL. (1996). *Science,* 274, 1389-1391.

Rudd MD, Gonzalez-Robayna I, Hernandez-Gonzalez I, Weigel NL, Bingman WE, III and Richards JS. (2007). *J Mol Endocrinol,* 38, 673-690.

Samuel VT, Choi CS, Phillips TG, Romanelli AJ, Geisler JG, Bhanot S, McKay R, Monia B, Shutter JR, Lindberg RA, Shulman GI and Veniant MM. (2006). *Diabetes,* 55, 2042-2050.

Sandri M, Lin J, Handschin C, Yang W, Arany ZP, Lecker SH, Goldberg AL and Spiegelman BM. (2006). *Proc Natl Acad Sci U S A,* 103, 16260-16265.

Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH and Goldberg AL. (2004). *Cell,* 117, 399-412.

Schilling MM, Oeser JK, Boustead JN, Flemming BP and O'Brien RM. (2006). *Nature,* 443, E10-E11.

Schuur ER, Loktev AV, Sharma M, Sun Z, Roth RA and Weigel RJ. (2001). *J Biol Chem,* 276, 33554-33560.

Sekine K, Chen YR, Kojima N, Ogata K, Fukamizu A and Miyajima A. (2007). *EMBO J,* 26, 3607-3615.

Seoane J, Le HV, Shen L, Anderson SA and Massague J. (2004). *Cell,* 117, 211-223.

Shi Y and Massague J. (2003). *Cell,* 113, 685-700.

So CW and Cleary ML. (2003). *Blood,* 101, 633-639.

van der Horst A and Burgering BM. (2007). *Nat Rev Mol Cell Biol,* 8, 440-450.

Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR and Darlington GJ. (1995). *Science,* 269, 1108-1112.

Wang Z, Wang DZ, Hockemeyer D, McAnally J, Nordheim A and Olson EN. (2004). *Nature,* 428, 185-189.

Yamamura Y, Lee WL, Inoue K, Ida H and Ito Y. (2006). *J Biol Chem,* 281, 5267-5276.

Zhao HH, Herrera RE, Coronado-Heinsohn E, Yang MC, Ludes-Meyers JH, Seybold-Tilson KJ, Nawaz Z, Yee D, Barr FG, Diab SG, Brown PH, Fuqua SA and Osborne CK. (2001). *J Biol Chem,* 276, 27907-27912.

CHAPTER 4

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

FOUR

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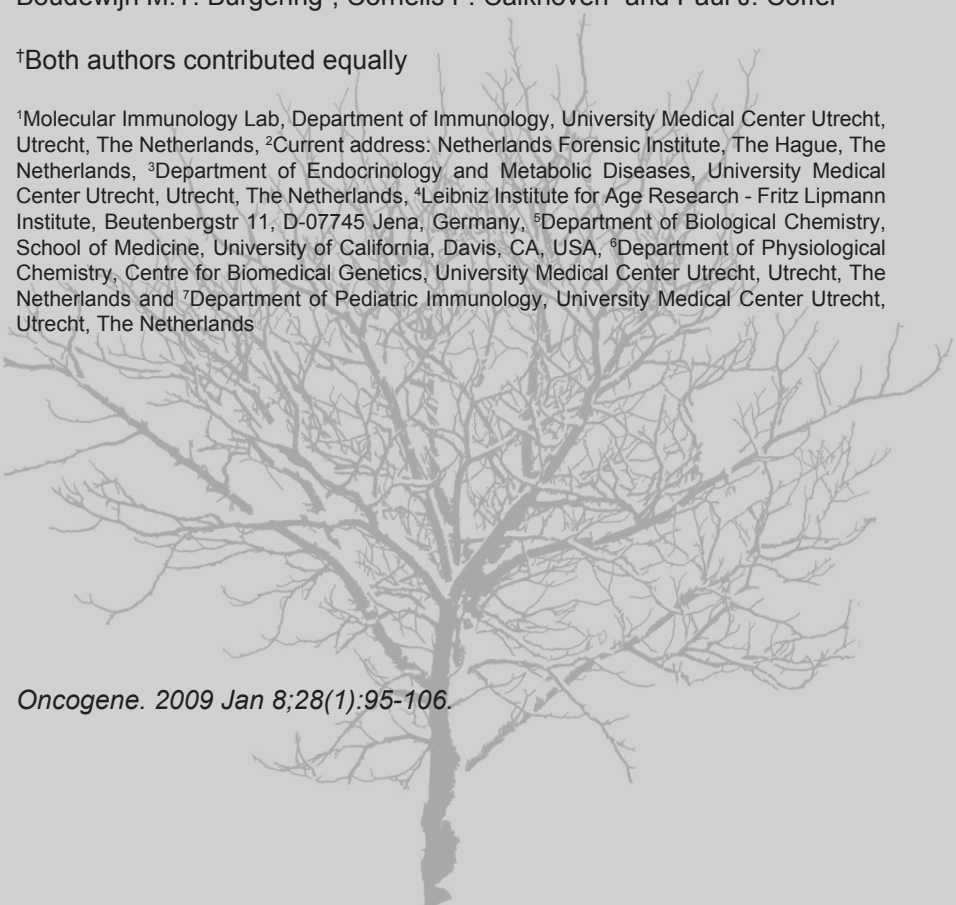

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ABSTRACT

Eukaryotic translation initiation factor 4B (eIF4B) plays a critical role during initiation of protein synthesis and its activity can be regulated by multiple phosphorylation events. In a search for novel Protein Kinase B (PKB/c-akt) substrates, we identified eIF4B as a potential target. Using an *in vitro* kinase assay we found that PKB can directly phosphorylate eIF4B on serine 422 (Ser422). Activation of a conditional PKB mutant, interleukin-3 (IL-3), or insulin stimulation resulted in PKB-dependent phosphorylation of this residue *in vivo*. This was prevented by pre-treatment of cells with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, or pharmacological inhibition of PKB. Pre-treatment of cells with rapamycin, inhibiting mTOR, or U0126 to inhibit MEK, had little effect on eIF4B Ser422 phosphorylation. In contrast, following amino acid refeeding, eIF4B Ser422 phosphorylation was found to be mTOR-dependent. We further identified eIF4B Ser406 as a novel mitogen-regulated phosphorylation site. Insulin induced phosphorylation of eIF4B Ser406 was dependent on both MEK and mTOR activity. Utilising a novel translational control luciferase assay, we could further demonstrate that phosphorylation of Ser406 or Ser422 is essential for optimal translational activity of eIF4B. These data provide novel insights into complex multi-kinase regulation of eIF4B phosphorylation and reveal an important mechanism by which PKB can regulate translation, potentially critical for the transforming capacity of this AGC kinase family member.

INTRODUCTION

Regulation of protein translation is crucial for the specific expression of proteins important for development, differentiation, cell growth and apoptosis (Holland *et al.*,2004b; Mamane *et al.*,2006). The ability of cells to regulate this process allows a rapid response to external stimuli without the necessity of mRNA synthesis, processing and transport. In transformed cells, components of the translation machinery are often deregulated or misexpressed and changes in the nucleolus; the suborganelle of the nucleus which functions as the centre of ribosome biogenesis, have long been recognized as a reliable marker of cellular transformation (Gani,1976; Pandolfi,2004).

Translational control mostly occurs at the level of initiation. The initiation phase of translation is regulated by a number of eukaryotic translation initiation factors (eIFs) (Gingras *et al.*,1999; Hershey *et al.*,2000). Initially, eIF4E binds to the cap structure at the 5' end of the mRNA. eIF4E is part of a trimeric complex, termed eIF4F, together with scaffolding protein eIF4G and ATPase/RNA helicase eIF4A. eIF4A unwinds the secondary structure of the 5'UTR allowing the 40S ribosomal subunit to bind to the mRNA. The helicase activity of eIF4A is significantly increased by the co-factor eIF4B (Lawson *et al.*,1989; Rogers, Jr. *et al.*,2001; Rozen *et al.*,1990). eIF4B itself has three functional domains, namely two mRNA binding domains (Methot *et al.*,1994; Naranda *et al.*,1994) and a DRYG domain necessary for dimerization and binding to eIF3 (Methot *et al.*,1996b). The two RNA binding domains have distinct affinities for RNA, the arginine rich motif (ARM) binds mRNA with higher affinity and is essential for RNA helicase activity (Methot *et al.*,1994). The RNA recognition motif (RRM) binds with high affinity to 18S rRNA (Methot *et al.*,1996a). Therefore, besides being a co-factor for eIF4A, eIF4B is thought to exhibit a bridge function between mRNA and rRNA (Methot *et al.*,1994).

Translational control is intimately connected to the regulation of intracellular signal transduction pathways. Phosphorylation of initiation factors provides an important means to control the rate of mRNA binding (Raught *et al.*,2000). The phosphorylation state of eIF4E, eIF4G, eIF4B and eIF3 positively correlates with both translation and growth rates of the cell. Changes in phosphorylation, and thus translation, occur in response to a wide variety of extracellular signals including, viral infection, heat-shock and in response to cellular growth factors and cytokines (Hershey *et al.*,2000; Mamane *et al.*,2006). Global changes in protein synthesis after these events are relatively small but a subgroup of mRNAs exhibits a dramatic change in their rate of translation. Rajasekhar *et al*. recently demonstrated that upon Protein Kinase B (PKB/c-akt) and RAS signalling the profile of mRNA associated to polysomes was drastically altered, although the underlying mechanism remains unclear (Rajasekhar *et al.*,2003). Interestingly, these mRNAs mainly encoded proteins involved in the regulation of growth, transcription, cell-cell interactions and morphology. Thus, by controlling translation efficiency, general stimuli, such as growth factors and cytokines, can selectively induce or suppress the translation of specific set of genes and deregulation of these cellular mechanisms controlling translation can lead to cellular transformation (Holland *et al.*,2004a).

Mammalian target of rapamycin (mTOR) plays a major role in the regulation of global and specific mRNA translation. mTOR is activated by phosphatidylinositol 3-kinase (PI3K) through PKB either by direct phosphorylation (Nave *et al.*,1999), or by phosphorylation of TSC2 which inactivates its GAP activity for the small G protein Rheb, a potent activator of mTOR (Inoki *et al.*,2002). The best-studied downstream targets of mTOR activation are those involved in translation regulation, namely p70S6kinase (p70S6K) and eIF4E-binding proteins, (4E-BPs). p70S6K phosphorylates ribosomal protein S6, whose hyperphosphorylation status correlates with translation activity. The phosphorylation of

the inhibitory 4E-BPs is required for their release of the proto-oncogene eIF4E resulting in increased cap-dependent translation (Richter *et al.*,2005; Ruggero *et al.*,2004). De-regulation of activation of the phosphatidylinositol 3-kinase (PI3K) pathway is found in a large variety of human cancers (Luo *et al.*,2003) and importantly, inhibition of translation by a specific mTOR inhibitor, rapamycin, can effectively block transformation initiated by perturbed PI3K signalling (Guertin *et al.*,2005). This indicates that PI3K/PKB/mTOR mediated regulation of translational control is crucial for maintenance of neoplasia.

eIF4B has long been known as a hyperphosphorylated protein (Duncan *et al.*,1984), and eIF4B phosphorylation is responsive to extracellular stimuli including serum, insulin and phorbol esters (Duncan *et al.*,1985). It had however remained elusive which kinase(s) are responsible for the phosphorylation of eIF4B. Recently, two reports have been published concerning the regulation of phosphorylation a specific serine residue (Ser422). Raught *et al.* (Raught *et al.*,2004) implicated p70S6K as the specific Ser422 kinase but subsequently Shahbazian and co-workers (Shahbazian *et al.*,2006) proposed that p70S6K and p90S6kinase (RSK) were both able to phosphorylate this residue. Both p70S6K and RSK are members of the AGC protein kinase family, which also contains PKB (Parker *et al.*,2001). This kinase family is defined by the high homology within their catalytic domains, resulting in similar substrate consensus sequences. The activity of these kinases, however, is differentially regulated, whereas PKB and p70S6K are components of the PI3K-mTOR pathway, RSK is activated by signalling through the small GTPase RAS.

Recent evidence that long-term rapamycin treatment can inhibit PKB activity (Sarbassov *et al.*,2006) made us re-examine the importance of mTOR signalling versus PKB signalling in the regulation of translation initiation. In this study, we show that PKB *in vitro* and *in vivo* can phosphorylate eIF4B within the RNA-binding domain at serine 422 (Ser422). We demonstrate that PKB is the dominant AGC protein kinase family member phosphorylating Ser422 upon insulin stimulation *in vivo*. We also demonstrate regulation of a novel phosphorylation site (Ser406) and show that phosphorylation of this residue is regulated by RSK and p70S6K *in vivo*. Utilising a novel reporter assay we demonstrate that mutation of these phosphorylation sites in eIF4B results in decreased translation initiation. These data provide novel insights into the complex regulation of eIF4B phosphorylation *in vivo*. In addition, we demonstrate for the first time that eIF4B phosphorylation is a novel mechanism by which PKB can regulate protein translation and may be critical for the transforming potential of this AGC kinase family member.

RESULTS

Identification of eIF4B as a PKB substrate

To identify novel PKB substrates, we made use of cytokine-dependent bone-marrow-derived Ba/F3 cells which are normally dependent on interleukin (IL-) 3 for their survival and proliferation. To specifically study the role of PKB in phosphorylation events following cellular activation by IL-3, a Ba/F3 cell line stably expressing conditionally active PKBα (myrPKB:ER) was generated, as previously described in van Gorp *et al*. (van Gorp *et al.*,2006). The activation of myrPKB:ER is, in the absence of 4-hydroxytamoxifen (4-OHT), inhibited by heat-shock and chaperone proteins that associate with the fused estrogen receptor (ER) hormone-binding domain. In the presence of 4-OHT these proteins dissociate, allowing PKB to become rapidly phosphorylated and activated.

myrPKB:ER cells were cytokine starved and the phosphorylation patterns of unstimulated cells were compared to those stimulated with 4-OHT for 15 minutes. Phosphorylated proteins were separated by phospho-Ser/Thr affinity purification, analyzed by 2D gel

electrophoreses and western blotting utilizing an antibody raised against the minimal PKB consensus phosphorylation site (RXRXXS/T) (Obata *et al.*,2000; Zhang *et al.*,2002). However since other members of the AGC kinase family have similar substrate consensus sequences, this antibody can perhaps best be viewed as a phospho-AGC kinase substrate antibody. We observed several proteins whose phosphorylation was upregulated upon

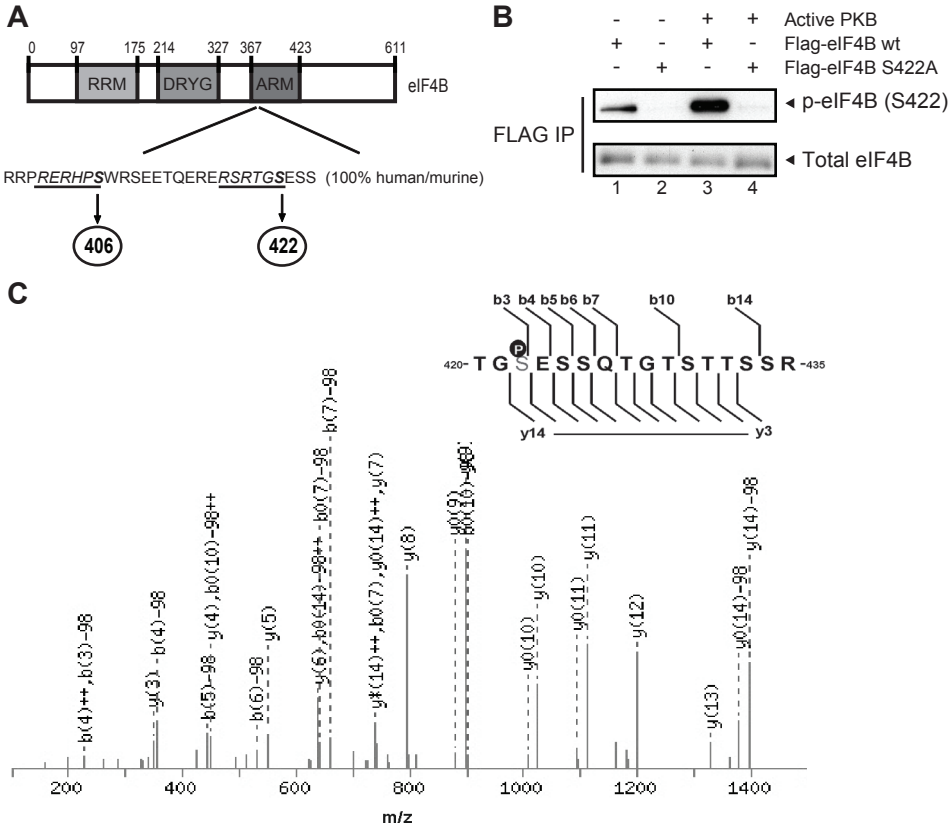

Figure 1. PKB phosphorylates eIF4B on Ser422 in vitro
(A) Schematic representation of the eIF4B protein. The three functional domains of eIF4B, the RRM, DRYG domain and the ARM are shown. Also shown are the two serines (Ser406 and Ser422, bold and italic) within their PKB phosphorylation consensus sequence (underlined) that were identified by in silico analysis by Scansite 2.0. Human and mouse eIF4B are 96% homologous. The region in which the two serines are localized is 100% conserved between these two species. **(B)** FLAG-tagged eIF4B or FLAG-tagged eIF4B in which Ser422 was mutated to alanine was phosphorylated by PKB in an in vitro kinase assay. Proteins were incubated without active PKB present as a control. Samples were analyzed for levels of phospho-eIF4B (S422) and FLAG. **(C)** Identification of the PKB phosphorylated Ser422 on eIF4B by mass spectrometry. Flag-tagged eIF4B protein was expressed in COS cells and immunoprecipitated protein was phosphorylated in vitro by PKB, separated on SDS-PAGE and trypsin digested. The resulting peptides were separated utilising TiO_2 phosphopeptide-enrichment columns and subjected to tandem mass spectrometry (LC-MS/MS). MS/MS spectrum (top) and sequence (bottom) of the Ser422 phosphorylated peptide of eIF4B (AA 420-435) as identified by MASCOT software (See experimental procedures). Identified b and y ions are indicated. The phosphorylated serine (Ser422) is indicated in grey.

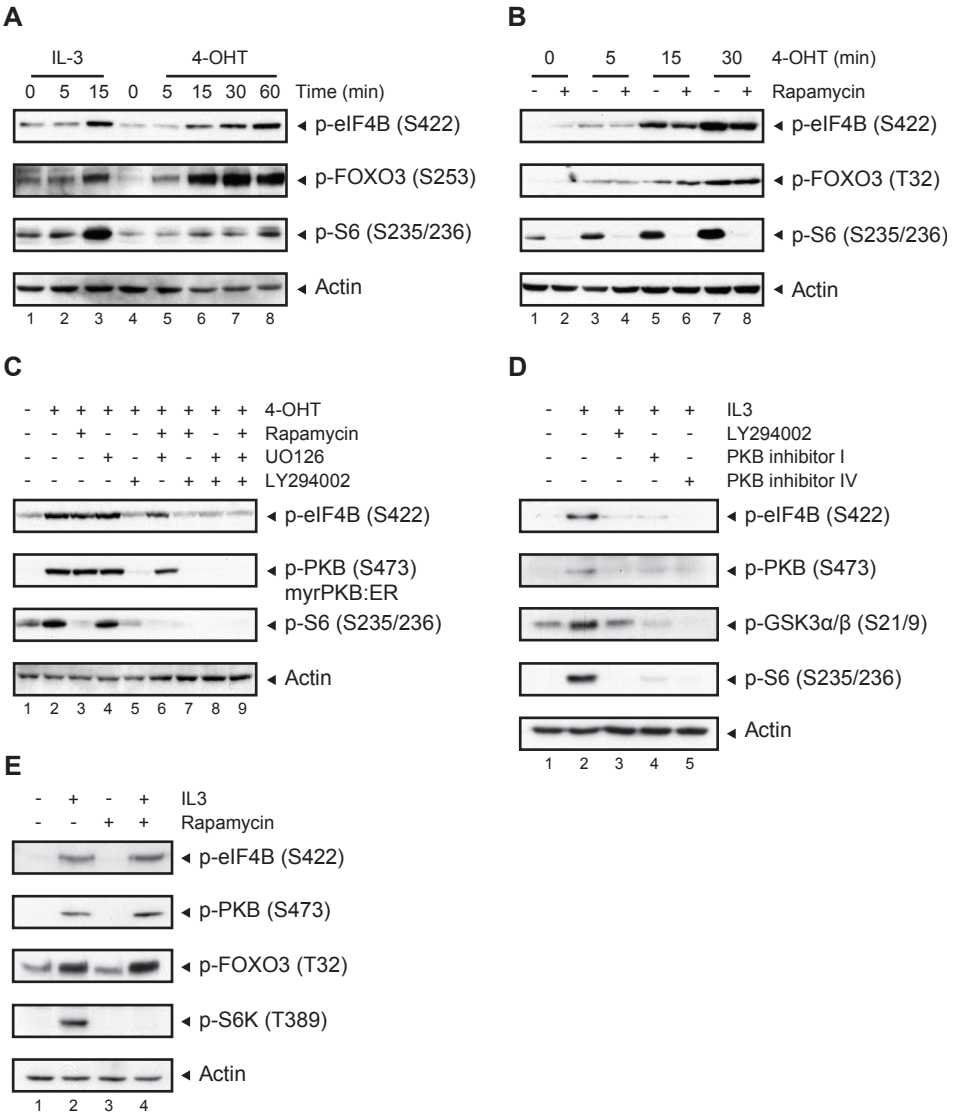

Figure 2. PKB activation sufficient and necessary for eIF4B phosphorylation of Ser422 in Ba/F3 myrPKB:ER cells

(A) Ba/F3-myrPKB:ER* cells were cytokine starved and left untreated or treated with either IL-3 (10ng/ml) or 4-OHT (100nM) for the indicated times, lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phopho-FOXO3 (S253), phospho-S6 (S235/S236) and actin. **(B)** Ba/F3-myrPKB:ER* cells were cytokine-starved and left untreated or treated with 4-OHT (100nM) for the indicated times after or without pre-treatment with rapamycin (20 ng/ml), lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phopho-FOXO3 (T32), phospho-S6 (S235/S236) and actin. **(C)** Ba/F3-myrPKB:ER* cells were cytokine-starved and left untreated or treated with 4-OHT for 15 minutes after or without pre-treatment for 2 hours with rapamycin (20 ng/ml), U0126 (15 μM), LY294002 (50 μM) or combinations of these inhibiters, lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-S6 (S235/S236) and actin. **(D)** Ba/F3-myrPKB:ER* cells were cytokine-starved and left untreated or

PKB activation. Tandem mass spectrometry identified one of these proteins as eIF4B (data not shown).

To identify potential PKB phosphorylation sites in eIF4B, we performed *in silico* analysis using Scansite 2.0 (Obenauer *et al.*,2003). A high stringency analysis of eIF4B identified two serines likely to be phosphorylated by PKB, serines 406 (Ser406) and 422 (Ser422) in the ARM region (Fig. 1A).

Due to the availability of a specific antibody raised against the phosphorylated Ser422 on eIF4B, we analyzed whether PKB activation resulted in phosphorylation of this residue. COS cells were transfected with FLAG-tagged eIF4B, or eIF4B in which Ser422 had been mutated to alanine. eIF4B was immunoprecipitated and incubated with active PKB. PKB was indeed able to directly phosphorylate eIF4B at Ser422 (Fig. 1B, compare lane 1 to 3) and mutation of this site abolished phosphorylation (Fig. 1B, lanes 2 and 4). Importantly, analysis of eIF4B phosphorylation sites after PKB-mediated *in vitro* phosphorylation by mass spectrometry unambigously demonstrated Ser422 as the primary phosphorylation site (Fig. 1C). Taken together, these data indicate that phosphorylation of Ser422 is an important phospho-acceptor site in eIF4B that can be regulated by PKB *in vitro*.

PKB phosphorylates eIF4B on Ser422 in vivo

To investigate the *in vivo* phosphorylation status of eIF4B, we again made use of the BaF3 myrPKB:ER cell line. BaF3 myrPKB:ER cells were cytokine starved overnight and stimulated with either IL-3 or 4-OHT for the times indicated. Both stimulation with IL-3 and 4-OHT induced phosphorylation of eIF4B on Ser422 as well as that of the PKB substrate forkhead transcription factor FOXO3 (Dijkers *et al.*,2002b) and the p70S6K substrate ribosomal protein S6 (Fig 2A). Since it has been previously demonstrated that phosphorylation of eIF4B on Ser422 can be mediated by p70S6K activity (Raught *et al.*,2004), we investigated whether inhibition of its upstream activator, the mTOR/Raptor complex by pre-incubation of the cells with rapamycin, could abolish phosphorylation on this site. Inhibition of the mTOR/-p70S6K pathway by rapamycin completely inhibited phosphorylation of p70S6K target S6 but not the phosphorylation of FOXO3 (Fig. 2B). eIF4B phosphorylation was only modestly reduced at later time points by pre-treating cells with rapamycin indicating that p70S6K is not responsible for PKB-mediated eIF4B Ser422 phosphorylation (Fig. 2B). Previously, Shahbazian and co-workers proposed that p70S6K and RSK can synergistically regulate eIF4B Ser422 phosphorylation in Hela cells when stimulated with serum (Shahbazian *et al.*,2006). We wished to determine whether eIF4B Ser422 phosphorylation was similarly regulated in Ba/F3 cells when PKB was specifically activated by addition of 4-OHT. Ba/F3 myrPKB:ER cells were cytokine starved overnight and the phosphorylation status of eIF4B at Ser422 was compared after PKB activation when cells were pre-incubated with either rapamycin, PI3K inhibitor LY294002, MEK inhibitor U0126 or combinations of these inhibitors. Pre-incubation with rapamycin abrogated phosphorylation of S6 but again only modestly inhibited eIF4B Ser422 phosphorylation (Fig. 2C, lane 3), whereas LY294002 completely abrogated this (Fig. 2C, lane 5). LY294002 also inhibited phosphorylation of both PKB and S6 (Fig. 2C, lane 5), indicating that the myrPKB:ER protein is still dependent on basal PI3K activity.

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treated with IL-3 (10ng/ml) for 15 minutes after or without pre-treatment with LY294002 (25μM), PKBinhibitor I (20μM) or PKBinhibitor IV (5μM), lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-GSK3α/β (S21/9), phospho-S6 (S235/S236) and actin. **(E)** Ba/F3-myrPKB:ER* cells were cytokine-starved and left untreated or treated with IL-3 (10ng/ml) for 15 minutes after or without pre-treatment with rapamycin (20 ng/ml), lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-FOXO3 (T32), phospho-S6K (T389) and actin.

Pre-treatment of cells with U0126 had no effect on the phosphorylation of eIF4B at Ser422 in response to 4-OHT (Fig. 2C, lane 4) even when combined with rapamycin (Fig. 2C, lane 6). Since LY294002 affects several PI3K effectors besides PKB we cytokine starved Ba/F3 myrPKB:ER cells overnight and analyzed IL3-induced eIF4B phosphorylation after pre-treatment with LY294002 and two specific PKB inhibitors. Incubation with either LY294002 or the PKB inhibitors completely inhibited the IL3-induced eIF4B Ser422 phosphorylation (Fig. 2D). In contrast, pre-treatment with rapamycin did not have any effect on the IL3-induced phosphorylation of eIF4B (Fig 2E). Taken together, these data indicate that PKB regulates eIF4B phosphorylation at Ser422 and this is not dependent on either p70S6K or MEK activity.

PKB-mediated phosphorylation of eIF4B in response to insulin

PKBβ null mutant mice have been shown to be defective in their insulin response and suffer from diabetes (Garofalo *et al.*,2003). This crucial role that PKB plays in mediating the effects of insulin, led us to investigate the role of PKB in regulating insulin-stimulated eIF4B phosphorylation. A14 cells were serum starved overnight and the phosphorylation status of eIF4B at Ser422 was compared after stimulation with insulin when cells were pre-incubated with rapamycin, LY294002 or U0126. eIF4B Ser422 phosphorylation was increased after insulin stimulation (Fig. 3A, lane 2) and pre-treatment with either rapamycin or U0126 had no effect on this phosphorylation (Fig. 3A, lane 3 and 4), while LY294002 treatment significantly abrogated eIF4B Ser422 phosphorylation (Fig. 3A, lane 5). In contrast to LY294002 (Fig. 3B, lane 4) a combination of rapamycin and U0126 again did not result in inhibition of Ser422 phosphorylation (Fig. 3B, lane 6). Taken together, these data indicate that insulin also utilizes PKB and not p70S6K and RSK to regulate phosphorylation of eIF4B at Ser422.

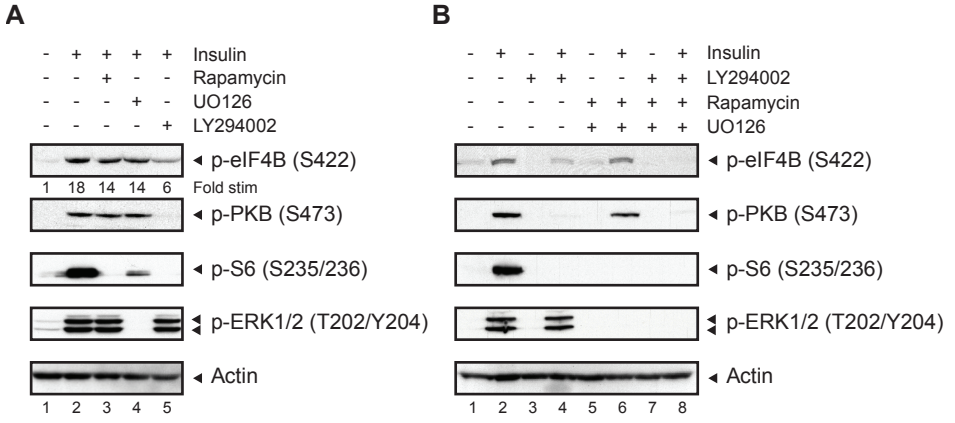

Figure 3. PKB activation sufficient for eIF4B phosphorylation of Ser422 after insulin stimulation of A14 cells

(A) A14 cells were serum starved overnight and left untreated or treated with insulin (1µg/ml) for 15 minutes after or without pre-treatment for 2 hours with rapamycin (20 ng/ml), U0126 (15 μM) or LY294002 (50 μM), lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-S6 (S235/S236) and phospho-ERK1/2 (T202/Y204) and actin. **(B)** A14 cells were serum starved overnight and left untreated or treated with insulin (1µg/ml) for 15 minutes after or without pre-treatment for 2 hours with LY294002 (50 μM), rapamycin (20 ng/ml) and U0126 (15 μM) or all of the before mentioned inhibitors together, lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-S6 (S235/S236) and phospho-ERK1/2 (T202/Y204) and actin.

mTOR regulates eIF4B phosphorylation in response to amino acid refeeding

Whereas insulin activates mTOR through Class I PI3K and PKB, amino acids can modulate mTOR activity utilising a distinct pathway which involves the Class III PI3K, hVps34, and this can occur independently of PKB activity (Nobukuni *et al.*,2005). To investigate the role of PKB in eIF4B phosphorylation after amino acid stimulation, we starved A14 cells in medium without amino acids followed by stimulation for 30 minutes with amino acids after pre-treatment with or without, LY294002, rapamycin or UO126. As a control cells were also stimulated with insulin. As shown in Figure 4 amino acid stimulation resulted in strong phosphorylation of eIF4B (Ser422) and p70S6K, while PKB phosphorylation was undetectable (Fig. 4, lane 8). Pre-incubation with either LY294002, which inhibits both Class I and III PI3K, or rapamycin inhibited the phosphorylation of eIF4B completely (Fig. 4, lanes 9, 10). These results indicate that in contrast to growth factor signalling, amino acid refeeding leads to eIF4B phosphorylation in a mTOR/p70S6K-dependent, PKB-independent manner.

Regulation of eIF4B Ser406 phosphorylation in response to insulin

To determine whether eIF4B could additionally be phosphorylated by PKB on Ser406, we generated a FLAG-tagged eIF4B in which this residue had been mutated to alanine. A14 cells were transfected with wild type or mutant eIF4B, serum starved overnight and subsequently stimulated with insulin before immunoprecipitating the FLAG-tagged protein. Insulin stimulation resulted in phosphorylation of eIF4B as detected by both the phospho-eIF4B Ser422 antibody as well as the phospho-PKB substrate antibody (Fig. 5A, lanes 1 and 2). However, when Ser406 was mutated to an alanine this abolished reactivity with the phospho-PKB substrate antibody (Fig. 5A, lanes 3 and 4), while there was no effect on Ser422 phosphorylation. This demonstrates that the phospho-PKB antibody specifically recognises eIF4B Ser406 allowing us to make use of this to analyse Ser406 phosphorylation *in vivo*.

To determine whether PKB activity is also required for insulin-induced eIF4B Ser406 phosphorylation, A14 cells were transfected with FLAG-tagged eIF4B, serum-starved overnight and subsequently treated with a specific PKB inhibitor before stimulation with insulin. Insulin stimulated robust phosphorylation of Ser422 and inhibition of PKB abrogated this, while phosphorylation of Ser406 was not affected by PKB inhibition (Fig. 5B, lane 4).This clearly indicates that while PKB activity is required for insulin induced Ser422 phosphorylation, other signalling pathways mediate the phosphorylation of Ser406. To define which signal transduction pathways regulate the eIF4B Ser406 phosphorylation

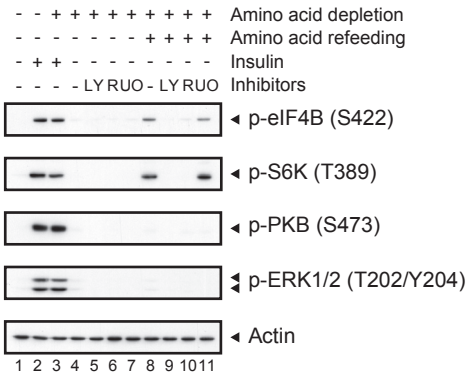

Figure 4. eIF4B Ser422 phosphorylation after amino acid refeeding is mTOR-dependent

A14 cells were serum-starved overnight and then for additional 4 hours starved in medium with or without amino acids. Cells were then stimulated with either 1 µg/ml insulin or amino acids for 30 minutes, after pre-treatment for 30 minutes with or without LY294002 (50 µM), rapamycin (20 ng/ml) or U0126 (15 µM). Cells were subsequently lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phospho-p70S6K (T389), phospho-PKB (S473), phospho-ERK1/2 (T202/Y204) and actin.

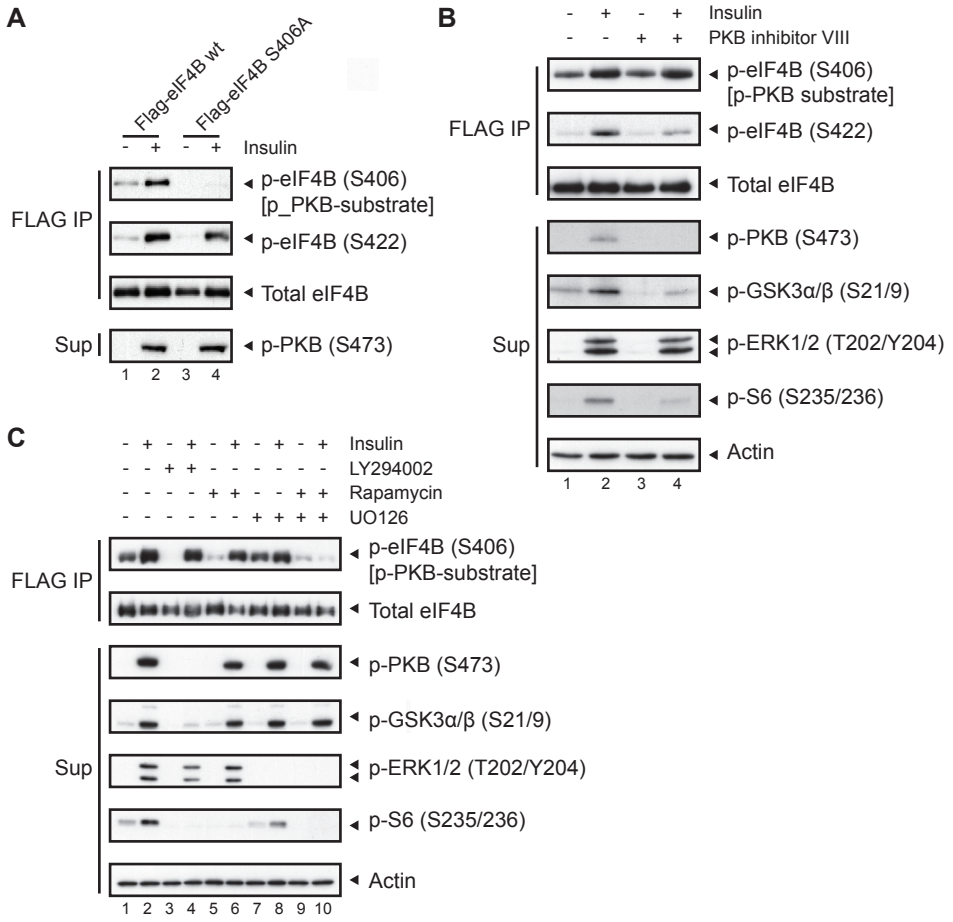

Figure 5. eIF4B Ser406 phosphorylation after insulin stimulation is MEK- and mTOR-dependent

(A) A14 cells were transfected with FLAG-tagged wild type eIF4B or mutant eIF4B in which Ser406 had been mutated to alanine. Cells were serum-starved overnight and subsequently stimulated for 15 minutes with insulin (1µg/ml) before immunoprecipitating the FLAG-tagged protein. The immunoprecipitated FLAG-eIF4B protein was analyzed for levels of phospho-PKB substrate, phospho-eIF4B (S422) and FLAG as a loading control. The whole cell lysate was analyzed for phospho-PKB (S473) as a control for insulin stimulation. **(B)** A14 cells were transfected with FLAG-tagged eIF4B. Cells were serum-starved overnight and subsequently stimulated for 15 minutes with insulin after pre-treatment for 30 minutes with or without PKBinhibitor VIII (10µM) before immunoprecipitating the FLAG-tagged protein. The immunoprecipitated FLAG-eIF4B protein was analyzed for levels of phospho-eIF4B (S422), phospho-PKB substrate and FLAG as a loading control. The whole cell lysate was analyzed for phospho-PKB (Ser473), phospho-GSK3α/β (S21/9), phospho-ERK1/2 (T202/Y204), phospho-S6 (S235/S236) and actin. **(C)** A14 cells were transfected with FLAG-tagged eIF4B. Cells were serum-starved overnight and subsequently stimulated for 15 minutes with insulin (1µg/ml) after or without pre-treatment for 30 minutes with either LY294002 (50 µM), rapamycin (20 ng/ml), U0126 (15 µM) or a combination of rapamycin (20 ng/ml) and U0126 (15 µM) before immunoprecipitating the FLAG-tagged protein. The immunoprecipitated FLAG-eIF4B protein was analyzed for levels of phospho-PKB substrate and FLAG as a loading control. The whole cell lysate was analyzed for phospho-PKB (Ser473), phospho-GSK3α/β (S21/9), phospho-ERK1/2 (T202/Y204), phospho-S6 (S235/S236) and actin.g

in vivo, A14 cells were transfected with FLAG-tagged eIF4B, serum starved overnight and treated with or without inhibitors, LY294002, rapamycin, UO126, or a combination of rapamycin and U0126 before stimulation with insulin. Pre-treatment with LY294002, rapamycin or UO126 had little effect on the phosphorylation of eIF4B on Ser406 (Fig 5C), whereas a combination of rapamycin and U0126 abrogated phosphorylation of this residue (Fig. 5C, lane 10). In contrast to eIF4B Ser422, inhibition of the PI3K/PKB signalling module by LY294002 has no effect on Ser406 phosphorylation. Our data suggests that Ser406 phosphorylation is a mTOR- and MEK-dependent event.

Phosphorylation of eIF4B regulates translation initiation in vivo

Taken together, our data suggests that phosphorylation of eIF4B Ser422 by PKB, and Ser406 through a MEK/mTOR-dependent pathway regulate translation initiation. In order to investigate this, we developed a novel translation control luciferase assay (TCLA) based on a system previously described by Wiesenthal et al (Wiesenthal *et al.*,2006). This TCLA makes use of an evolutionary conserved upstream open reading frame (uORF) of

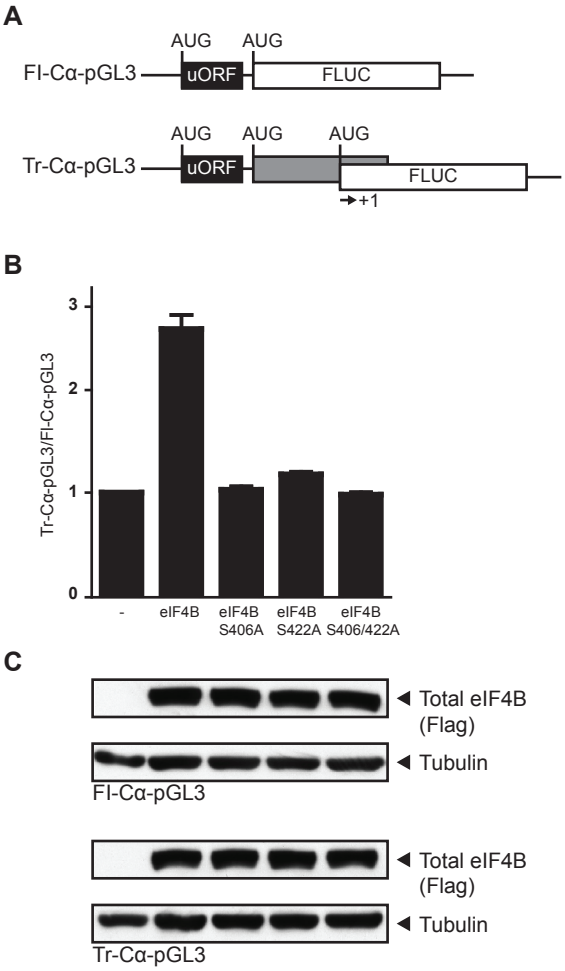

Figure 6. eIF4B Ser406 and Ser422 phosphorylation is required for optimal translational activity

(A) Schematic representation of reporter constructs. Fl-Cα-Gl3 encodes the uORF from C/EBPα with the first startcodon followed by the Firefly luciferase, while Tr-Cα-Gl3 contains the uORF and the second AUG followed by the Firefly luciferase gene. AUG1 lies out-of-frame with the luciferase AUG2. Fl-Cα-Gl3 measures translation initiation and Tr-Cα-Gl3 measures translation reinitiation. **(B)** Cells were transiently transfected with the various mutant forms of Flag-tagged eIF4B together with Fl-Cα-pGL3 or Tr-Cα-pGL3 constructs and pGL4.74 Renilla luciferase expressing vector for normalization. After 24 hours Firefly and Renilla luciferase activity in whole-cell lysates was determined by luminescence. After normalization, the ratio of normalized Tr-Cα-pGL3 to Fl-Cα-pGL3 luciferase activity was calculated. Three independent transfection studies were performed. Statistical significance was determined using Student's T-test. **(C)** Samples from luciferase assay were analyzed for expression levels of eIF4B with a FLAG antibody and tubulin as a loading control.

the transcription factor C/EBPα. This uORF controls the ratio of two proteins expressed from a single mRNA by regulated re-initiation (Calkhoven *et al.*,2000): a full-length protein expressed from a proximal initiation site and a expressed amino-terminally truncated protein from a different reading frame at a distal site. At high translational activity the uORF is recognized and the truncated protein is translated as a result of efficient re-initiation. The uORF of C/EBPα was used for the generation of a reporter assay, which is able to measure translation and translation re-initiation. Two plasmids were generated: one vector encodes the uORF from C/EBPα with the first startcodon followed by the firefly luciferase (Fl-Cα-Gl3), which measures translation, and a second vector which contains the uORF and the second AUG followed by the luciferase gene (Tr-Cα-Gl3), which measures translation re-initiation (Fig. 6A). The levels of the luciferase signal from the Tr-Cα-Gl3 are therefore directly proportional to the translational activity of the cell and an increase in Tr-Cα-Gl3/Fl-Cα-Gl3 ratio correlates with an increase in this activity.
To determine whether Ser406 and Ser422 phosphorylation affects the translational activity of cells, cells were transiently transfected to express various mutant forms of Flag-tagged eIF4B together with Fl-Cα-Gl3 or Tr-Cα-Gl3. As shown in Figure 6B, expression of wt eIF4B resulted in an increased ratio of the two luciferase signals, reflecting a higher translational activity, while mutation of Ser406, Ser422 or both phosphorylation sites abrogated this effect. Taken together, these data indicate that phosphorylation of eIF4B on both Ser406 and Ser422 is an important mechanism by which AGC kinase family members can positively regulate translational activity.

DISCUSSION

In this study, we have demonstrated that PKB can phosphorylate eIF4B on Ser422 *in vitro*. Upon mitogen-stimulation phosphorylation on Ser442 is also regulated by PKB *in vivo*, since blocking PKB activity either by addition of a specific PKB inhibitor or the PI3K inhibitor LY294002 was sufficient to abolish phosphorylation on this residue. In contrast mTOR inhibitor rapamycin and MEK inhibitor U0126 had no effect on Ser422 phosphorylation *in vivo*. Furthermore, we have identified a novel eIF4B phosphorylation site, Ser406, which is also phosphorylated upon mitogen-stimulation. Since Ser406 represents a consensus phosphorylation motif for AGC kinase family members and its phosphorylation can be abrogated by inhibition of both MEK and mTOR, we propose that the phosphorylation of this residue is regulated by RSK and p70S6K (Fig. 7). Importantly using a novel translational control luciferase assay (TCLA) phosphorylation of both residues was found to modulate the translational activity of eIF4B.
Previously, Shahbazian and co-workers reported that the phosphorylation of eIF4B on Ser422 is synergistically regulated by p70S6K and RSK upon serum-stimulation (Shahbazian *et al.*,2006). Phosphorylation of this residue upon insulin-stimulation was reported to be solely dependent on p70S6K. However, in this study cells were pre-treated with rapamycin for an extended period of time (up to 18 hours). Recently, it has become clear that prolonged treatment with rapamycin can inhibit PKB activity in a cell type specific manner (Sarbassov *et al.*,2006). Sarbassov and co-workers provided compelling evidence that mTOR in complex with Rictor (mTORC2) was sensitive to long-term rapamycin treatment and this complex was previously reported by the same group as the long sought after PDK2 kinase which phosphorylates PKB at Ser473 leading to its activation (Sarbassov *et al.*,2006; Sarbassov *et al.*,2005). In our study, cells were pre-treated with rapamycin for only a short period of time (less than 2 hours) to ensure that only mTOR in complex with Raptor (mTORC1), the upstream activator of p70S6K and not mTORC2

was inhibited. This had no effect on PKB activation as shown in Fig. 3A allowing us to specifically analyse the effect of mTOR/p70S6K inhibition.

Members of the AGC protein kinase family have highly homologous kinase domains and similar substrate specificities, and can therefore be considered as potentially "promiscuous" when it comes to phosphorylation of target proteins. Care must therefore be taken in drawing conclusions from *in vitro* assays where, it is likely that various members of the AGC kinase family may phosphorylate substrates at the same site. *In vivo*, however, phosphorylation of substrates is likely to be a highly regulated process. In the case of eIF4B, three AGC kinase family members have now been shown to phosphorylate Ser422 *in vitro*, p70S6K, RSK and PKB respectively. Whereas insulin specifically utilizes PKB to phosphorylate this residue, serum may also utilize RSK to regulate Ser422 phosphorylation. Shahbazian and co-workers show after serum stimulation a temporal effect of MEK inhibitor U0126 and mTOR inhibitor rapamycin (Shahbazian *et al.*,2006). U0126 effects the early phase of eIF4B phosphorylation whereas rapamycin effects the late phase. In this study, we have shown that for insulin stimulation RSK does not play a role in Ser422 phosphorylation but this kinase is crucial in Ser406 phosphorylation. Therefore, it is safe to conclude that eIF4B phosphorylation and activation are regulated in a stimulus- and cell type-dependent manner, and this could be the reason why the Ser406 site was not identified by Raught and co-workers in their phosphomapping experiment after serum stimulation (Raught *et al.*,2004).

While we have shown that PKB is the dominant kinase regulating Ser422 phosphorylation after insulin stimulation, this does not discount a role for other AGC family members in eIF4B phosphorylation. Indeed, while our data demonstrate that mTOR activity is not required for insulin-mediated Ser422 phosphorylation, it is required for phosphorylation of Ser422 after amino acid refeeding. Regulation of eIF4B phosphorylation may be a fundamental process in the regulation of protein translation in response to diverse extracellular stimuli. Our data suggests that utilizing various AGC kinase family members allows this mechanism of translational control to be regulated through distinct stimulus-specific intracellular signalling pathways.

The effects of eIF4B phosphorylation on translation have, to a limited degree, been studied previously and eIF4B phosphorylation has been reported to correlate with high

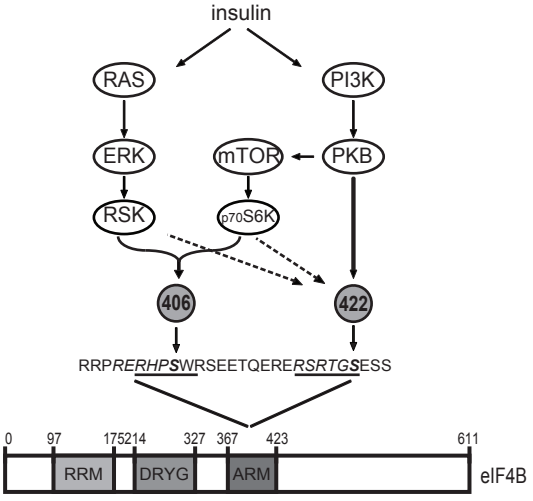

Figure 7. Signaling pathways regulating eIF4B Ser406 and Ser422 phosphorylation

Insulin activates the PI3K/PKB/mTOR pathway as well as the RAS/MEK/ERK pathway. Upon activation by insulin the RAS/MEK/ERK and mTOR pathways are required for regulating eIF4B Ser406 phosphorylation whereas PKB phosphorylates Ser422. Both residues are important in the regulation of translation activation.

translational activity. In accordance with this, phosphorylation of eIF4B on Ser422 has been shown to stimulate its interaction with eIF3, an important player in translation initiation (Holz *et al.*,2005; Shahbazian *et al.*,2006). In addition Holz *et al*. found that expression of an eIF4B S422D mutant increased cap-dependent translation (Holz *et al.*,2005). Here we have developed a novel readout for translation initiation using the translation control luciferase assay (TCLA). Our data reveal that mutation of the 422 or 406 residues abrogates the eIF4B-mediated increase in translational activity. These results demonstrate that phosphorylation of eIF4B by AGC protein kinase family members in the ARM region indeed activates eIF4B, resulting in a positive effect on translation initiation.

In this study, we provide evidence that phosphorylation of eIF4B can be regulated by both RAS and PKB signalling. Both pathways have been shown to be deregulated in a plethora of neoplasias. What role could eIF4B phosphorylation play in the process of transformation? eIF4B has been shown to play a critical role in stimulating the helicase activity of eIF4A to unwind inhibitory secondary structures in the 5' untranslated region of mRNAs. These highly structured mRNAs are poorly translated when the translation initiation activity is decreased (Lodish,1976). Highly structured mRNAs often encode those proteins that are components of pathways critical to cell growth, such as growth factors, transcription factors, tyrosine kinases and receptors (Ruggero *et al.*,2003). Rajasekhar *et al*. recently demonstrated that upon PKB and RAS signalling the profile of mRNA associated to polysomes was drastically altered, these mRNAs mainly encoded for the proteins mentioned (Rajasekhar *et al.*,2003). Therefore activation of eIF4B by dysregulated RAS and PKB signalling may be critical in the induction of cellular transformation.

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

MATERIALS AND METHODS

Cell culture

Ba/F3 cells were cultured in RPMI 1640 medium with 8% Hyclone serum (Gibco, Paisley, UK) and recombinant mouse IL-3 produced in COS cells (Dijkers et al.,2002a). For the generation of clonal Ba/F3 cells stably expressing myrPKB:ER*, the SRα-myrPKB:ER* construct was electroporated into Ba/F3 cells together with pSG5 conferring neomycin resistance and maintained in the presence of 1mg/ml G418 (Gibco, Paisley, UK) and IL-3. Clonal cell lines were generated by limited dilution. For cytokine withdrawal experiments, cells were washed twice with PBS and resuspended in AimV medium (Gibco, Paisley, UK). A14 cells and COS cells were cultured in Dulbecco's modified Eagles Medium (Gibco, Paisley, UK) with 8% FCS (Gibco, Paisley, UK). A14 cells are NIH 3T3 derived cells that overexpress the insulin receptor (Burgering et al.,1991). A14 cells were serum starved in DMEM supplemented with 0.1% FCS. For amino acid refeeding A14 cells were serum-starved overnight and then for additional 4 hours starved in medium without amino acids, followed by stimulation with MEM Amino Acids Solution (Gibco, Paisley, UK).

Constructs

Pc-DNA3-FLAG-eIF4B and pcDNA3-FLAG-eIF4BS422A have been previously published (Raught et al.,2004). pcDNA3-FLAG-eIF4BS406A was generated from pcDNA3-FLAG-eIF4B by side-directed mutagenesis. For the construction of Fl-Cα-pGL3 the 135-nt rat C/EBPα 5'UTR was cut (EcoRI

blunted / NcoI) from rC/EBPαwt-pcDNA3 (Calkhoven et al., 2000), and cloned into pGL3-Promoter Firefly luciferase vector (HindIII blunted / NcoI) (Promega). This construct was used to emulate Full-length C/EBPα translation.

For Tr-Cα-pGL3, a plasmid was constructed containing 485 nt of rat C/EBPα-cDNA ranging from the Cap-site to the AUG start codon normally used for Tr-C/EBPα expression(Calkhoven et al.,2000): An NcoI fragment was produced by PCR covering the sequence between Fl-C/EBPα start codon to the Tr-C/EBPα start codon. At the Tr-C/EBPα initiation site the start codon for luciferase expression was shifted (+1) out-of-frame with the Fl-C/EBPα frame. The PCR fragment was cloned into (NcoI) Fl-Cα-pGL3. PCR primers: 5'-gtggatagcggtttgactcacg-3' (binding to CMV promoter of C/EBPα-pcDNA3) and 5'-ttccatggggcaccgccggggcc-3' (+1 mutation and NcoI-site). This construct was used to emulate Truncated C/EBPα translation.

Antibodies and reagents
Monoclonal antibodies against phospho-PKB (Ser473) and the polyclonal antibodies against phospho-eIF4B (Ser422), phospho-PKB substrate, phospho-GSK3α/β (S21/9) and phospho-p70S6K (T389) were from Cell Signaling Technologies (Hitchin, UK). Actin antibody was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The phospho-FOXO3 (Thr32) and phospho-FOXO3 (Ser253) antibodies were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Phospho-MAPK42/44(Thr202/Tyr204) and phosphor-S6 (S235/S236) were from New England Biolabs (Hitchin, UK). The Anti-FLAG M2 monoclonal antibody peroxidase conjugate, 4-hydroxytamoxifen (4-OHT) and insulin were purchased from Sigma (Seelze, Germany). LY294002, U0126 and rapamycin were obtained from Biomol International LP (Hamburg, Germany) and PKB inhibitors IV, V and VIII were from Calbiochem (San Diego, CA).

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

Immunoprecipitation
For the immunoprecipitation assays either COS or A14 cells (9 cm dishes) were transfected with a total of 10 μg of plasmid DNA by the calcium phosphate or Polyethyleneimine (PEI) precipitation method. The following morning the cells were washed with PBS and fresh medium was added to the cells. For serum starvation cells were again washed with PBS at the end of the day and DMEM containing 0.1% FCS was added to the cells. After another 24 hours of growth cells were stimulated as indicated and lysed in RIPA lysis buffer (20 mM Tris pH 7.8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodiumdeoxycholin, 5mM EDTA, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF). Lysates were centrifuged at maximum speed for 10 minutes to remove DNA and cellular debris. A part of the lysate was taken as a control for stimulations, 5x sample buffer was added to a final concentration of 1x (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue) and boiled for 5 minutes. The rest of the lysate was incubated for at least 2 hours with FLAG M2 agarose beads from Sigma (Seelze, Germany) at 4°C, subsequently beads were washed four times with RIPA lysis buffer and boiled in 1x sample buffer (60 mM Tris pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol and bromophenol blue).

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

Tandem Mass spectrometry
Immunoprecipitated eIF4B was digested with Trypsin (Roche) and enriched for phosphorylated

peptides using a ~5mm length TiO_2 microcolumn, packed in GE-Loader tip with a 3M Empore C8 plug from an extraction disc, essentially as described (Larsen *et al.*,2005). Peptides were loaded onto this column in buffer A (80% acetonitrile, 0.1% trifluoric acid)/ 200g/l DHB(2,5-dihydroxibenzoic acid). Columns were washed once in bufferA/DHB followed by a wash in buffer A. The bound peptides were eluted with 20μl 1% ammonia in 5μl 10% Formic acid. Samples were directly subjected to nanoflow liquid (LC) chromatography (Agilent 1100 series) and concentrated on a C18 precolumn (100um ID, 2cm). Peptides were separated on an aqua™ C18 reversed phase column (kind gift of Prof. A. Heck, dimensions; 75μM ID, 20 cm) at a flow rate of 200nl/min with a 60 min. linear acetonitrile gradient from 0 to 90%. The LC system was directly coupled to a QTOF Micro tandem mass spectrometer (Micromass Waters, UK). A survey scan was performed from 400-1200 amu s^{-1} and precursor ions were sequenced in MS/MS mode at a threshold of 150 counts. Data were processed and subjected to database searches using MASCOT software (Matrixscience) against SWISSPROT and the NCBI non-redundant database, allowing for the detection of phosphorylation residues, with a 0.25 Da mass tolerance for both precursor ion and fragment ion. The identified peptides were confirmed by manual interpretation of the spectra.

Translational control luciferase assay
Cells were cultured in DMEM supplemented with 10% FCS. Transient transfections were performed using FUGENE (Roche Diagnostics) according to the manufactures instructions. Briefly, cells were seeded at a density of $4x10^4$ cells per well of a 96-well plate and grown to 80-90% confluency. The cells were cotransfected with 0.1μg DNA of Fl-Cα-pGL3 or Tr-Cα-pGL3 constructs/well, 0.2μg eIF4B-pcDNA3 vector and 0.1μg pGL4.74 Renilla luciferase expressing vector for normalization (Promega). Fresh media was added after 12h and the cells were grown for another 24h. Firefly and Renilla luciferase activity in whole-cell lysates was determined by luminescence (Mithras, Berthold). After normalization, the ratio of normalized Fl-Cα-pGL3 to Tr-Cα-pGL3 luciferase activity was calculated.

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REFERENCES

Burgering BM, Medema RH, Maassen JA, van de Wetering ML, van der Eb AJ, McCormick F *et al*. (1991). Insulin stimulation of gene expression mediated by p21ras activation. *EMBO J.* 10: 1103-1109.

Calkhoven CF, Muller C, Leutz A. (2000). Translational control of C/EBPalpha and C/EBPbeta isoform expression. *Genes Dev.* 14: 1920-1932.

Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L *et al*. (2002b). FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *J.Cell Biol.* 156: 531-542.

Dijkers PF, Birkenkamp KU, Lam EW, Thomas NS, Lammers JW, Koenderman L *et al*. (2002a). FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. *J.Cell Biol.* 156: 531-542.

Duncan R, Hershey JW. (1984). Heat shock-induced translational alterations in HeLa cells. Initiation factor modifications and the inhibition of translation. *J.Biol.Chem.* 259: 11882-11889.

Duncan R, Hershey JW. (1985). Regulation of initiation factors during translational repression caused by serum depletion. Covalent modification. *J.Biol.Chem.* 260: 5493-5497.

Gani R. (1976). The nucleoli of cultured human lymphocytes. I. Nucleolar morphology in relation to

transformation and the DNA cycle. *Exp.Cell Res.* 97: 249-258.

Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL *et al*. (2003). Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. *J.Clin.Invest* 112: 197-208.

Gingras AC, Raught B, Sonenberg N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu.Rev.Biochem* 68: 913-963.

Guertin DA, Sabatini DM. (2005). An expanding role for mTOR in cancer. *Trends Mol.Med.* 11: 353-361.

Hershey JW, Merrick WC. (2000). Pathway and mechanism of initiation of protein synthesis. 33-88.

Holland EC, Sonenberg N, Pandolfi PP, Thomas G. (2004b). Signaling control of mRNA translation in cancer pathogenesis. *Oncogene* 23: 3138-3144.

Holland EC, Sonenberg N, Pandolfi PP, Thomas G. (2004a). Signaling control of mRNA translation in cancer pathogenesis. *Oncogene* 23: 3138-3144.

Holz MK, Ballif BA, Gygi SP, Blenis J. (2005). mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell* 123: 569-580.

Inoki K, Li Y, Zhu T, Wu J, Guan KL. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat.Cell Biol.* 4: 648-657.

Larsen MR, Thingholm TE, Jensen ON, Roepstorff P, Jorgensen TJ. (2005). Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. *Mol.Cell Proteomics.* 4: 873-886.

Lawson TG, Lee KA, Maimone MM, Abramson RD, Dever TE, Merrick WC *et al*. (1989). Dissociation of double-stranded polynucleotide helical structures by eukaryotic initiation factors, as revealed by a novel assay. *Biochemistry* 28: 4729-4734.

Lodish HF. (1976). Translational control of protein synthesis. *Annu.Rev.Biochem* 45: 39-72.

Luo J, Manning BD, Cantley LC. (2003). Targeting the PI3K-Akt pathway in human cancer: rationale and promise. *Cancer Cell* 4: 257-262.

Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N. (2006). mTOR, translation initiation and cancer. *Oncogene* 25: 6416-6422.

Methot N, Pause A, Hershey JW, Sonenberg N. (1994). The translation initiation factor eIF-4B contains an RNA-binding region that is distinct and independent from its ribonucleoprotein consensus sequence. *Mol.Cell Biol.* 14: 2307-2316.

Methot N, Pickett G, Keene JD, Sonenberg N. (1996a). In vitro RNA selection identifies RNA ligands that specifically bind to eukaryotic translation initiation factor 4B: the role of the RNA remotif. *RNA.* 2: 38-50.

Methot N, Song MS, Sonenberg N. (1996b). A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. *Mol.Cell Biol.* 16: 5328-5334.

Naranda T, Strong WB, Menaya J, Fabbri BJ, Hershey JW. (1994). Two structural domains of initiation factor eIF-4B are involved in binding to RNA. *J.Biol.Chem.* 269: 14465-14472.

Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. (1999). Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J.* 344 Pt 2: 427-431.

Nobukuni T, Joaquin M, Roccio M, Dann SG, Kim SY, Gulati P *et al*. (2005). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. *Proc.Natl.Acad.*

Sci.U.S.A 102: 14238-14243.

Obata T, Yaffe MB, Leparc GG, Piro ET, Maegawa H, Kashiwagi A *et al*. (2000). Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *J.Biol.Chem.* 275: 36108-36115.

Obenauer JC, Cantley LC, Yaffe MB. (2003). Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic Acids Res.* 31: 3635-3641.

Pandolfi PP. (2004). Aberrant mRNA translation in cancer pathogenesis: an old concept revisited comes finally of age. *Oncogene* 23: 3134-3137.

Parker PJ, Parkinson SJ. (2001). AGC protein kinase phosphorylation and protein kinase C. *Biochem Soc.Trans.* 29: 860-863.

Rajasekhar VK, Viale A, Socci ND, Wiedmann M, Hu X, Holland EC. (2003). Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. *Mol.Cell* 12: 889-901.

Raught B, Gingras AC, Sonenberg N. (2000). Regulation of ribosomal recruitment in eukaryotes. 245-295.

Raught B, Peiretti F, Gingras AC, Livingstone M, Shahbazian D, Mayeur GL *et al*. (2004). Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. *EMBO J.* 23: 1761-1769.

Richter JD, Sonenberg N. (2005). Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature* 433: 477-480.

Rogers GW, Jr., Richter NJ, Lima WF, Merrick WC. (2001). Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F. *J.Biol.Chem.* 276: 30914-30922.

Rozen F, Edery I, Meerovitch K, Dever TE, Merrick WC, Sonenberg N. (1990). Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. *Mol.Cell Biol.* 10: 1134-1144.

Ruggero D, Montanaro L, Ma L, Xu W, Londei P, Cordon-Cardo C *et al*. (2004). The translation factor eIF-4E promotes tumor formation and cooperates with c-Myc in lymphomagenesis. *Nat.Med.* 10: 484-486.

Ruggero D, Pandolfi PP. (2003). Does the ribosome translate cancer? *Nat.Rev.Cancer* 3: 179-192.

Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF *et al*. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol.Cell* 22: 159-168.

Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* 307: 1098-1101.

Shahbazian D, Roux PP, Mieulet V, Cohen MS, Raught B, Taunton J *et al*. (2006). The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. *EMBO J.* 25: 2781-2791.

van Gorp AG, Pomeranz KM, Birkenkamp KU, Hui RC, Lam EW, Coffer PJ. (2006). Chronic Protein Kinase B (PKB/c-akt) Activation Leads to Apoptosis Induced by Oxidative Stress-Mediated Foxo3a Transcriptional Up-regulation. *Cancer Res.* 66: 10760-10769.

Wiesenthal V, Leutz A, Calkhoven CF. (2006). A translation control reporter system (TCRS) for the analysis of translationally controlled processes in the vertebrate cell. *Nucleic Acids Res.* 34: e23-

Zhang H, Zha X, Tan Y, Hornbeck PV, Mastrangelo AJ, Alessi DR *et al*. (2002). Phosphoprotein analysis using antibodies broadly reactive against phosphorylated motifs. *J.Biol.Chem.* 277: 39379-39387.

SUPPLEMENTARY DATA

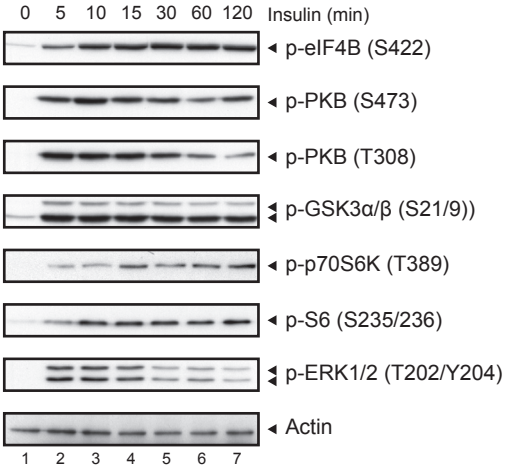

Figure S1. Insulin stimulation results in activation of PI3K/PKB/mTOR pathway as well as the RAS/MEK/ERK pathway.
A14 cells were serum starved overnight and left untreated or treated with insulin (1μg/ml) for the times indicated, lysed and equal amounts of protein were analyzed for levels of phospho-eIF4B (S422), phospho-PKB (S473), phospho-PKB (T308), phospho-GSK3α/β (S21/9), phospho-p70S6K (T389), phospho-S6 (S235/S236), phospho-ERK1/2 (T202/Y204) and actin.

CHAPTER 5

Comparative analysis of FOXO3- and FOXO4-regulated genes by microarrays

Transcriptional feedback control of IL-3R surface expression by FOXO3

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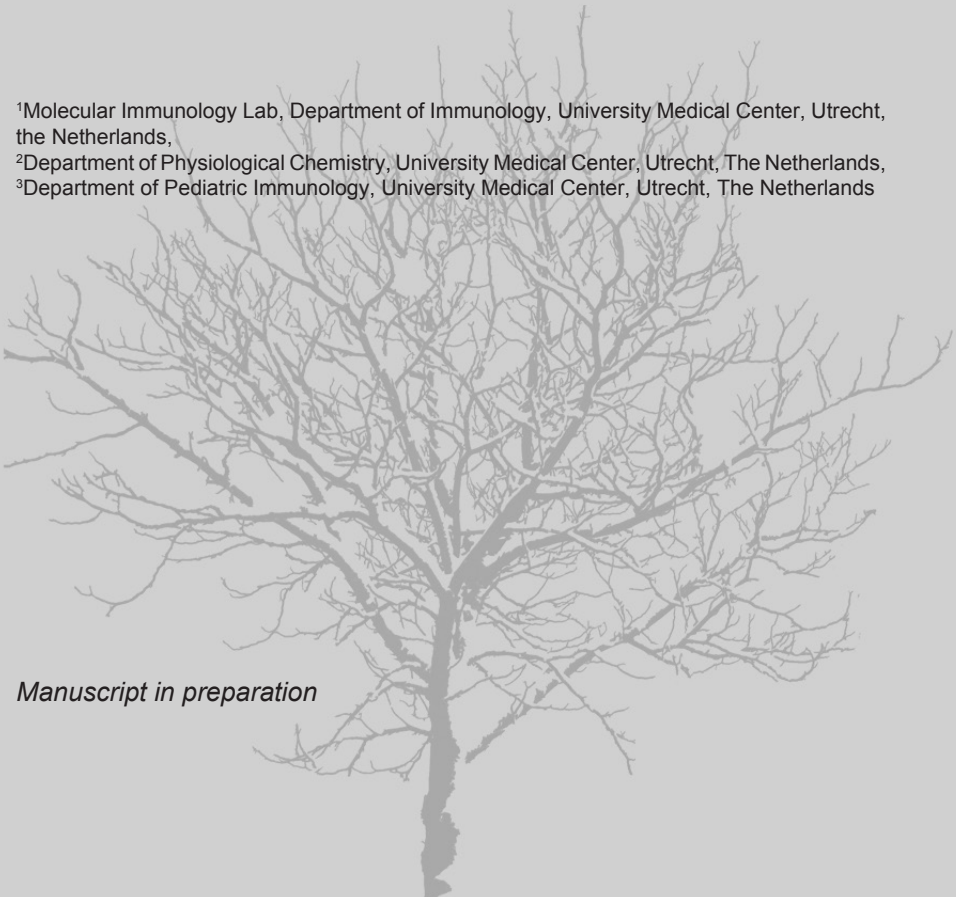


Manuscript in preparation

ABSTRACT

The Forkhead box O (FOXO) transcription factors; FOXO1, FOXO3 and FOXO4 can regulate a plethora of biological processes including cell cycle regulation, stress resistance, development, reproduction and ageing. Despite their diverse roles in development and physiology *in vivo,* it remains unclear whether they can function in a non-redundant manner within a single cell type. To identify novel transcriptional targets differentially regulated by FOXO3 and FOXO4 we generated Ba/F3 cell lines expressing inducible constitutively active FOXO3 and FOXO4 (FOXO3(A3)-ER and FOXO4(A3)-ER) and examined differential gene expression by microarray analysis. Activation of FOXO3 and FOXO4 altered the expression of more than 1000 genes and comparative analysis revealed that FOXO3 and FOXO4 regulate a non-redundant but overlapping set of transcriptional targets. Quantitative RT-PCR of selected genes after activation of FOXO3 and FOXO4 validated the microarray analysis and suggest that FOXO3 and FOXO4 may have both redundant and non-redundant functions within a single cell type. In addition, we identified JAK2 as a putative FOXO target. Activation of FOXO3 and FOXO4 resulted in a upregulation of JAK2 mRNA and protein expression. Cytokine deprivation or treatment with the specific PI3K inhibitor LY294002 also resulted in increased JAK2 expression. JAK2 has been demonstrated to stabilise membrane expression of cytokine receptors, and surface expression of the IL-3Rα was significantly increased after activation of FOXO3. This suggests that FOXO3 may increase IL3-R signalling through a transcriptionally regulated feedback loop.

INTRODUCTION

The IL-3, IL-5, GM-CSF cytokine family are critical mediators of the survival, proliferation and differentiation of haematopoietic cells (reviewed in Geijsen *et al.*, 2001). Binding of these cytokines to their cognate receptors results in activation of multiple signal transduction cascades including the Ras-MEK-ERK, JAK-STAT and PI3K-PKB pathways (reviewed in Martinez-Moczygemba and Huston, 2003). Activation of phosphoinositol-3-kinase (PI3K) by growth factors and cytokines regulates multiple cellular processes including survival, proliferation, growth and cytoskeletal rearrangement. Once activated, PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP_2) in the cell membrane resulting in formation of PIP_3. The formation of phosphatidylinositol-3,4,5-triphosphate (PIP_3) subsequently results in recruitment of proteins containing a pleckstrin homology (PH) domain, through which they bind to PIP_3 (Engelman, 2009). Among those are the serine-threonine kinase protein kinase B (also known as c-akt) and phosphoinositide dependent kinase 1 (PDK1), which phosphorylates PKB on Thr 308 resulting in its activation and release from the plasma membrane (Burgering and Coffer, 1995; Ananthanarayanan *et al.*, 2007; Stephens *et al.*, 1998). The activation of PKB results in increased proliferation and survival through regulation of key regulators of cell cycle, apoptosis and metabolism (Manning and Cantley, 2007). Many of the effects of PKB activation are mediated by phosphorylation and inhibition of Forkhead box O (FOXO) transcription factors; FOXO1, FOXO3 and FOXO4. Phosphorylation of FOXOs by PKB prevents DNA binding, induces binding to 14-3-3 proteins and results in nuclear export and consequently inhibition of FOXO function (Brunet *et al.*, 1999; Kops *et al.*, 1999).
Sequence alignment analysis reveals that several regions in FOXO1, FOXO3 and FOXO4 are highly conserved, including the first PKB phosphorylation site, the DNA binding domain and a region containing the (nuclear localisation sequence) NLS (reviewed in Obsil and Obsilova, 2008). Due to the high conservation within the DBD FOXOs share similar affinity for the DNA binding consensus sequence: TTGTTTAC (Furuyama *et al.*, 2000). Dephosphorylation of FOXOs in the absence of growth factor signalling stimulates nuclear entry leading to activation or repression of a variety of transcriptional targets.
FOXO3 and FOXO4 are ubiquitously expressed but with varying expression levels. While FOXO3 is mainly expressed in muscle, heart, spleen and ovaries, FOXO4 shows the highest expression in heart brain spleen and lung (Anderson *et al.*, 1998; Furuyama *et al.*, 2000; Greer and Brunet, 2005). Depending on the cell-type, activation of FOXOs can have influence on a wide range of biological processes including cell cycle regulation, stress resistance, development, reproduction and ageing. In haematopoietic cells and neurons activation of FOXOs increases the expression of pro-apoptotic genes including Bim and FasL resulting in induction of apoptosis (Brunet *et al.*, 1999; Dijkers *et al.*, 2000). In contrast, in other cell types FOXOs increase expression of cell cycle inhibitors such as p27 thereby causing a block in cell cycle progression (Medema *et al.*, 2000). After cell cycle arrest FOXO activation results in upregulating of target genes involved in protection against oxidative stress such as manganese superoxide dismutase (MnSOD) and Growth arrest and DNA damage response gene (Gadd45) a protein involved in DNA repair mechanisms (Kops *et al.*, 2002a; Tran *et al.*, 2002).

Despite transcriptional redundancy often being reported for FOXOs *in* vitro, their *in vivo* roles in development and physiology are diverse, with individual disruption of *Foxo3* and *Foxo4* genes in mice resulting in distinct phenotypes (Hosaka *et al.*, 2004). *Foxo3*$^{-/-}$ females show an age-dependent infertility with an abnormal ovarian follicular development (Hosaka *et al.*, 2004). The *Foxo3*$^{-/-}$ female mice exhibit global follicular

activation leading to oocyte death, resulting in early depletion of functional ovarian follicles and subsequently infertility (Castrillon *et al.*, 2003). In addition, examination of lymphoid compartment revealed that *Foxo3* deficiency leads to spontaneous lymphoproliferation and wide-spread organ inflammation, due to hyperactivation of helper T cells (Lin *et al.*, 2004). In contrast to *Foxo3*-deficient mice, *Foxo4*$^{-/-}$ mice are normal in appearance and do not show any consistent abnormalities (Hosaka *et al.*, 2004). The development of an inducible *Foxo1*$^{-/-}$, *Foxo3*$^{-/-}$ and *Foxo4*$^{-/-}$ mouse model demonstrated the redundant role of FOXOs in oncogenesis and stem cell homeostasis (Paik *et al.*, 2007; Tothova *et al.*, 2007). Analysis of the haematopoietic system after loss of *Foxo1*, *Foxo3* and *Foxo4* demonstrated increased numbers of myeloid progenitors in peripheral blood, while in the bone marrow the number of haematopoietic stem cells (HSCs) was reduced. Further analysis revealed that FOXOs are required for haematopoietic stem cell renewal by decreasing reactive oxygen species levels (Tothova *et al.*, 2007). Recently, it has been demonstrated that deletion of *Foxo1*, *Foxo3* and *Foxo4* has a similar effect on neural stem cells (NSCs). *Foxo*-deficient mice showed a decline in the NSC pool, due to increased proliferation and loss of self renewal, indicating that FOXOs play a critical role in stem cell homeostasis (Paik *et al.*, 2009). A similar phenotype was also detected after loss of only *Foxo3* demonstrating the importance of FOXO3 in neural stem cell homeostasis (Renault *et al.*, 2009). In addition, after conditional deletion of *Foxo1*, *Foxo3* and *Foxo4,* mice developed lymphoblastic thymic lymphomas and hemangiomas, demonstrating that FOXOs act as functional redundant tumour suppressors (Paik *et al.*, 2007). Surprisingly, despite wide-spread expression of FOXOs, the tumour phenotype was restricted to thymocytes and endothelial-derived cells. Moreover, not all tissues containing endothelial cells were affected and microarray analysis of differentially affected endothelium revealed non-overlapping lists of putative FOXO targets, indicating that the regulation of FOXO targets is highly context-dependent (Paik *et al.*, 2007).

Several studies have utilised microarray analysis to globally identify novel FOXO targets genes and this has implicated the involvement of FOXOs in a variety of cellular processes, including cell cycle progression, DNA repair and apoptosis (Delpuech *et al.*, 2007; Modur *et al.*, 2002; Ramaswamy *et al.*, 2002; Tran *et al.*, 2002). However no studies have been performed that critically compare transcriptional targets after FOXO3 and FOXO4 activation within a single cell type. In this study we performed microarray analyses after inducible activation of inducible active FOXO3 and FOXO4 to identify transcriptional targets, which are differentially regulated by FOXO3 and FOXO4. Using this approach, we identified subsets of genes which were differentially regulated by FOXO3 and FOXO4, indicating that FOXO3 and FOXO4 indeed have non-redundant functions. In addition, we have identified JAK2 as a novel FOXO target. Activation of either FOXO3 or FOXO4 induces an upregulation of JAK2 mRNA and protein expression. JAK2 has been implicated in stabilisation of cytokine receptors and the FOXO3-mediated upregulation of JAK2 correlated with increased IL-3 receptor surface expression. Taken together, these results suggest that FOXO may increase IL-3 sensitivity by upregulating JAK2.

RESULTS

Identification of FOXO3 and FOXO4 transcriptional targets

To identify novel transcriptional targets differentially regulated by FOXO3 and FOXO4 we generated Ba/F3 cell lines expressing inducible constitutively active FOXO3 and

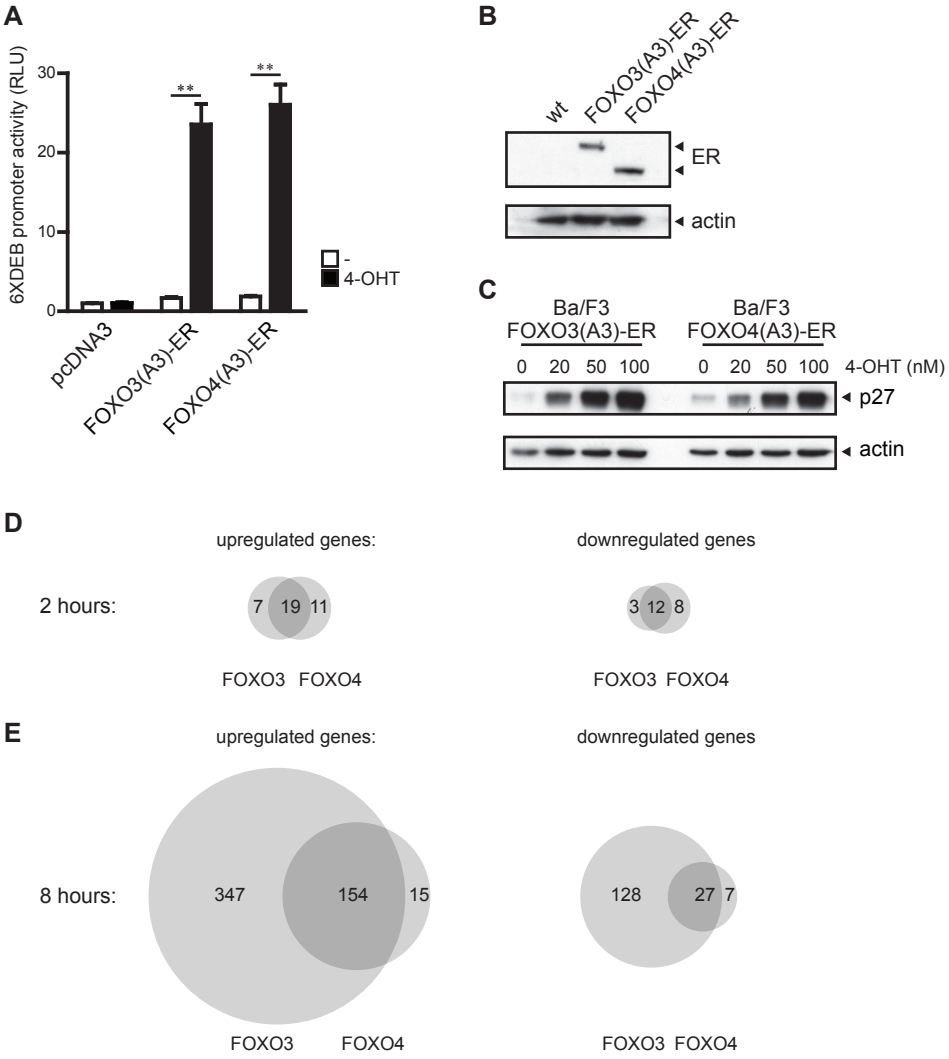


Figure 1. Activation of FOXO3 and FOXO4 results in transcriptional regulation of a large set of genes

(A) HEK 293 cells were transfected with a 6x DBE reporter plasmid together with Renilla and FOXO3(A3) as indicated. The next day cells were stimulated with 4-OHT (100 nM) and and luciferase activity was measured after 16 hours Data are depicted as relative luciferase units (RLU) compared to control. Shown are mean ± SEM values of two independent experiments performed with triplicate samples. **(B)** Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were lysed and equal amounts of proteins were analyzed for expression levels of the FOXO constructs with an ER antibody and actin served as a loading control. **(C)** Ba/F3 cells expressing FOXO3(A3)-ER cells or FOXO4(A3)-ER were stimulated with 4-OHT in the presence of mIL-3 (5 ng/ml) for 24 hours. Cells were lysed and equal amounts of proteins were analyzed for levels of p27 and actin. **(D,E)** Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT (100 nM) in the presence of mIL-3 (5 ng/ml) for 2 **(D)** and 8 **(E)** hours, RNA was isolated and microarray analyses performed. Shown are the number of genes, which were more than 1.7 fold up- or downregulated in response to FOXO3 and FOXO4 activation. Shown are the results of one experiment performed in quadruplicate.

FOXO4 (FOXO3(A3)-ER and FOXO4(A3)-ER). Constitutively active FOXO3 and FOXO4 proteins were generated by mutating the three PKB phosphorylation sites to alanines thereby rendering these proteins insensitive to inhibition via PKB. Coupling of these constitutively active FOXOs to the hormone binding domain of the estrogen receptor (ER) causes recruitment of heat shock proteins maintaining the proteins in an inactive state. Addition of the estrogen analogue 4-hydroxytamoxifen (4-OHT), results in rapid dissociation of heat shock proteins and activation of the signalling molecules (Littlewood *et al.*, 1995). To demonstrate that activation of FOXO3(A3)-ER and FOXO4(A3)-ER with 4-OHT results in similar FOXO promoter activity, cells were transiently transfected with the FOXO constructs together with a promoter construct expressing six canonical FOXO binding elements (6xDBE) and stimulated with 4-OHT for 16 hours. As shown in Figure 1A activation of FOXO3 and FOXO4 resulted in similar levels of luciferase activity indicating that the transcriptional activity of FOXO3 and FOXO4 constructs was comparable. Next, the bone-marrow-derived Ba/F3 cell line was transduced with either FOXO3(A3)-ER or FOXO4(A3)-ER and clonal lines were generated by limited dilution.

Table 1. Microarray data of selected genes for qRT-PCR
Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT for 2 and 8 hours, RNA was isolated and microarray analyses were performed. Shown are the fold changes relative to untreated cells of selected genes. Data are represented as mean values of one experiment performed in quadruplicate.

Symbol	Name	Function	**FOXO3 2h**	**FOXO4 2h**	**FOXO3 8h**	**FOXO4 8h**
Crem	cAMP responsive element modulator	cAMP signalling	-	-	3.6	-
Eps8	epidermal growth factor receptor pathway substrate 8	Cytoskeletal rearrangements	-	-	2.0	-
Ahrgap9	Rho GTPase activating protein 9	Cytoskeletal rearrangements	-	-	1.9	-
Nsbp1	nucleosomal binding protein 1	Differentiation	-	-	1.9	-
Stk38	serine/threonine kinase 38	MAPK signalling	-	-	1.8	-
Cdca7	cell division cycle associated 7	Cell cycle	-	-	-2.3	-
Pmch	pro-melanin-concentrating hormone	Metabolism	-	-	-	2.1
Clk1	CDC-like kinase 1	mRNA splicing	3.0	2.3	3.7	2.1
JAK2	Janus kinase 2	Cytokine signalling	-	-	2.4	1.9
JAK1	Janus kinase 2	Cytokine signalling	-	-	1.9	-
Uhrf1	ubiquitin-like, containing PHD and RING finger domains, 1	Cell cycle	-	-	-2.0	-

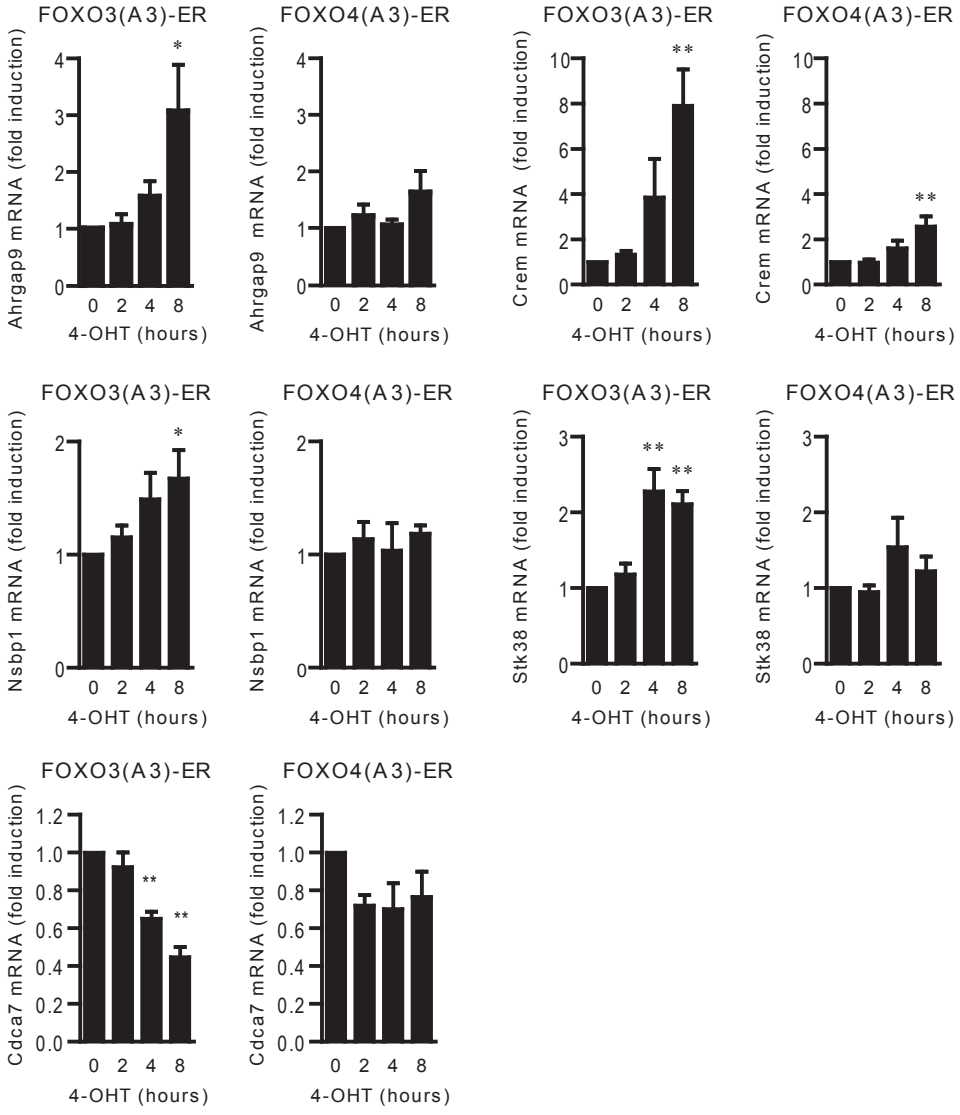


Figure 2. Arhgap9, Crem, Nsbp1, Stk38 and Cdca7 mRNA expression is specifically regulated by FOXO3

Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 100 nM 4-OHT in the presence of mIL-3 (5 ng/ml) for the indicated times. RNA was isolated and relative mRNA levels of Arhgap9, Crem, Nsbp1, Stk38 and Cdca7 were analyzed using quantitative PCR. Data are represented as mean ± SEM values normalized for B_2M of at least three experiments performed with technical duplicates. * $p < 0.05$ and ** $p < 0.01$

In order to compare functional differences between FOXO3 and FOXO4 two clones were selected with similar ER expression levels (Fig. 1B). In addition, Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT To demonstrate that activation of FOXO3(A3)-ER and FOXO4(A3)-ER with 4-OHT results in similar levels of FOXO promoter transcriptional activity and protein expression levels of the previously described FOXO3 and FOXO4 target p27 were analyzed by Western blotting. As shown in Figure 1C, activation of FOXO3 and FOXO4 resulted in a clear upregulation of p27 expression, which was comparable after activation of either FOXO3 or FOXO4.

To characterise transcriptional targets regulated by FOXO3 and FOXO4 activation Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT and microarray analyses were performed. In Figure 1D and E the number of genes more than 1.7 fold changed after FOXO activation is depicted compared to untreated cells. As shown in Figure 1D, after 2 hours of FOXO activation both FOXO3 and FOXO4 regulated a small overlapping subset of genes. In contrast, after 8 hours of stimulation expression of 1040 transcripts were changed, with a much larger subset of genes regulated by FOXO3 than FOXO4 (Fig. 1E). The FOXO3 and FOXO4 data sets demonstrated considerable overlap, with only a small number of genes, which were specifically regulated by FOXO4. To further investigate the differential effects of FOXO3 and FOXO4 activation, a selection was made from the genes identified in the arrays (Table 1). These genes were selected for their differential regulation by FOXO3 and FOXO4 and their involvement in cellular processes not previously ascribed to FOXO transcription factors. To validate the microarray results and to investigate the regulation of the selected genes over time, Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT, RNA was isolated and mRNA expression of the selected genes was analyzed by quantitative RT-PCR (qRT-PCR). As shown in Figure 2, Ahrgap9, Crem, Nsbp1, Stk38 and Uhrf1 were regulated by FOXO3, while activation of FOXO4 had no or very little effect on transcript levels (Fig. 2). In contrast, Pmch mRNA expression was increased exclusively after activation of FOXO4 (Fig. 3). Furthermore, mRNA expression of Hbp1, Clk1, Eps8, and Uhrf1 was regulated by both FOXO3 and FOXO4 (Fig. 4). These results confirm and extend the microarray analysis and suggest that FOXO3 and FOXO4 have both redundant and non-redundant functions within a single cell type.

To better understand the functional consequences of FOXO activation, transcripts that were regulated by FOXO3 and FOXO4 were grouped into subcategories according to

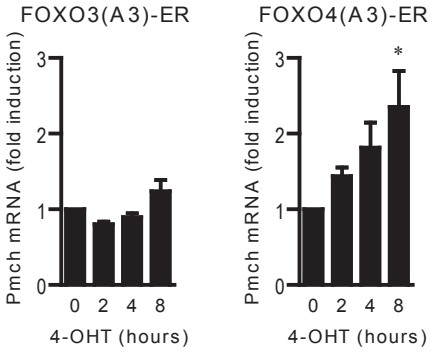


Figure 3. Pmch mRNA expression is specifically upregulated by FOXO4
Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 100 nM 4-OHT in the presence of mIL-3 (5 ng/ml) for the indicated times. RNA was isolated and relative mRNA levels of Pmch were analyzed using quantitative PCR. Data are represented as mean ± SEM values normalized for B_2M of of at least three experiments performed with technical duplicates. * $p < 0.05$

their function using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). The functional classification of genes regulated by FOXO activation revealed that a large number of genes encode for basic cellular processes, including proliferation, cell growth, and cell death that have been previously attributed to FOXO function (Fig 5A). In addition, we analysed signalling pathways that were significantly associated with the FOXO-regulated genes. Depicted is the percentage of genes in a pathway that were found to be regulated in the FOXO3 and FOXO4 dataset. As shown in Figure 5B, FOXO-regulated genes were most associated with the p53 signalling pathway and with interferon signalling. Interestingly, FOXO-regulated transcripts were also involved in the JAK-STAT signalling pathway, which is an important upstream modulator of PI3K-PKB-FOXO activity in response to haematopoietic cytokines.

JAK2 mRNA expression is regulated by FOXO3 and FOXO4

The Janus kinases (JAK) are an unique family of cytoplasmic tyrosine kinases that play a pivotal role in signal transduction via cytokine receptors (reviewed in Ihle and Gilliland,

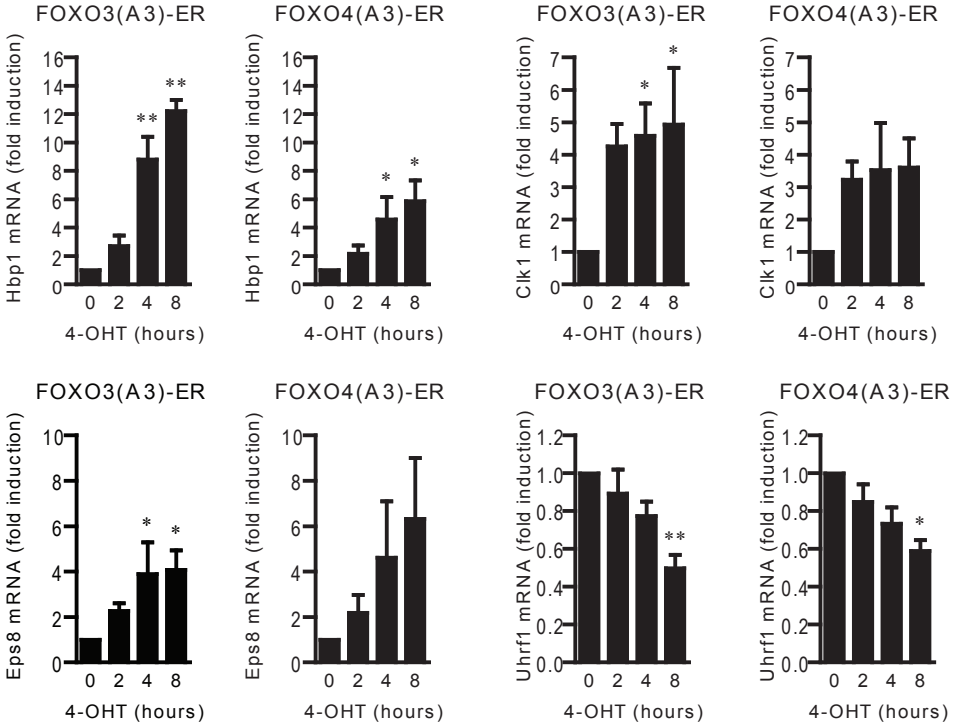
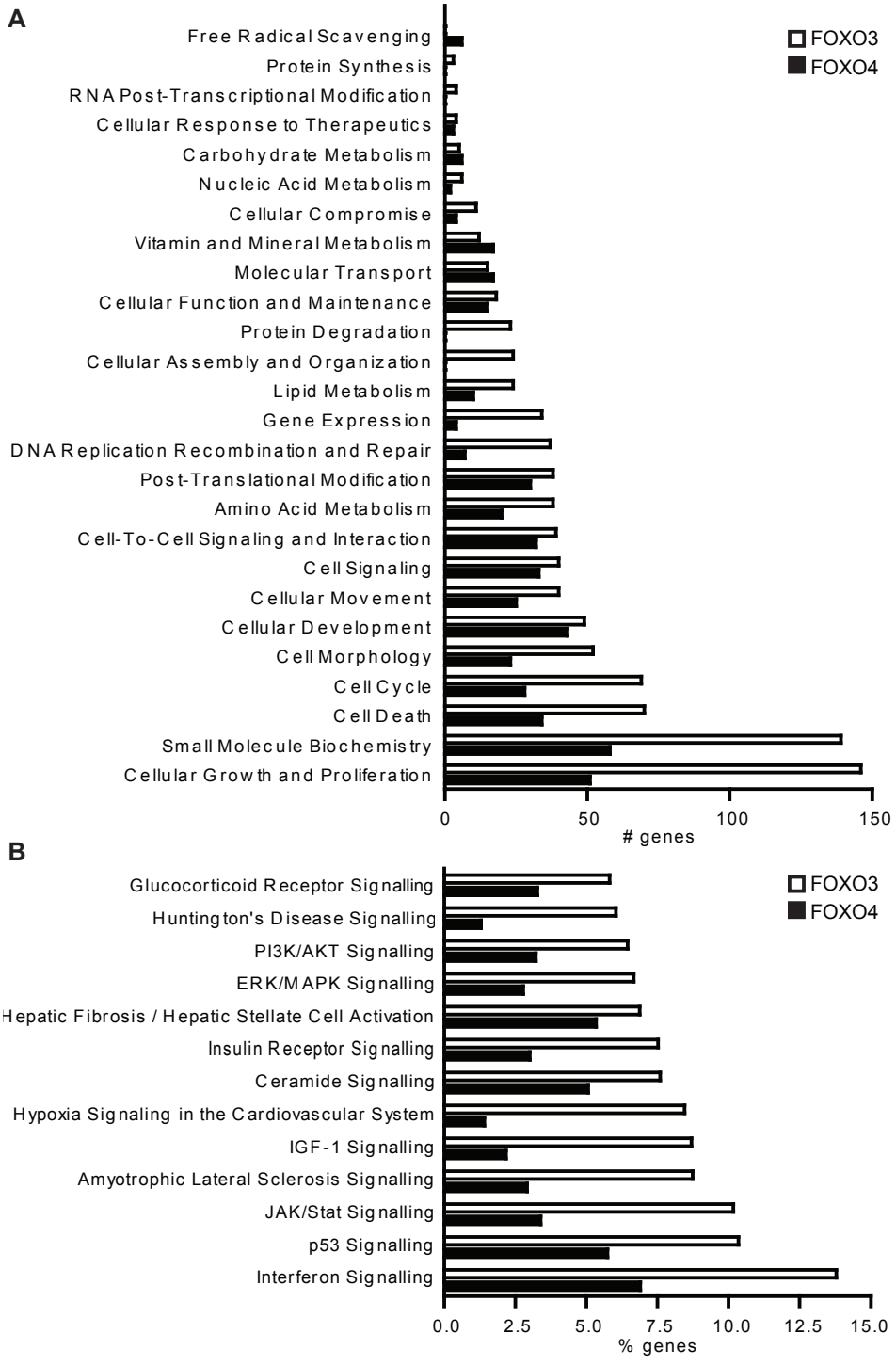


Figure 4. Hbp1, Clk1, Eps8, and Uhrf1 mRNA expression is regulated by both FOXO3 and FOXO4

Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 100 nM 4-OHT in the presence of mIL-3 (5 ng/ml) for the indicated times. RNA was isolated and relative mRNA levels of Hbp1, Clk1, Eps8, and Uhrf1 were analyzed using quantitative PCR. Data are represented as mean ± SEM values normalized for B_2M of of at least three experiments performed with technical duplicates. * $p < 0.05$ and ** $p < 0.01$


A
Free Radical Scavenging
Protein Synthesis
RNA Post-Transcriptional Modification
Cellular Response to Therapeutics
Carbohydrate Metabolism
Nucleic Acid Metabolism
Cellular Compromise
Vitamin and Mineral Metabolism
Molecular Transport
Cellular Function and Maintenance
Protein Degradation
Cellular Assembly and Organization
Lipid Metabolism
Gene Expression
DNA Replication Recombination and Repair
Post-Translational Modification
Amino Acid Metabolism
Cell-To-Cell Signaling and Interaction
Cell Signaling
Cellular Movement
Cellular Development
Cell Morphology
Cell Cycle
Cell Death
Small Molecule Biochemistry
Cellular Growth and Proliferation
FOXO3
FOXO4
0
50
100
150
genes
B
Glucocorticoid Receptor Signalling
Huntington's Disease Signalling
PI3K/AKT Signalling
ERK/MAPK Signalling
Hepatic Fibrosis / Hepatic Stellate Cell Activation
Insulin Receptor Signalling
Ceramide Signalling
Hypoxia Signaling in the Cardiovascular System
IGF-1 Signalling
Amyotrophic Lateral Sclerosis Signalling
JAK/Stat Signalling
p53 Signalling
Interferon Signalling
FOXO3
FOXO4
0.0
2.5
5.0
7.5
10.0
12.5
15.0
% genes

2007). JAKs are associated with cytokine receptors and become phosphorylated after ligand binding and dimerisation of the receptors (Witthuhn *et al.*, 1993). Upon phosphorylation JAKs become active and phosphorylate tyrosine residues at the distal domains of cytokine receptors thereby generating docking sites for signal transducers and activators of transcription (STATs) and other SH2-containing signalling molecules (Ihle and Gilliland, 2007). By microarray analysis, JAK2 was found to be regulated by both FOXO3 and FOXO4, while JAK1 was only upregulated by FOXO3. To validate this observation, Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT, RNA was isolated and mRNA expression of the selected genes was analyzed by quantitative RT-PCR (qRT-PCR). As shown in Figure 6A, activation of either FOXO3 or FOXO4 resulted in an upregulation of JAK2 mRNA. In addition, JAK1 mRNA was also upregulated after activation of FOXO3 (Fig. 6B). The family of JAK kinases consist of four members: JAK1, JAK2, JAK3 and TYK2, which associate with specific cytokine receptors and mediate downstream signalling. To determine whether JAK3 and TYK2 expression levels were also regulated by FOXO3, mRNA expression of the selected genes was analyzed by q/RT-PCR. After stimulation of Ba/F3 cells expressing FOXO3(A3)-ER with 4-OHT, no change in JAK3 and TYK2 mRNA levels was observed (Fig. 6C,D). These results indicate that FOXO3 specifically regulates JAK1 and JAK2 mRNA expression. To confirm that JAK2 expression is directly regulated by FOXO-mediated transcription, Ba/F3 FOXO3(A3)-ER cells were stimulated with 4-OHT in the presence of the general transcriptional inhibitor actinomycin D. Addition of actinomycin D completely abrogated FOXO-induced JAK2 upregulation as well as the upregulation of p27, indicating that JAK2 expression is regulated at the level of transcription (Fig. 6E).

JAK2 protein expression is regulated by FOXO3 and FOXO4

To further characterise the regulation of JAK2 expression by FOXOs, Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT, lysed and protein expression levels were analyzed by Western blotting. As shown in Figure 7A activation of FOXO3 and FOXO4 for 8 hours resulted in a clear upregulation of JAK2 expression, which was further increased after 24 and 48 hours stimulation with 4-OHT. In addition, previously described FOXO targets Id1, Bim and p27 were concomitantly regulated by FOXO activation (Medema *et al.*, 2000; Dijkers *et al.*, 2000; Birkenkamp *et al.*, 2007). Ba/F3 cells expressing FOXO3(A3)-ER or Ba/F3 wildtype cells were also either deprived of IL-3, or stimulated with IL-3 in the presence or absence of 4-OHT for 24 hours. Cytokine-starvation of Ba/F3 wildtype cells resulted in increased expression of JAK2, Bim, and p27 and decreased expression of Id1 (Fig. 7B). As an additional control, 4-OHT stimulation increased JAK2 expression in Ba/F3 cells expressing FOXO3(A3)-ER but not in wildtype cells (Fig. 7B). To further evaluate the effect of cytokine deprivation, which results in activation of endogenous FOXO transcription factors, we deprived wildtype Ba/F3 cells from IL-3 and evaluated JAK2 expression. Ba/F3 cells lysed at various time points after cytokine starvation showed a clear upregulation of JAK2 expression, which increased over time (Fig. 7C). Ba/F3 cells were also incubated with the specific PI3K

Figure 5. Functional classification of FOXO-regulated genes by pathway analysis

Functional classification of genes that were regulated more than 1.7 fold in response to FOXO3 and FOXO4 activation for 8 hours by Ingenuity Pathway Analysis. **(A)** Graph represents the number of genes within categories based on molecular and cellular functions, which were significantly associated with the FOXO data sets. ($p<0.05$) **(B)** Graph represents the percentage of FOXO-regulated genes within the signalling pathways, which were significantly associated with the FOXO data sets. ($p<0.05$)

inhibitor LY294002 to analyse whether inactivation of PI3K could also regulate JAK2 expression. As shown in Figure 7D, treatment of Ba/F3 cells with LY294002 also resulted in increased JAK2 expression. Finally, to determine whether FOXO3-mediated regulation of JAK2 expression was conserved between species, the human colon carcinoma cell line DLD1 expressing FOXO3(A3)-ER was stimulated with 4-OHT and JAK2 expression was analysed by Western blotting. Activation of FOXO3 in DLDL1 cells resulted in a clear upregulation of JAK2 expression (Fig. 7E). These results indicate that the upregulation of JAK2 expression by FOXO3 is conserved between cell types and across species.

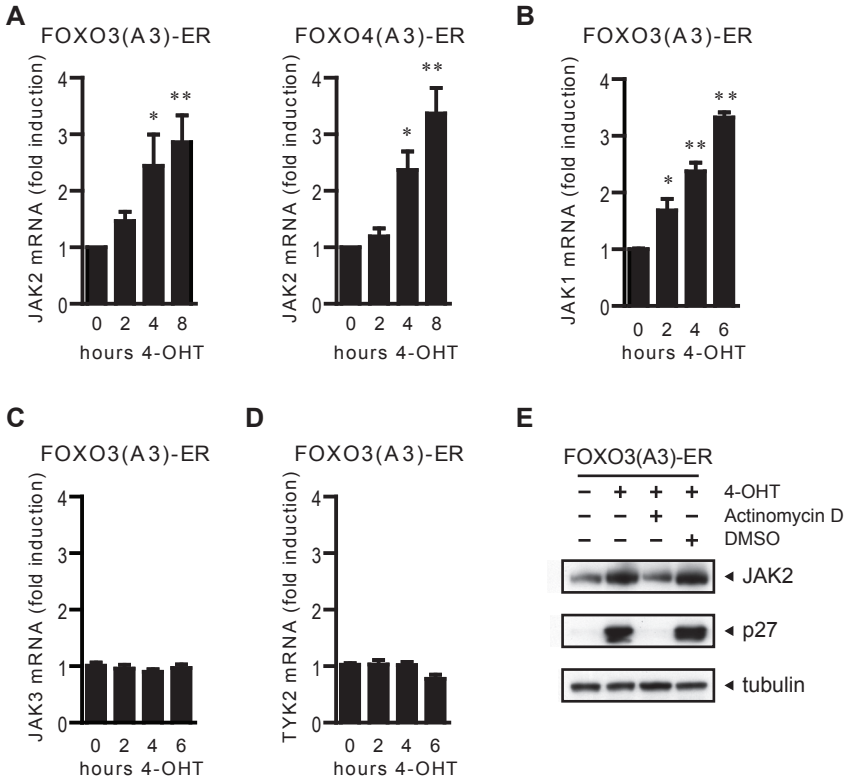


Figure 6. JAK2 and JAK1 mRNA levels are upregulated by activation of FOXO3
(A) Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 100 nM 4-OHT in the presence of mIL-3 (5 ng/ml) for the indicated times. RNA was isolated and relative mRNA levels of JAK2 were analyzed using quantitative PCR. Data are represented as mean ± SEM values normalized for B_2M of four experiments performed with technical duplicates. * $p < 0.05$ and ** $p < 0.01$
(B,C,D) Ba/F3 cells expressing FOXO3(A3)-ER were stimulated with 100 nM 4-OHT in the presence of mIL-3 (5 ng/ml) for the indicated times. RNA was isolated and relative mRNA levels of JAK1, JAK3 and Tyk2 were analyzed using quantitative PCR. Data are represented as mean ± SEM values normalized for B_2M of two experiments performed with technical duplicates. * $p < 0.05$ and ** $p < 0.01$
(E) Ba/F3 cells expressing FOXO3(A3)-ER were cultured in the presence of IL3 and stimulated with 4-OHT (100 nM) for 16 hours with or without Actinomycin D (1 µg/ml) or DMSO as a control. Cells were lysed and equal amounts of proteins were analyzed for levels of Jak2, p27 and tubulin. Shown are representative blots of 3 independent experiments.

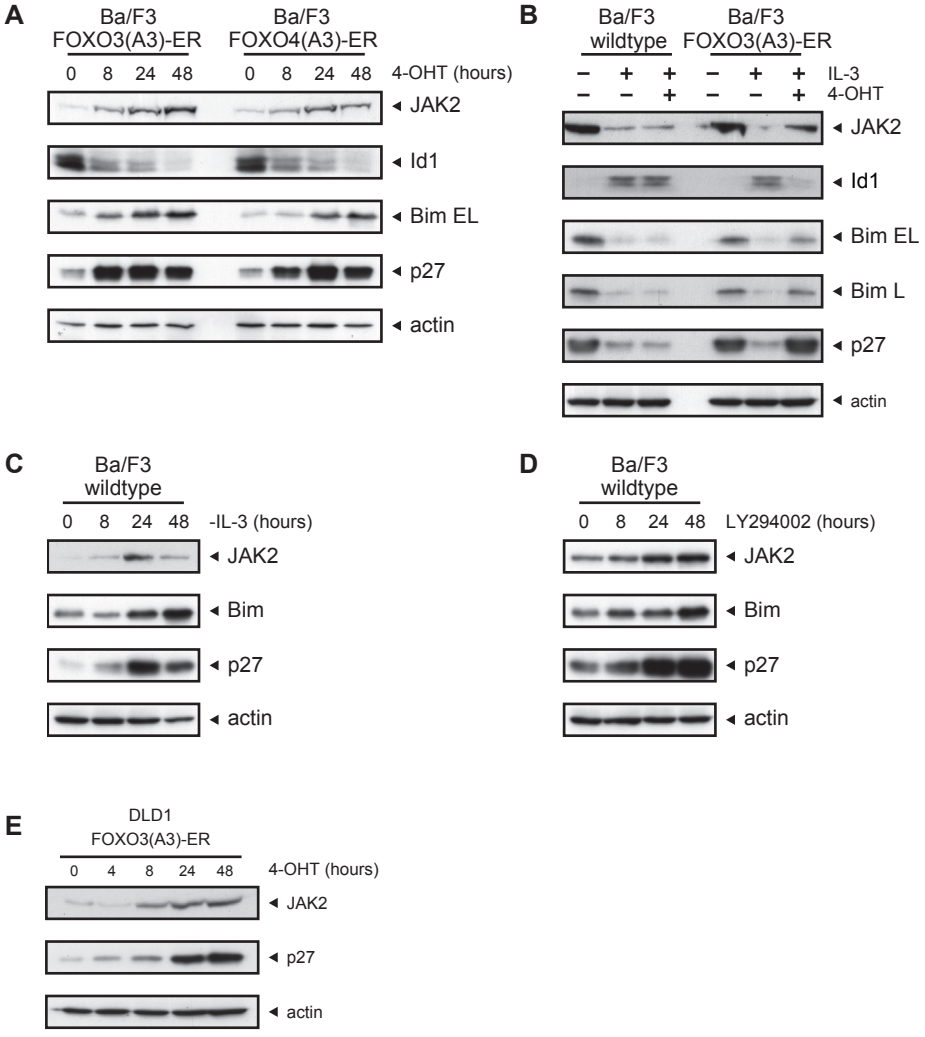


Figure 7. JAK2 protein levels are upregulated by activation of FOXO3 or FOXO4
(A) Ba/F3 cells expressing FOXO3(A3)-ER cells or FOXO4(A3)-ER were stimulated with 4-OHT (100 nM) in the presence of mIL-3 (5 ng/ml) for indicated time points. Cells were lysed and equal amounts of proteins were analyzed for levels of Jak2, Id1, Bim, p27 and actin. **(B)** Wild-type Ba/F3 cells and Ba/F3 cells expressing FOXO3(A3)-ER were cultured either in the presence of IL-3 , stimulated with 4-OHT (100 nM) or starved from IL-3 for 24 hours. Cells were lysed and equal amounts of proteins were analyzed for levels of Jak2, Id1, Bim, p27 and actin. Shown are representative blots of 3 independent experiments. **(C)** Wild-type Ba/F3 cells were cytokine-starved for the indicated time points. Cells were lysed and equal amounts of proteins were analyzed for levels of Jak2, Bim, p27 and actin. Shown are representative blots of 3 independent experiments. **(D)** Wild- type Ba/F3 cells were cultured in the presence of LY294002 (10 μM) for indicated time points. Cells were lysed and equal amounts of proteins were analyzed for levels of Jak2, Bim, p27 and actin. Shown are representative blots of 3 independent experiments. **(E)** DL23 cells were stimulated with 4-OHT (500 nM) for indicated time points. Cells were lysed and equal amounts of proteins were analyzed for levels of Jak2, p27 and actin. Shown are representative blots of 4 independent experiments.

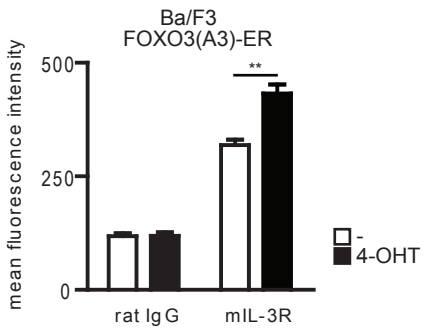


Figure 8. Activation of FOXO3 increases IL-3 receptor surface expression
Ba/F3 cells expressing FOXO3(A3)-ER cells were cultured stimulated with 4-OHT (100 nM) in the presence of mIL-3 (5 ng/ml) for indicated 24 hours. IL-3R expression was determined by FACS analysis. Mean fluorescence intesisnties are depicted for IL-3R staining and rat IgG as a isotype control. Shown are the mean ± SEM of three independent experiments. ** $p < 0.01$

IL-3 receptor α expression is increased after FOXO3 activation
JAK2 plays a critical role in cytokine receptor-mediated signalling and has been demonstrated to be required for stabilisation of plasma membrane expression of the erythropoietin receptor (EpoR), thrombopoietin receptor (Tpo-R), growth hormone receptor (GHR) and granulocyte colony-stimulating factor receptor (G-CSFR) (He *et al.*, 2005; Huang *et al.*, 2001; Tong *et al.*, 2006; Meenhuis *et al.*, 2009). After ectopic expression of EpoR in a JAK2 deficient cell line, the majority of EpoR is retained in the endoplasmic reticulum. However, cotransfection of the EpoR with JAK2 induced a twelve-fold increase of the EpoR on the plasma membrane, indicating that JAK2 is required for surface expression (Huang *et al.*, 2001).
To investigate whether FOXO3-mediated JAK2 expression might similarly act to increase IL-3R expression at the plasma membrane, Ba/F3 FOXO3(A3)-ER cells were stimulated with 4-OHT for 24 hours and the IL3-Rα expression was determined by FACS. As shown in Figure 8, IL-3Rα expression was significantly increased after activation of FOXO3. This result suggests that FOXO3 increases IL3-R signalling via upregulation of IL3-R at the plasma membrane.

DISCUSSION

FOXO transcription factors have been found to function as important transcriptional regulators of multiple cellular processes in a redundant manner. Specific deletion of *Foxo* genes in mice has revealed both redundant and non-redundant roles in development and physiology. *Foxo3*$^{-/-}$ an *Foxo4*$^{-/-}$ mice show distinct phenotypes, suggesting that *in vivo* FOXO3 and FOXO4 have unique non-overlapping transcriptional targets (Hosaka *et al.*, 2004). However, the generation of conditional *Foxo1,3,4*$^{-/-}$ mice revealed that FOXOs do have a redundant role in oncogenesis and stem cell homeostasis (Paik *et al.*, 2007; Paik *et al.*, 2009; Tothova *et al.*, 2007). Several studies have used microarray analysis for the identification of novel FOXO targets genes *in vitro*, however these studies did not compare FOXO3 and FOXO4 activation within a single cell type (Delpuech *et al.*, 2007; Modur *et al.*, 2002; Ramaswamy *et al.*, 2002; Tran *et al.*, 2002). In this study we have performed microarray analysis after inducible activation of FOXO3 and FOXO4 to identify differentially regulated target genes. Using this approach, we have for the first time identified subsets of genes which are differentially regulated by FOXO3 and FOXO4 within one cell type, indicating that FOXO3 and FOXO4 indeed have non-redundant functions.

FOXOs have been described to interact with a wide range of binding partners allowing for a much broader transcriptional response (reviewed in van der Vos and Coffer, 2008). These interactions play an important role in the regulation of the transcriptional program after FOXO activation. The importance of FOXO binding partners has been illustrated by a study from Ramaswamy *et al.* Transcriptional profiling and chromatin immunoprecipitation experiments using a FOXO1 mutant, in which DNA-binding was abolished, demonstrated that FOXO1 was still able to regulate a subset of transcripts involved in cell cycle regulation (Ramaswamy *et al.*, 2002). This result indicates that FOXOs can mediate the regulation of a variety of cellular processes through direct association with diverse transcription factor families. Since FOXOs show very little homology outside their DNA-binding domain the binding of distinct binding partners to specific FOXO isoforms may result in unique transcriptional programmes. In addition, FOXO proteins are regulated by a variety of post-translational modifications, including phosphorylation, acetylation and ubiquitination, which are responsible for another level of FOXO regulation (reviewed in Calnan and Brunet, 2008). Coexpression of the deacetylase SIRT1 has been proposed to switch the FOXO transcriptional program from apoptosis towards stress resistance and survival (Brunet *et al.*, 2004), suggesting that acetylation can influence the outcome of FOXO activation. Furthermore, oxidative stress can trigger phosphorylation of FOXO4 by JNK resulting in nuclear localization and activation of its transcriptional activity (Essers *et al.*, 2004). This phosphorylation site is not conserved in other FOXO isoforms, suggesting that JNK can specifically regulate FOXO4-mediated transcription These results indicate that post-translational modifications on specific non-conserved residues might influence functional consequences of FOXO activation and might be responsible for FOXO isoform specific gene regulation .

Among the differentially regulated genes we identified was Rho GTPase activating protein 9 (Ahrgap9), which was specifically upregulated after FOXO3 activation. Further examination of the microarray data revealed that Ahrgap1, Ahrgap 25, Rho associated protein kinase (Rock) 1 and Rock2 mRNA transcripts were also increased by activation of FOXO3, but not by FOXO4. The Rho family of GTPases is involved in adhesion and migration of cells through modulation of the actin cytoskeleton (reviewed in Buchsbaum, 2007). Rock1 and Rock2 are protein kinases and downstream effectors of Rho signalling, which mediate Rho-induced actin reorganisation by phosphorylating various cytoskeleton organization regulating proteins (Schmandke *et al.*, 2007). Active GTP-bound Rho proteins are inactivated by Rho GTP-ase activating proteins (RhoGAPs or Ahrgap's) (Buchsbaum, 2007). It has been reported that FOXO3 but not FOXO4 can influence migration of endothelial cells. Overexpression of constitutively active FOXO3 inhibited endothelial cell migration and tube formation *in vitro,* while FOXO4 had no effect (Potente *et al.*, 2005). Further experiments demonstrated that knockdown of FOXO1 and FOXO3 resulted in a overlapping non-redundant set of genes involved in migration, thereby explaining the difference in functional outcome of FOXO3 activation (Potente *et al.*, 2005). The selective regulation of the Rho pathway by FOXO3 that we observe might provide further insight in the regulation of adhesion and migration by FOXO3 and awaits further research.

Furthermore we identified JAK2 as a novel FOXO transcriptional target. Cytokine receptors lack intrinsic catalytic activities and rely on JAK kinases for signal transduction (reviewed in Ihle and Gilliland, 2007). Myeloid progenitors from *JAK2-/-* mice fail to respond to erythropoietin, thrombopoietin, IL-3 and granulocyte/macrophage colony-stimulating factor (Parganas *et al.*, 1998). Although much much has been reported concerning JAK2 function, the regulatory steps that control its expression level remain almost completely undefined.

Here we have demonstrated that cytokine signalling regulates JAK2 mRNA and protein expression (Fig. 6,7). JAK2 expression is upregulated by FOXO3, suggesting a potential transcriptional feedback mechanism for IL-3-mediated signalling. JAK2 plays an essential role in the development of leukemia and an activating mutation in JAK2 (V617F) has been found in the majority of patients with polycythemia vera and a significant proportion of patients with other myeloproliferative disorders, including essential thrombocytopenia and idiopathic myelofybrosis (Baxter *et al.*, 2005; James *et al.*, 2005; Kralovics *et al.*, 2005; Levine *et al.*, 2005; Zhao *et al.*, 2005). The mutation is positioned in the autoinhibitory domain of JAK2 and renders the kinase constitutively active resulting in constitutive activation of STAT and PI3K pathways and transformation of haematopoietic progenitors (reviewed in Ihle and Gilliland, 2007). However, alternative mutations that result in activation of JAK2 signalling might contribute to the pathogenesis of JAK2V617F-negative myeloproliferative disorders. It has been demonstrated that overexpression of JAK2 promotes *in vitro* cell

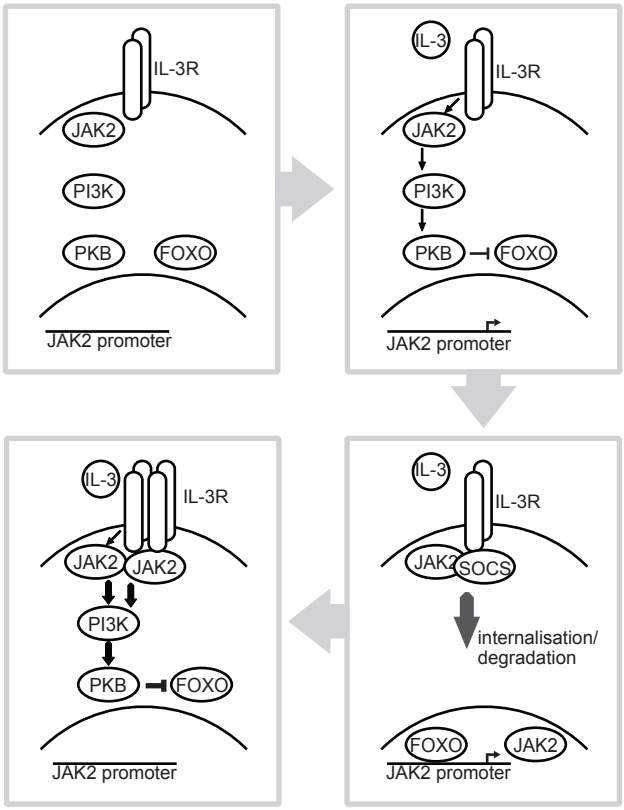


Figure 9. The role of FOXO-induced JAK2 expression in feedback regulation of cytokine signalling

In the presence of cytokines the PI3K-PKB pathway ensures survival and proliferation by inhibiting FOXO transcription factors. After IL-3 stimulation, SOCS mediates the internalisation and degradation of the receptor complex thereby terminating the signal. This results in activation of FOXOs and subsequent upregulation of JAK2 expression. In addition, the increased JAK2 expression increases IL-3R plasma membrane expression. After restimulation of the IL-3R with IL-3 the increased JAK2 protein levels ensure a fast response.

transformation by increasing JAK2 kinase activity and increasing STAT5 activation, suggesting that deregulation of JAK2 expression might contribute to oncogenesis (Knoops *et al.*, 2008). Here we provide evidence that FOXO3 can upregulate JAK2 expression and this might provide novel targets for therapeutic intervention in JAK2V617F-negative myeloproliferative disorders.

In addition we also found that FOXO3 activation resulted in increased IL-3Rα surface expression, suggesting that FOXO3-mediated JAK2 expression increases IL3 sensitivity. It has been demonstrated that JAK2 stabilises surface expression of the EpoR, Tpo-R, GHR and G-CSFR (He *et al.*, 2005; Huang *et al.*, 2001; Tong *et al.*, 2006; Meenhuis *et al.*, 2009). The stabilisation of Epo-R expression depends on the interaction of JAK2 with four residues in the cytosolic domain of the immature Epo-R in the endoplasmic reticulum (Huang *et al.*, 2001). Three of these residues constitute a hydrophobic motif which is highly conserved among various cytokine receptors (Constantinescu *et al.*, 2001). However, since this motif is not present in the IL3-R, the mechanism of JAK2-mediated stabilisation of IL3-R expression awaits further research. Our finding that FOXO3 is involved in feedback signalling provides a novel mechanism for haematopoietic cells to regulate proliferation by responding rapidly to changes in cytokine levels. When IL-3 is abundant, activation of the IL3-R and subsequent activation of PI3K and PKB promote proliferation by inhibiting FOXO3. However, during IL-3 deprivation inactivation of PI3K and PKB triggers activation of FOXO3 thereby inhibiting proliferation. Meanwhile, the activation of FOXO3 upregulates JAK2 expression and increases IL-3R surface expression, which act to “prime” the cells for further activation allowing them to respond rapidly when IL-3 is again available (Fig. 9).

FOXO-mediated feedback control of growth factor signalling pathways has previously been described to play a role in insulin signalling in *D melanogaster* (Puig *et al.*, 2003). The PI3K-PKB-FOXO signalling module is evolutionary conserved and in the fruit fly activation of the conserved insulin receptor DAF-2 activates PI3K-PKB signalling resulting in inhibition of the FOXO orthologue dFOXO. In the fruit fly dFOXO has been found to directly regulate insulin receptor (InR) expression. The dFOXO-mediated control of InR expression creates a feedback loop, which allows a rapid response to nutrients after fasting (Puig *et al.*, 2003; Puig and Tjian, 2005). This observation is reminiscent of our data showing that FOXO-mediated upregulation of JAK2 in mammalian cells.

In conclusion, utilising comparative microarray analyses after inducible activation of inducible active FOXO3 and FOXO4 we have identified subsets of genes which were differentially regulated by FOXO3 and FOXO4. qRT-PCR results confirmed and extended the microarray analysis and indicate that FOXO3 and FOXO4 modulate the expression of both overlapping and non-overlapping transcriptional targets within a single cell type. These results show that FOXO3 and FOXO4 have both redundant and non-redundant functions, which are important for multiple cellular processes.

MATERIALS AND METHODS

Constructs and cell lines

pcDNA3-HA-FOXO3(A3)-ER has been described previously (Dijkers *et al.*, 2000). pcDNA3-HA-FOXO4(A3)-ER was generated by cloning FOXO4(A3) without the stopcodon into pcDNA3 containing the hormone-binding domain of the estrogen receptor (pcDNA3-ER). Ba/F3 cells were cultured in RPMI-1640 containing glutamax (Invitrogen), 10% fetal bovine serum (Hyclone, Logan, UT), penicillin and streptomycin (Invitrogen), 0.5 mM β-mercaptoethanol (Merck, Darmstadt, Germany) and recombinant mouse IL-3 produced in Cos cells (Caldenhoven *et al.*, 1995). For the generation of clonal Ba/F3 cells stably expressing FOXO3(A3)-ER or FOXO4(A3)-ER, the pcDNA3-HA-FOXO3(A3)-ER construct

and the pcDNA3-HA-FOXO3(A3)-ER construct were electroporated into Ba/F3 cells. Cells were maintained in the presence of 1 mg/ml G418 (Gibco, Paisley, UK) and clonal lines were generated by limited dilution. DLD1 cells were cultured in DMEM containing glutamax (Invitrogen), 10% fetal bovine serum and penicillin and streptomycin (Invitrogen). DLD1 cells expressing FOXO3(A3)-ER have been described previously (Kops *et al.*, 2002b).

RNA isolation, microarray analysis and quantitative RT-PCR

Cells were stimulated as indicated an harvested at the respective times, washed twice with PBS, lysed in 1 ml Trizol (Invitrogen, Breda, The Netherlands). For isolation of total RNA samples were incubated at room temperature for 3 minutes, 0.2 ml chloroform was added, vortexed and centrifuged for 15 minutes at 8000 rpm at 4°C. Subsequently, the aqueous phase was transferred to new tubes, 0.5 ml isopropanol was added and incubated for minimal 30 minutes at -20°C. Samples were centrifuged for 10 minutes at 14000 rpm at 4°C. The pellet was washed with 70% ethanol and dissolved in water. DNAse treatment and purification was performed using Qiagen's RNeasy kit (Qiagen Inc., Valencia, CA).

For microarray analysis, RNA was amplified, labeled and hybridised on Corning UltraGAPS slides containing Mouse 70-mer oligos (Operon, Mouse V2 AROS) as previously described (Raaben *et al.*, 2007). Hybridized slides were scanned on an Agilent scanner (G2565AA) at 100% laser power, 30% PMT. After data extraction using Imagene 8.0 (BioDiscovery), printtip Loess normalization was performed (Yang *et al.*, 2002) on mean spot-intensities. Data was analysed using ANOVA (R version 2.2.1/MAANOVA version 0.98-7)(http://www.r-project.org/). In a fixed effect analysis, sample, array and dye effects were modeled. P-values were determined by a permutation F2-test, in which residuals were shuffled 5000 times globally. Genes with $p < 0.05$ after family wise error correction were considered significantly changed.

For qRT-PCr analysis equal amounts of total RNA were reversed transcribed with SuperScript III reverse transcriptase (Invitrogen, Breda, the Netherlands). The resulting cDNA was amplified using an Biorad Icycler with the primer pairs for mouse Arhgap9, Crem, Nsbp1, Cdca7, Stk38, Pmch, Hbp1, Clk1, Eps8, JAK2, Uhrf1 and B2M a nonregulated housekeeping gene (primers sequences can be found in supplemental data).

Functional Analysis of the FOXO3 and FOXO4 regulated genes

Functional Analysis identified the biological functions and/or diseases that were significantly associated to the data set. Genes from the dataset that were 1.7 fold changed upon FOXO3 and FOXO4 activation and were significantly associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

Antibodies and reagents

Antibodies against actin and Id1 were from Santa Cruz (Palo Alto, CA). Antibody against p27 was from BD Biosciences (San Diego, CA). JAK2 antibody was from Cell Signalling and Bim antibody was from ABR. 4-OHT was obtained from Sigma-Aldrich (Missouri, USA). Murine IL-3 was obtained from Peprotech (New Jersey, USA). LY294002 was obtained from Cayman Chemical (Ann Arbor, MI).

Western blotting

Western blot analysis was performed using standard techniques. In brief, Ba/F3 cells were lysed in Laemmli buffer (0.12 M Tris HCl pH 6.8, 4% SDS and 20% glycerol, 35 mM β-mercaptoethanol and bromophenol blue) and boiled for 5 min. DLD1 cells were lysed in 1x sample buffer (2% SDS, 10%glycerol, 2% β-mercaptoethanol, 60 mM Tris pH 6.8 and bromophenolblue) Equal amounts of total lysate were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with the appropriate antibodies according to the manufacturer's conditions. Membranes were washed, incubated with appropriate secondary antibodies and developed by ECL (Amersham Pharmacia, Amersham, UK).

Reporter assay

For transient reporter assays HEK293 cells were transfected with 6xDBE-luciferase together with pcDNA3-HA-FOXO3(A3)-ER and pcDNA3-HA-FOXO4(A3)-ER) and renilla to normalize for transfection efficiency by calcium phosphate precipitation method. After 24 hours stimulation with 4-OHT (100nM), cells were washed twice with PBS, lysed in 100 µL passive lysis buffer and assayed for luciferase activity using Dual-Luciferase Reporter Assay System (Promega).

Analysis of IL-3Rα cell surface expression
Ba/F3 cells expressing FOXO3(A3)-ER were stimulated as indicated, washed and stained with an IL-3R antibody (Santa Cruz). Cells were again washed and FACS analysis was performed (FACS Calibur, Beckton Dickinson, Alphen a/d Rijn, The Netherlands).

Statistical analysis
Data are expressed as mean ± SEM. Significant differences between mean values were evaluated using 1-way ANOVA followed by Dunnet test (for serial analysis of one variable) or 2-way ANOVA followed by Bonferoni post test (for serial analysis of two variables). * $p < 0.05$ and ** $p < 0.01$

REFERENCES

Ananthanarayanan,B., Fosbrink,M., Rahdar,M., and Zhang,J. (2007). Live-cell molecular analysis of Akt activation reveals roles for activation loop phosphorylation. J. Biol. Chem. *282*, 36634-36641.

Anderson,M.J., Viars,C.S., Czekay,S., Cavenee,W.K., and Arden,K.C. (1998). Cloning and characterization of three human forkhead genes that comprise an FKHR-like gene subfamily. Genomics *47*, 187-199.

Baxter,E.J., Scott,L.M., Campbell,P.J., East,C., Fourouclas,N., Swanton,S., Vassiliou,G.S., Bench,A. J., Boyd,E.M., Curtin,N., Scott,M.A., Erber,W.N., and Green,A.R. (2005). Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet *365*, 1054-1061.

Birkenkamp,K.U., Essafi,A., van,d., V, da,C.M., Hui,R.C., Holstege,F., Koenderman,L., Lam,E. W., and Coffer,P.J. (2007). FOXO3a induces differentiation of Bcr-Abl-transformed cells through transcriptional down-regulation of Id1. J. Biol. Chem. *282*, 2211-2220.

Brunet,A., Bonni,A., Zigmond,M.J., Lin,M.Z., Juo,P., Hu,L.S., Anderson,M.J., Arden,K.C., Blenis,J., and Greenberg,M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.

Brunet,A., Sweeney,L.B., Sturgill,J.F., Chua,K.F., Greer,P.L., Lin,Y., Tran,H., Ross,S.E., Mostoslavsky,R., Cohen,H.Y., Hu,L.S., Cheng,H.L., Jedrychowski,M.P., Gygi,S.P., Sinclair,D.A., Alt,F.W., and Greenberg,M.E. (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.

Buchsbaum,R.J. (2007). Rho activation at a glance. J. Cell Sci. *120*, 1149-1152.

Burgering,B.M. and Coffer,P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature *376*, 599-602.

Caldenhoven,E., van,D.T., Raaijmakers,J.A., Lammers,J.W., Koenderman,L., and De Groot,R.P. (1995). Activation of the STAT3/acute phase response factor transcription factor by interleukin-5. J. Biol. Chem. *270*, 25778-25784.

Calnan,D.R. and Brunet,A. (2008). The FoxO code. Oncogene *27*, 2276-2288.

Castrillon,D.H., Miao,L., Kollipara,R., Horner,J.W., and DePinho,R.A. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science *301*, 215-218.

Constantinescu,S.N., Huang,L.J., Nam,H., and Lodish,H.F. (2001). The erythropoietin receptor cytosolic juxtamembrane domain contains an essential, precisely oriented, hydrophobic motif. Mol. Cell *7*, 377-385.

Delpuech,O., Griffiths,B., East,P., Essafi,A., Lam,E.W., Burgering,B., Downward,J., and Schulze,A. (2007). Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression. Mol. Cell Biol. *27*, 4917-4930.

Dijkers,P.F., Medema,R.H., Lammers,J.W., Koenderman,L., and Coffer,P.J. (2000). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1.

Curr. Biol. *10*, 1201-1204.

Engelman,J.A. (2009). Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat. Rev. Cancer *9*, 550-562.

Essers,M.A., Weijzen,S., De Vries-Smits,A.M., Saarloos,I., de Ruiter,N.D., Bos,J.L., and Burgering,B. M. (2004). FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J. *23*, 4802-4812.

Furuyama,T., Nakazawa,T., Nakano,I., and Mori,N. (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem. J. *349*, 629-634.

Geijsen,N., Koenderman,L., and Coffer,P.J. (2001). Specificity in cytokine signal transduction: lessons learned from the IL-3/IL-5/GM-CSF receptor family. Cytokine Growth Factor Rev. *12*, 19-25.

Greer,E.L. and Brunet,A. (2005). FOXO transcription factors at the interface between longevity and tumor suppression. Oncogene *24*, 7410-7425.

He,K., Loesch,K., Cowan,J.W., Li,X., Deng,L., Wang,X., Jiang,J., and Frank,S.J. (2005). Janus kinase 2 enhances the stability of the mature growth hormone receptor. Endocrinology *146*, 4755-4765.

Hosaka,T., Biggs,W.H., III, Tieu,D., Boyer,A.D., Varki,N.M., Cavenee,W.K., and Arden,K.C. (2004). Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc. Natl. Acad. Sci. U. S. A *101*, 2975-2980.

Huang,L.J., Constantinescu,S.N., and Lodish,H.F. (2001). The N-terminal domain of Janus kinase 2 is required for Golgi processing and cell surface expression of erythropoietin receptor. Mol. Cell *8*, 1327-1338.

Ihle,J.N. and Gilliland,D.G. (2007). Jak2: normal function and role in hematopoietic disorders. Curr. Opin. Genet. Dev. *17*, 8-14.

James,C., Ugo,V., Le Couedic,J.P., Staerk,J., Delhommeau,F., Lacout,C., Garcon,L., Raslova,H., Berger,R., naceur-Griscelli,A., Villeval,J.L., Constantinescu,S.N., Casadevall,N., and Vainchenker,W. (2005). A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature *434*, 1144-1148.

Knoops,L., Hornakova,T., Royer,Y., Constantinescu,S.N., and Renauld,J.C. (2008). JAK kinases overexpression promotes in vitro cell transformation. Oncogene *27*, 1511-1519.

Kops,G.J., Dansen,T.B., Polderman,P.E., Saarloos,I., Wirtz,K.W., Coffer,P.J., Huang,T.T., Bos,J.L., Medema,R.H., and Burgering,B.M. (2002a). Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature *419*, 316-321.

Kops,G.J., de Ruiter,N.D., De Vries-Smits,A.M., Powell,D.R., Bos,J.L., and Burgering,B.M. (1999). Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature *398*, 630-634.

Kops,G.J., Medema,R.H., Glassford,J., Essers,M.A., Dijkers,P.F., Coffer,P.J., Lam,E.W., and Burgering,B.M. (2002b). Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol. Cell Biol. *22*, 2025-2036.

Kralovics,R., Passamonti,F., Buser,A.S., Teo,S.S., Tiedt,R., Passweg,J.R., Tichelli,A., Cazzola,M., and Skoda,R.C. (2005). A gain-of-function mutation of JAK2 in myeloproliferative disorders. N. Engl. J. Med. *352*, 1779-1790.

Levine,R.L., Wadleigh,M., Cools,J., Ebert,B.L., Wernig,G., Huntly,B.J., Boggon,T.J., Wlodarska,I., Clark,J.J., Moore,S., Adelsperger,J., Koo,S., Lee,J.C., Gabriel,S., Mercher,T., D'Andrea,A., Frohling,S., Dohner,K., Marynen,P., Vandenberghe,P., Mesa,R.A., Tefferi,A., Griffin,J.D., Eck,M.J., Sellers,W.R., Meyerson,M., Golub,T.R., Lee,S.J., and Gilliland,D.G. (2005). Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell *7*, 387-397.

Lin,L., Hron,J.D., and Peng,S.L. (2004). Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. Immunity. *21*, 203-213.

Littlewood,T.D., Hancock,D.C., Danielian,P.S., Parker,M.G., and Evan,G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res. *23*, 1686-1690.

Manning,B.D. and Cantley,L.C. (2007). AKT/PKB signaling: navigating downstream. Cell *129*, 1261-1274.

Martinez-Moczygemba,M. and Huston,D.P. (2003). Biology of common beta receptor-signaling cytokines: IL-3, IL-5, and GM-CSF. J. Allergy Clin. Immunol. *112*, 653-665.

Medema,R.H., Kops,G.J., Bos,J.L., and Burgering,B.M. (2000). AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. Nature *404*, 782-787.

Meenhuis,A., Irandoust,M., Wolfler,A., Roovers,O., Valkhof,M., and Touw,I.P. (2009). Janus kinases promote cell-surface expression and provoke autonomous signalling from routing-defective G-CSF receptors. Biochem. J. *417*, 737-746.

Modur,V., Nagarajan,R., Evers,B.M., and Milbrandt,J. (2002). FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. J. Biol. Chem. *277*, 47928-47937.

Obsil,T. and Obsilova,V. (2008). Structure/function relationships underlying regulation of FOXO transcription factors. Oncogene *27*, 2263-2275.

Paik,J.H., Ding,Z., Narurkar,R., Ramkissoon,S., Muller,F., Kamoun,W.S., Chae,S.S., Zheng,H., Ying,H., Mahoney,J., Hiller,D., Jiang,S., Protopopov,A., Wong,W.H., Chin,L., Ligon,K.L., and DePinho,R.A. (2009). FoxOs cooperatively regulate diverse pathways governing neural stem cell homeostasis. Cell Stem Cell *5*, 540-553.

Paik,J.H., Kollipara,R., Chu,G., Ji,H., Xiao,Y., Ding,Z., Miao,L., Tothova,Z., Horner,J.W., Carrasco,D. R., Jiang,S., Gilliland,D.G., Chin,L., Wong,W.H., Castrillon,D.H., and DePinho,R.A. (2007). FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell *128*, 309-323.

Parganas,E., Wang,D., Stravopodis,D., Topham,D.J., Marine,J.C., Teglund,S., Vanin,E.F., Bodner,S., Colamonici,O.R., van Deursen,J.M., Grosveld,G., and Ihle,J.N. (1998). Jak2 is essential for signaling through a variety of cytokine receptors. Cell *93*, 385-395.

Potente,M., Urbich,C., Sasaki,K., Hofmann,W.K., Heeschen,C., Aicher,A., Kollipara,R., DePinho,R. A., Zeiher,A.M., and Dimmeler,S. (2005). Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. J. Clin. Invest *115*, 2382-2392.

Puig,O., Marr,M.T., Ruhf,M.L., and Tjian,R. (2003). Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. Genes Dev. *17*, 2006-2020.

Puig,O. and Tjian,R. (2005). Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. Genes Dev. *19*, 2435-2446.

Raaben,M., Groot Koerkamp,M.J., Rottier,P.J., and de Haan,C.A. (2007). Mouse hepatitis coronavirus replication induces host translational shutoff and mRNA decay, with concomitant formation of stress granules and processing bodies. Cell Microbiol. *9*, 2218-2229.

Ramaswamy,S., Nakamura,N., Sansal,I., Bergeron,L., and Sellers,W.R. (2002). A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. Cancer Cell *2*, 81-91.

Renault,V.M., Rafalski,V.A., Morgan,A.A., Salih,D.A., Brett,J.O., Webb,A.E., Villeda,S.A., Thekkat,P. U., Guillerey,C., Denko,N.C., Palmer,T.D., Butte,A.J., and Brunet,A. (2009). FoxO3 regulates neural stem cell homeostasis. Cell Stem Cell *5*, 527-539.

Schmandke,A., Schmandke,A., and Strittmatter,S.M. (2007). ROCK and Rho: biochemistry and

neuronal functions of Rho-associated protein kinases. Neuroscientist. *13*, 454-469.

Stephens,L., Anderson,K., Stokoe,D., Erdjument-Bromage,H., Painter,G.F., Holmes,A.B., Gaffney,P. R., Reese,C.B., McCormick,F., Tempst,P., Coadwell,J., and Hawkins,P.T. (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. Science *279*, 710-714.

Tong,W., Sulahian,R., Gross,A.W., Hendon,N., Lodish,H.F., and Huang,L.J. (2006). The membrane-proximal region of the thrombopoietin receptor confers its high surface expression by JAK2-dependent and -independent mechanisms. J. Biol. Chem. *281*, 38930-38940.

Tothova,Z., Kollipara,R., Huntly,B.J., Lee,B.H., Castrillon,D.H., Cullen,D.E., McDowell,E.P., Lazo-Kallanian,S., Williams,I.R., Sears,C., Armstrong,S.A., Passegue,E., DePinho,R.A., and Gilliland,D. G. (2007). FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell *128*, 325-339.

Tran,H., Brunet,A., Grenier,J.M., Datta,S.R., Fornace,A.J., Jr., DiStefano,P.S., Chiang,L.W., and Greenberg,M.E. (2002). DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science *296*, 530-534.

van der Vos and Coffer,P.J. (2008). FOXO-binding partners: it takes two to tango. Oncogene *27*, 2289-2299.

Witthuhn,B.A., Quelle,F.W., Silvennoinen,O., Yi,T., Tang,B., Miura,O., and Ihle,J.N. (1993). JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell *74*, 227-236.

Yang,Y.H., Dudoit,S., Luu,P., Lin,D.M., Peng,V., Ngai,J., and Speed,T.P. (2002). Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Res. *30*, e15.

Zhao,R., Xing,S., Li,Z., Fu,X., Li,Q., Krantz,S.B., and Zhao,Z.J. (2005). Identification of an acquired JAK2 mutation in polycythemia vera. J. Biol. Chem. *280*, 22788-22792.

SUPPLEMENTAL DATA:

Primers used for qRT-PCR:

Gene	Forward primer	Reverse primer
Arhgap9	CAGACAGAGACTTTGACTAGGGA	GTGGATCGAGCCATTCCTGAG
Cdca7	AGAACTCGACCTCTTACCAGG	CGTCGTTCATGTAGCTGTCCAT
Clk1	TCAGAGTGGAGACGTACTAAGTG	TGGGGGTCTGTTGTATTCAAGT
Crem	ATGTCTTGAAAATCGTGTGGCT	TGGCAATAAAGGTCTTTGAGGG
Eps8	TCTTCACCACCCTATTCCCAG	CATCTTTCCGATCCAGCACGA
GS	TGAACAAAGGCATCAAGCAAATG	CAGTCCAGGGTACGGGTCTT
Hbp1	ACAAATCAGATGCCTAATGCAGT	TAGGGTCACTTTGAACAGCCT
JAK1	GAAGGGCCGCTACAGCCTGC	AGGCTGCCACTCCTGGGCTT
JAK2	GAAGCTCCTCTGCTTGATGAC	AACGCACTTTGGTAAGAATGTCT
JAK3	CTGCATAGAGGACGTGGACA	CCAAGTCCAGCACGGCCAGG
Nsbp1	CCCGACTGTCTGCTATGCC	CCACGTTGCATTCGTCTTTAAC
Pmch	GTCTGGCTGTAAAACCTTACCTC	CCTGAGCATGTCAAAATCTCTCC
Stk38	CCAGAGGTGTTCATGCAGACG	GGAGCAGAATGGTGGGTAGC
TYK2	CGGTGGACTTCCAGCGGCTC	TGGCTGGCAGTGCGTCTTGG
Uhrf1	CCACACCGTGAACTCTCTGTC	GGCGCACATCATAATCGAAGA

CHAPTER 5 - APPENDIX

Identification of transcriptional pathway targets through comparative microarray analysis after specific activation of multiple components of the PI3K-PKB-FOXO pathway

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Phosphoinsositol-3- kinase has been linked to a variety of cellular functions, including cell growth, proliferation, differentiation, motility, survival and intracellular trafficking (reviewed in Engelman, 2009). Many of these functions relate to the ability of PI3K to activate protein kinase B (PKB, also know as c- Akt). After activation by growth factors and cytokines, PI3K activation results in phosphorylation and activation of PKB (Burgering and Coffer, 1995). The activation of PKB causes an increased proliferation and survival by regulation of proteins involved in cell cycle regulation, apoptosis and metabolism (reviewed in Manning and Cantley, 2007). The effect of PKB on survival and proliferation is mediated by inhibitory phosphorylation of Forkhead Box O (FOXO) transcription factors; FOXO1, FOXO3 and FOXO4 (Brunet *et al.*, 1999; Kops *et al.*, 1999). Phosphorylation of FOXOs by PKB induces binding of 14-3-3 proteins thereby blocking DNA binding and nuclear import and consequently inhibition of FOXO function (reviewed in Obsil and Obsilova, 2008).

In order to identify novel transcriptional targets regulated by the PI3K-PKB-FOXO pathway we performed microarray analyses after inducible activation of the key components in this pathway. Microarray technology can measure the differential expression of large numbers of genes and thereby identify downstream effectors, which expression is altered upon activation of a specific protein. Typically, microarray experiments produce long lists of genes that are differentially expressed between two different situations. To identify novel transcriptional targets of the PI3K-PKB-FOXO pathway, we have generated several cell lines expressing key inducibly active components in this pathway. By comparative analysis of PI3K, PKB and FOXO-regulated transcripts we were able to focus on transcriptional target genes that are regulated in a true pathway-dependent manner, thereby reducing the amount of genes and providing more insight in the biological processes regulated by true pathway target genes.

To be able to selectively activate PI3K and PKB, cell lines were made stably expressing an inducible active PI3K (myrPI3K-ER) or expressing inducible active PKBα (myrPKB-ER) coupled to the hormone binding domain of the estrogen receptor. In addition, cell lines expressing inducible constitutively active FOXO3 and FOXO4 constructs were made by mutating the three inhibitory PKB phosphorylation sites to alanines (FOXO3(A3)-ER and FOXO4(A3)-ER) and fusion to the hormone binding domain of the estrogen receptor.

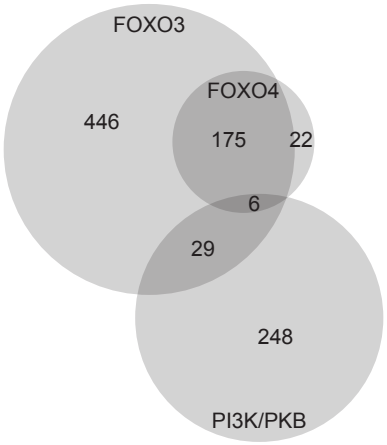

Figure 1. The number of genes regulated by activation of PI3K, PKB, FOXO3 and FOXO4
Ba/F3 cells expressing myrPI3K-ER, myrPKB-ER, FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT (100 nM) for 4 hours (PI3K, PKB) or 8 hours (FOXO3, FOXO4), RNA was isolated and microarray analyses performed. Shown are the number of genes, which were either upregulated by both PI3K and PKB while downregulated by either FOXO3 or FOXO4 or vice versa (more than 1.7 fold). Shown are the results of one experiment performed in quadruplicate.

Coupling of these signalling molecules to the hormone binding domain of the estrogen receptor (ER) causes recruitment of heat shock proteins maintaining the proteins in an inactive state. Addition of the estrogen analogue 4-hydroxytamoxifen (4-OHT), results in rapid dissociation of heat shock proteins and activation of the signalling molecules (Littlewood *et al.*, 1995). To characterise transcriptional targets of the PI3K-PKB-FOXO pathway we have performed microarray analyses after activation of either PI3K, PKB, FOXO3 or FOXO4 with 4-hydroxy-tamoxifen (4-OHT). To identify transcripts which are true targets of the PI3K-PKB-FOXO module we have focussed on genes which were more than 1.7 fold changed and were either upregulated by both PI3K and PKB while downregulated by either FOXO3 or FOXO4 or *vice versa*. Using this unique pathway approach we have identified 35 genes whose expression was regulated in a PI3K-PKB-FOXO dependent manner (Fig. 1). Among the identified transcriptional targets were Mxi1 and PTEN-induced kinase1 (PINK1), which have both been recently described to be regulated by FOXOs (Delpuech *et al.*, 2007; Mei *et al.*, 2009).

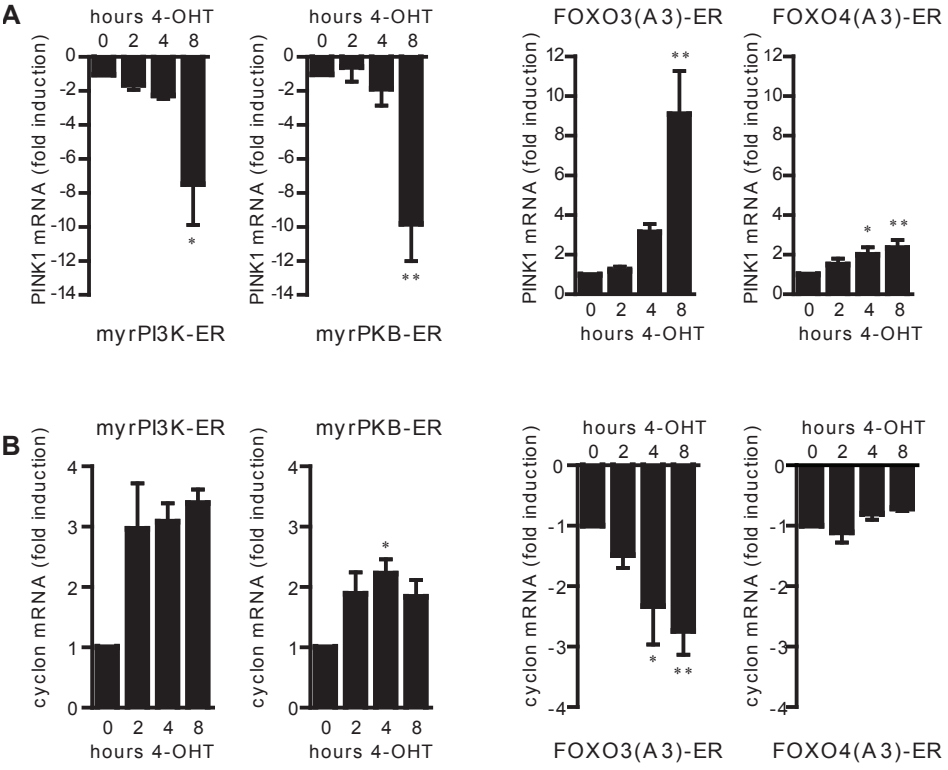

Figure 2. PINK1 and cyclon mRNA expression is regulated by the PI3K-PKB-FOXO signalling module

(A,B) Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 100 nM 4-OHT in the presence of mIL-3 (5 ng/ml) for the indicated times. RNA was isolated and relative mRNA levels of PINK1 **(A)** and cyclon **(B)** were analyzed using quantitative PCR. Data are represented as mean ± SEM values normalized for B_2M of at least three experiments performed with technical duplicates. * $p < 0.05$ and ** $p < 0.01$

To validate the microarray results and to further examine the regulation of the selected genes over time, Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT, RNA was isolated and mRNA expression of PINK1 and cyclon was analyzed by quantitative RT-PCR (qRT-PCR). As shown in Figure 2A activation of either PI3K or PKB resulted in a clear downregulation of PINK1 mRNA levels. In contrast, FOXO3 activation resulted in an large increase in PINK1 mRNA expression, while FOXO4 activation resulted in a small but significant increase. The *PINK1* gene encodes a serine/threonine protein kinase and mutations in this gene have been found in patients with autosomal recessive early-onset Parkinson disease. PINK1 localizes to mitochondria and is involved in survival of neuronal cells possibly by protecting cells from stress-induced mitochondrial dysfunction (reviewed in Bueler, 2009). Recently, it was found that in T cells FOXO3 regulates PINK1 mRNA expression through direct binding to its promoter (Mei *et al.*, 2009). Knockdown of PINK1 expression sensitized cells to IL-2 withdrawal-induced cell death, suggesting that in lymphocytes regulation of Pink1 expression by FOXOs is important in cellular survival after IL-2 deprivation (Mei *et al.*, 2009). These results are in agreement with our data, indicating that FOXO3 regulates PINK1 expression in both human and murine lymphoid cells. Since mutations in this gene are linked to Parkinson, further research is required to elucidate the role of regulation of PINK1 expression by FOXO3 in neuronal cells.
Furthermore, we looked at mRNA expression levels of cytokine-induced protein with coiled-coil domain (cyclon). Cyclon was identified as a cytokine-inducible gene which expression was upregulated after IL-3 stimulation of Ba/F3 cells (Hoshino and Fujii, 2007). Ba/F3 cells expressing myrPI3K-ER and myrPKB-ER were stimulated with 4-OHT and the mRNA expression of cyclon was examined by qRT-PCR analysis. Activation of either PI3K or PKB resulted in an increase in cyclon mRNA expression. In contrast, FOXO3 activation downregulated cyclon mRNA expression, while FOXO4 had no effect on expression levels, suggesting that cyclon is specifically regulated by FOXO3.

To further investigate the regulation of cyclon expression by FOXOs, Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were transduced with a cyclon promoter reporter construct that contains the cyclon promoter in front of GFP and a CMV promoter in front of CD2 as a control for transduction efficiency. The cells were either deprived from IL-3 or stimulated with IL-3 in the presence or absence of 4-OHT for 24 hours. Stimulation of Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER with IL-3 resulted in an increase of cyclon promoter activity which was suppressed by activation of FOXO3. Surprisingly, although after FOXO4 activation no change in cyclon mRNA expression was observed, the promoter reporter assay did show suppression of cyclon promoter activity by FOXO4. In the microarray analysis cyclon expression was found to be only 1.3 fold changed by FOXO4 activation, which might be below the threshold required for detection by q/RT-PCR, but still sufficient to downregulate the promoter activity. Until now, no function has been discovered for cyclon and the role of this protein in cytokine signalling awaits further research.

Here we describe for the first time a global microarray analysis of multiple components of one signal transduction cascade in a single cell type. By comparative analysis of the PI3K, PKB, FOXO3 and FOXO4 datasets and focussing on genes which are regulated in a reciprocal manner by all components of the pathway we generated a list of true “pathway” targets. Utilising this unique pathway approach we have identified 35 genes which expression was regulated in a PI3K-PKB-FOXO dependent manner. The finding of previously described transcriptional targets indicates that this approach is a valid method to identify

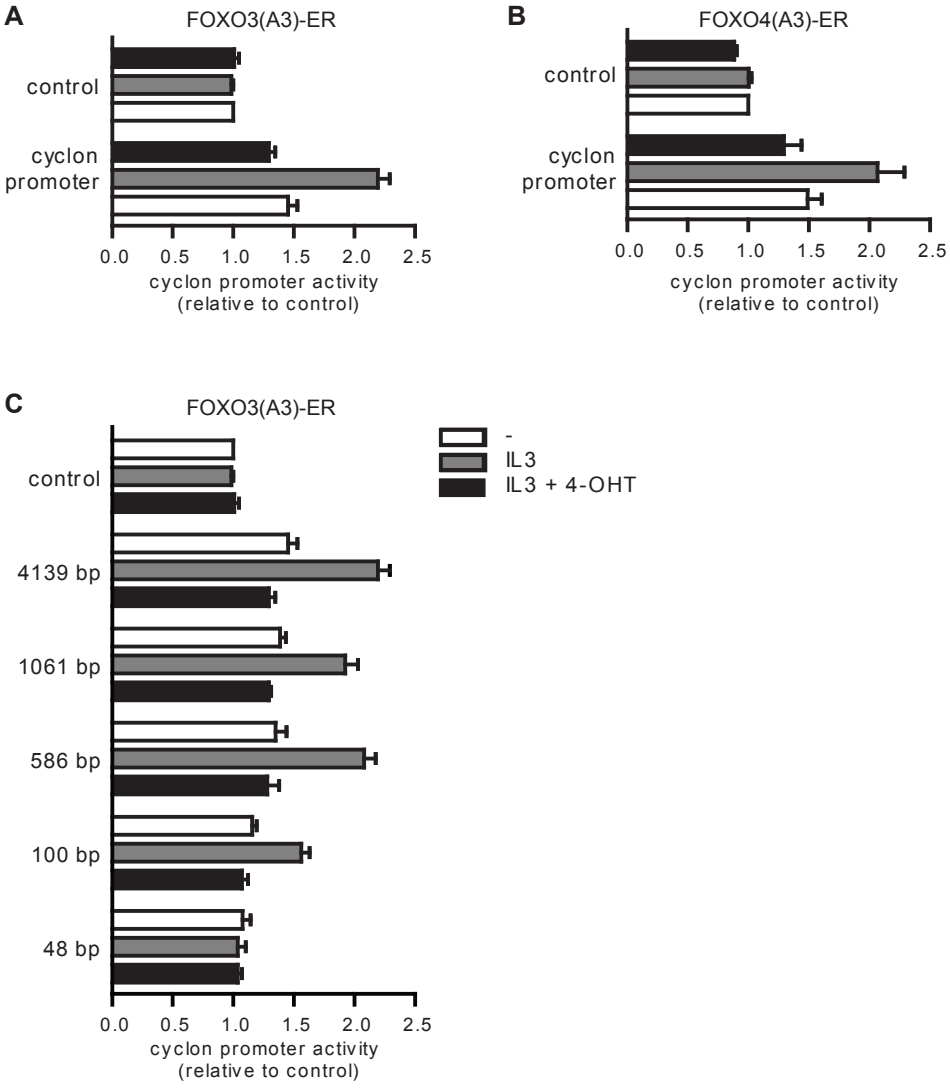

Figure 3. FOXO3 suppresses the cyclon promoter
(A,B,C) Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were transduced with a cyclon promoter reporter construct that contains the cyclon promoter in front of GFP and a CMV promoter in front of CD2. Subsequently, cells were cultured either in the presence of IL3 , stimulated with 4-OHT (100 nM) or starved from IL3. After 24 hours GFP and CD2 expression were determined by FACS analysis. The geomean fluorescence intensity of GFP from CD2 positive cells was divided by the geomean fluorescence of CD2 to correct for transduction efficiency. Shown are the mean ± SEM of three independent experiments.

FOXO targets. In conclusion, the comparative analysis of regulated transcripts after inducible activation of the key components in the PI3K-PKB-FOXO pathway is a powerful approach to identify promising novel transcriptional targets of this signalling module and characterisation of these might provide new insights in the downstream biological processes.

METHODS

Constructs and cell culture
SRα-myrp110α-ER, SRα-myrPKB-ER and pcDNA3-HA-FOXO3(A3)-ER have been described previously (Dijkers *et al.*, 2000a; Dijkers *et al.*, 2000b). pcDNA3-HA-FOXO4(A3)-ER was generated by cloning FOXO4(A3) without the stopcodon into pcDNA3 containing the hormone-binding domain of the estrogen receptor (pcDNA3-ER). Ba/F3 cells were cultured in RPMI-1640 containing glutamax (Invitrogen), 10% fetal bovine serum (Hyclone, Logan, UT), penicillin and streptomycin (Invitrogen), 0.5 mM β-mercaptoethanol (Merck, Darmstadt, Germany) and recombinant mouse IL-3 produced in Cos cells. For the generation of clonal Ba/F3 cells stably expressing myrPI3K-ER or myrPKB-ER, the SRα-myrp110α-ER construct and the SRα-myrPKB-ER construct were electroporated into Ba/F3 cells together with pSG5 conferring neomycin resistance. For the generation of clonal Ba/F3 cells stably expressing FOXO3(A3)-ER or FOXO4(A3)-ER, the pcDNA3-HA-FOXO3(A3)-ER construct and the pcDNA3-HA-FOXO3(A3)-ER construct were electroporated into Ba/F3 cells. Cells were maintained in the presence of 1 mg/ml G418 (Gibco, Paisley, UK) and clonal lines were generated by limited dilution. The pd1EGFP/EF-CD2 construct containing different lengths of the cyclon promoter has been described previously (Hoshino and Fujii, 2007).

RNA isolation, microarray analysis and qRT-PCR
Ba/F3 cells expressing myrPI3K-ER and myrPKB-ER were cytokine starved overnight and stimulated with 4-OHT (100 nM). Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT (100 nM) in the presence of mIL-3 (5 ng/ml). At the respective time cell were washed twice with PBS and lysed in 1 ml Trizol (Invitrogen, Breda, The Netherlands). RNA was isolated and microarray analysis was performed as described in Chapter 5.
Equal amounts of total RNA were reversed transcribed with SuperScript III reverse transcriptase (Invitrogen, Breda, the Netherlands). The resulting cDNA was amplified using an Biorad Icycler with the primer pairs for mouse PINK1 and cyclon and B2M, a nonregulated housekeeping gene, was used as an internal control to normalize input RNA.

Cyclon promoter reporter assay
Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were transfected with cyclone promoter reporter. After 24 hours cells were washed with PBS, split in three and were left untreated or stimulated with IL-3 (5 ng/ml) in the presence or absence of 4-OHT (100 nM). After 8 hours cells were stained for CD2 expression or isotype control for 30 minutes at 4C and FACS analysis was performed (FACS Calibur, Beckton Dickinson, Alphen a/d Rijn, The Netherlands).

Statistical analysis
Data are expressed as mean ± SEM. Significant differences between mean values were evaluated using 1-way ANOVA followed by Dunnet test. * $p < 0.05$ and ** $p < 0.01$

REFERENCE LIST

Brunet,A., Bonni,A., Zigmond,M.J., Lin,M.Z., Juo,P., Hu,L.S., Anderson,M.J., Arden,K.C., Blenis,J., and Greenberg,M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell *96*, 857-868.

Bueler,H. (2009). Impaired mitochondrial dynamics and function in the pathogenesis of Parkinson's disease. Exp. Neurol. *218*, 235-246.

Burgering,B.M. and Coffer,P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature *376*, 599-602.

Delpuech,O., Griffiths,B., East,P., Essafi,A., Lam,E.W., Burgering,B., Downward,J., and Schulze,A. (2007). Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression. Mol. Cell Biol. *27*, 4917-4930.

Dijkers,P.F., Medema,R.H., Lammers,J.W., Koenderman,L., and Coffer,P.J. (2000a). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol. *10*, 1201-1204.

Dijkers,P.F., Medema,R.H., Pals,C., Banerji,L., Thomas,N.S., Lam,E.W., Burgering,B.M., Raaijmakers,J.A., Lammers,J.W., Koenderman,L., and Coffer,P.J. (2000b). Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol. Cell Biol. *20*, 9138-9148.

Engelman,J.A. (2009). Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat. Rev. Cancer *9*, 550-562.

Hoshino,A. and Fujii,H. (2007). Redundant promoter elements mediate IL-3-induced expression of a novel cytokine-inducible gene, cyclon. FEBS Lett. *581*, 975-980.

Kops,G.J., de Ruiter,N.D., De Vries-Smits,A.M., Powell,D.R., Bos,J.L., and Burgering,B.M. (1999). Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature *398*, 630-634.

Littlewood,T.D., Hancock,D.C., Danielian,P.S., Parker,M.G., and Evan,G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res. *23*, 1686-1690.

Manning,B.D. and Cantley,L.C. (2007). AKT/PKB signaling: navigating downstream. Cell *129*, 1261-1274.

Mei,Y., Zhang,Y., Yamamoto,K., Xie,W., Mak,T.W., and You,H. (2009). FOXO3a-dependent regulation of Pink1 (Park6) mediates survival signaling in response to cytokine deprivation. Proc. Natl. Acad. Sci. U. S. A *106*, 5153-5158.

Obsil,T. and Obsilova,V. (2008). Structure/function relationships underlying regulation of FOXO transcription factors. Oncogene *27*, 2263-2275.

CHAPTER 6

FOXO3 induces differentiation of Bcr-Abl transformed cells through transcriptional down-regulation of Id1

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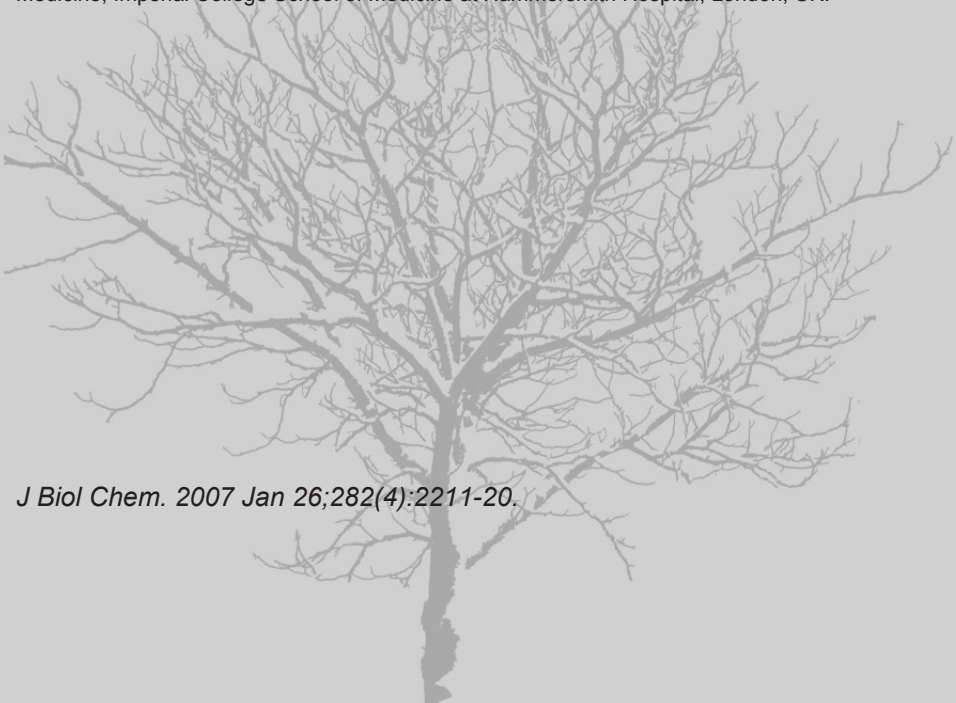

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ABSTRACT

Leukemic transformation often requires activation of protein kinase B (PKB/c-akt) and is characterized by increased proliferation, decreased apoptosis and a differentiation block. PKB phosphorylates and inactivates members of the FOXO subfamily of Forkhead transcription factors. It has been suggested that hyperactivation of PKB maintains the leukemic phenotype through actively repressing FOXO-mediated regulation of specific genes. We have found expression of the transcriptional repressor Id1 (inhibitor of DNA-binding 1) to be abrogated by FOXO3 activation. Inhibition of PKB activation or growth factor deprivation also resulted in strong down-regulation of Id1 promoter activity, Id1 mRNA and protein expression. Id1 is highly expressed in Bcr-Abl transformed K562 cells, correlating with high PKB activation and FOXO3 phosphorylation. Inhibition of Bcr-Abl by the chemical inhibitor STI571 resulted in activation of FOXO3 and down-regulation of Id1 expression. By performing ChIP assays and promoter-mutation-analysis, we demonstrate that FOXO3 acts as a transcriptional repressor by direct binding to the Id1 promoter. STI571 treatment, or expression of constitutively active FOXO3, resulted in erythroid differentiation of K562 cells, which was inhibited by ectopic expression of Id1. Taken together our data strongly suggest that high expression of Id1, through PKB-mediated inhibition of FOXO3, is critical for maintenance of the leukemic phenotype.

INTRODUCTION

Homeostasis of the hematopoietic system requires tight control of proliferation, differentiation and survival of progenitor cells (1-5). Combinations of cytokines acting on a progenitor cell, initiate a specific developmental program through activation of distinct downstream signal transduction pathways (6). Interference with this highly regulated process can lead to the development of hematopoietic malignancies. Myeloid transformation is often associated with chromosomal translocations and somatic mutations, affecting gene expression in ways that lead to defects in normal programs of cell proliferation, differentiation and survival (7-12). For instance, Chronic myeloid leukemia (CML) is a lethal hematopoietic stem cell malignancy characterized by the t(9;22) chromosomal translocation, a translocation between the long arms of chromosomes 9 and 22, resulting in the formation of the Philadelphia (ph) chromosome and the fusion of a truncated *bcr* gene to the 5'-upstream sequences of the second exon of *c-abl* ((9;13). The *bcr-abl* fusion gene is known to be essential to the pathogenesis of CML and the Bcr-Abl protein demonstrates constitutively active kinase activity, which is essential and sufficient for malignant transformation (9;13;14). Bcr-Abl exerts diverse actions on hematopoietic cells in terms of cellular transforming activity; inhibition of apoptosis, cell cycle progression, altered cell migration and adhesion to extracellular matrix (9;13;14). Expression of Bcr-Abl results in growth factor independence of cells and activates multiple signaling cascades, including the phosphatidylinositol 3-kinase (PI3K)/ protein kinase B (PKB/c-akt) signaling pathway (15;16).

In non-malignant cells activation of the PI3K/PKB signaling module is stimulated by growth factors and cytokines and has been linked to regulation of cellular proliferation and survival in a diverse variety of cell systems (17-19). Recently, it has been demonstrated that the members of the FOXO subfamily of transcription factors FOXO1, FOXO3 and FOXO4 are directly phosphorylated by PKB (20;21). In the absence of growth or survival factors FOXOs are unphosphorylated, localized in the nucleus, and transcriptionally active. Upon stimulation with growth factors or cytokines, PKB activity is induced, it translocates to the nucleus and phosphorylates FOXOs, leading to inhibition of transcriptional activity and nuclear export (22). We and others have demonstrated that FOXO transcription factors can regulate a variety of genes that influence cellular proliferation (e.g. $p27^{Kip1}$ and cyclinD), survival (e.g.FasL and Bim), metabolism (e.g. PEPCK and G6Pase) and responses to stress (e.g. MnSOD and catalase) (21;22).

Myeloid leukemic cells are characterized not only by uncontrolled proliferation and resistance to apoptosis, but also by a block in differentiation (7;10). Since the PI3K/PKB pathway is constitutively activated in leukemic cells (23;24), this suggests that hyperactivation of the PI3K/PKB pathway maintains the leukemic phenotype not only by regulating proliferation and apoptosis but also by actively repressing a set of genes regulating hematopoiesis. Importantly, PI3K signaling is crucial for transformation of Bcr-Abl. Skorski *et al* demonstrated that inhibition of PI3K downstream signaling by ectopic expression of dominant-negative PKB inhibited Bcr-Abl-dependent transformation of murine bone marrow cells *in vitro* and suppressed leukemia development in SCID mice (25). In addition, recently it has been shown that FOXO3 was constitutively phosphorylated and therefore inactive in cell lines expressing Bcr-Abl (26;27). This suggests that inhibition of FOXO3 transcriptional activity may be required to maintain the leukemic phenotype. Therefore, we looked for novel target genes of FOXO3 that might indeed play a role in regulating hematopoiesis.

In order to identify novel transcriptional target genes of the FOXO transcription factor FOXO3, cDNA microarray analysis was performed using a bone marrow derived cell line stably expressing an inducible active FOXO3 mutant. We found the transcriptional repressor Id1 (inhibitor of differentiation) (28) to be a direct transcriptional target of FOXO3. Here we show that the transcriptional down-regulation of Id1 by FOXO3 is required for the induction of differentiation of leukemic cells. We show that expression of a constitutively active FOXO3 mutant induced differentiation of Bcr-Abl transformed cells. Conversely, constitutive expression of Id1 inhibited differentiation. Taken together, our data strongly suggest that the high expression of Id1, through a PI3K/PKB-mediated inhibition of FOXO3 is critical for maintenance of the leukemic phenotype.

RESULTS

Identification of Id1 as a novel transcriptional target of FOXO3

We and others have found that enforced expression of FOXO activity can induce either cell cycle arrest or apoptosis, depending on the cell type (22;35-37). However, recently a role for FOXOs in regulating differentiation of several cell types has been described (36;38). In order to identify novel transcriptional targets of FOXO3 that might be involved in hematopoiesis, we made use of a bone marrow-derived cell line (Ba/F3 cells) stably expressing an inducible form of active FOXO3, in which all three PKB-phosphorylation sites were mutated to alanine, FOXO3(A3):ER* (29). Addition of 4-hydroxy tamoxifen (4-OHT) to these cells results in the rapid induction of FOXO3 transcriptional activity, promoting induction of FOXO target genes. To identify novel transcriptional target genes of FOXO3, cDNA derived from the FOXO3(A3):ER* expressing cells stimulated for 0 or 2 hours with 100nM 4-OHT were hybridized to custom-made DNA microarrays containing 15.000 cDNAs (data not shown) (39). Use of this short time point increases the chance of identifying direct FOXO targets. Interestingly, we identified the transcriptional repressor Id1 (Inhibitor of DNA binding) as one of the genes that was most prominently down-regulated (11.6-fold; data not shown). Id1 transcript levels were confirmed by real-time PCR. We analyzed the kinetics of FOXO3-mediated Id1 down-regulation by analyzing FOXO3(A3):ER* cells that were treated with 4-OHT for the times indicated (Fig. 1). FOXO3 activation resulted in a very rapid down-regulation of Id1 mRNA, that was maintained for at least 24 hours.

Id1 protein levels are down-regulated by FOXO3 activation

Next we examined whether the effect of FOXO3 on Id1 mRNA was also reflected at the protein level. Ba/F3-FOXO3(A3):ER* cells were stimulated with 4-OHT as indicated,

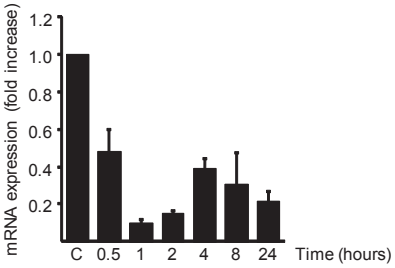


Figure 1. Id1 mRNA expression is inhibited by FOXO3 activation.
Ba/F3-FOXO3(A3):ER* cells were treated for the indicated times with 4-OHT (100nM), RNA was isolated, cDNA synthesized and real time PCR was performed using specific primers for Id1. Results were normalized for the housekeeping gene GAPDH. The results reflect the relative change of Id1 from three independent experiments ± S.E.M.

protein was isolated and subsequently analyzed for Id1 expression. As shown in Figure 2a, addition of 4-OHT resulted in a striking and rapid down-regulation of Id1 protein levels. In contrast, another target gene of FOXO3, the cell cycle inhibitor p27 was strongly up-regulated upon 4-OHT treatment. Furthermore, other treatments resulting in FOXO3 activation, such as cytokine deprivation (Fig. 2B), or inhibition of PI3K by the chemical inhibitor LY294002 (Fig. 2C), had similar effects on Id1 protein expression, although with distinct kinetics reflecting activation of FOXO3.
These data demonstrate that FOXO3-induced inhibition of Id1 mRNA is also reflected at the protein level. The very rapid down-regulation of Id1 protein is to be expected since it has been described to be very unstable; Id1 having a half-life of approximately 30 minutes (40).

Activation of FOXO3 inhibits transcriptional activity of the Id1 promoter
Although we have demonstrated regulation of Id1 mRNA by FOXO3, we wished to determine if FOXO3 can also modulate Id1 promoter activity. COS cells were transiently transfected with a luciferase reporter construct under the control of the Id1 promoter, together with increasing concentrations of a constitutively active FOXO3 mutant. As shown in Figure 3A, ectopic expression of the active FOXO3(A3) mutant strongly inhibited Id1 promoter activity in a dose-dependent manner. Similarly, in Ba/F3 cells stably expressing FOXO3(A3):ER* addition of 4-OHT resulted in a strong down-regulation of Id1 promoter activity (Fig. 3B). Deprivation of IL-3 also resulted in down-regulation of Id1 promoter activity in Ba/F3 wild type cells (Fig. 3B). Together these data strongly suggest that the FOXO3-induced inhibition of Id1 expression is through direct inhibition of Id1 promoter activity.

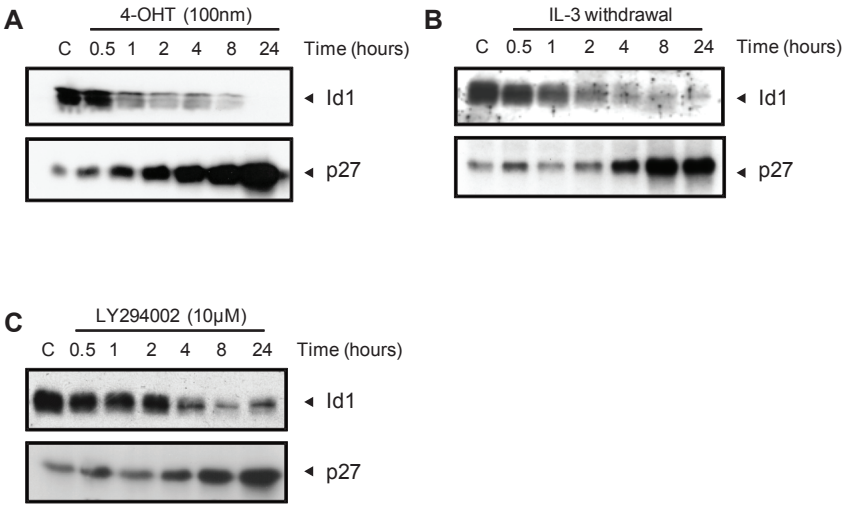


Figure 2. Id1 protein levels are inhibited upon FOXO3 activation.
(A) Ba/F3-FOXO3(A3):ER* cells were cultured in the presence of IL-3, treated with 4-OHT (100nM) for the indicated times, lysed and equal amounts of protein were analyzed for levels of Id1 or p27kip1. **(B)** Ba/F3 cells were cytokine-starved for the indicated times, lysed and analyzed as above. **(C)** Ba/F3 cells were cultured in the presence of IL-3, treated with LY294002 (10μM) for the indicated times, lysed and analyzed as above.

Constitutive Id1 expression contributes to the leukemic phenotype

The Id transcriptional repressors (Id1 – Id4) have been suggested to act at the checkpoint at which undifferentiated progenitor cells make the commitment to terminal differentiation (28;41;42). Indeed, we have recently observed that the down-regulation of Id1 is required during myelopoiesis (30). It is now well established that members of the Id family are over-expressed in a range of human tumors, and Id1 is the family member most widely over-expressed in human cancers. However, a direct link between Id1 expression and chronic myeloid leukemia has not yet been clearly proven. Therefore, we examined whether hyperactivation of the PI3K/PKB signaling module might contribute to the chronic myeloid leukemic (CML) phenotype. We questioned whether the constitutive inhibition of FOXO3, which would result in high expression of Id1 may be responsible for maintaining cells in an undifferentiated state. In accordance with recently published data (26;27), we found that in the human Bcr-Abl expressing leukemic cell line K562, PKB was strongly activated as shown by its high phosphorylation status, while its downstream target FOXO3 was inactive (Fig. 4A). Treatment of cells with the chemical inhibitor STI571 (43), which specifically inhibits Bcr-Abl kinase activity, resulted in dephosphorylation and therefore inhibition of PKB (Fig. 4A). In addition, upon STI571 treatment FOXO3 was dephosphorylated and therefore activated (Fig. 4A), as shown by up-regulation of p27, a direct target gene (Fig. 4B). Importantly, activation of FOXO3 was accompanied by a dramatic down-regulation of Id1 protein levels (Fig. 4B), which was also reflected at mRNA (Fig. 4C). In addition, stimulation of K562 cells stably expressing FOXO3(A3):ER* (29), with 4-OHT resulted in strong down-regulation of Id1 mRNA (data not shown) and protein expression (Fig. 4D), while in cells expressing the empty vector no effect on Id1 expression was observed, demonstrating that sole activation of FOXO3 is enough to down-regulate Id1 expression. These data demonstrate that indeed in leukemic Bcr-Abl expressing cells high Id1 expression is observed correlating with high PI3K/PKB activity.

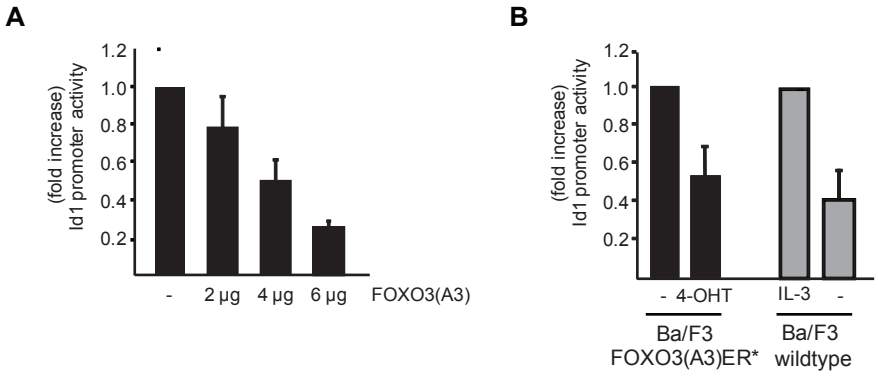


Figure 3. FOXO3 down-regulates Id1 promoter activity.
(A) COS cells were transfected with 2µg of pGL3-Id1 together with the indicated concentrations of pECE-FOXO3(A3). 24 hours after transfection luciferase activity was analyzed as described in materials and methods. **(B)** BaF3-FOXO3(A3):ER* cells(left) or BaF3 cells (right) were electroporated with 16 µg of pGL3-Id1 plasmid together with 500ng of pRL-TK plasmid as an internal transfection control. BaF3-FOXO3(A3):ER* cells were cultured for 24 hours with IL-3 in the absence or presence of 4-OHT (100nM) and BaF3 cells in the absence or presence of IL-3 before analyzing luciferase activity as described in material and methods. The results reflect the relative luciferase activity from 3 or more independent experiments ± S.E.M.

FOXO3 directly regulates expression of Id1

To investigate whether FOXO3 regulates Id1 expression through direct binding to its promoter, we analysed the Id1 promoter region for putative FOXO-binding sites. We found four potential sites at positions -134bp to -128 bp upstream of the ATG (site 1), one at position -565bp to -559bp (site 2), and one at position -1509bp to -1503bp (site 3). One nearly perfect sequence at position -1627bp to -1621bp (site 4) was found allowing a 2–bp mismatch compared with the consensus sequence TTGTTTAC (37) (Fig. 5A).

To determine whether addition of STI571 was also sufficient to regulate Id1 promoter activity, K562 cells were transfected with either a full-length, or a truncated, Id1 promoter reporter construct. Addition of STI571 resulted in inhibition of both full-length and truncated Id1 promoter activity (Fig. 5B). The shorter promoter construct (-353) only contained a single FOXO binding element (site 1). This suggests that site 1 is the critical binding

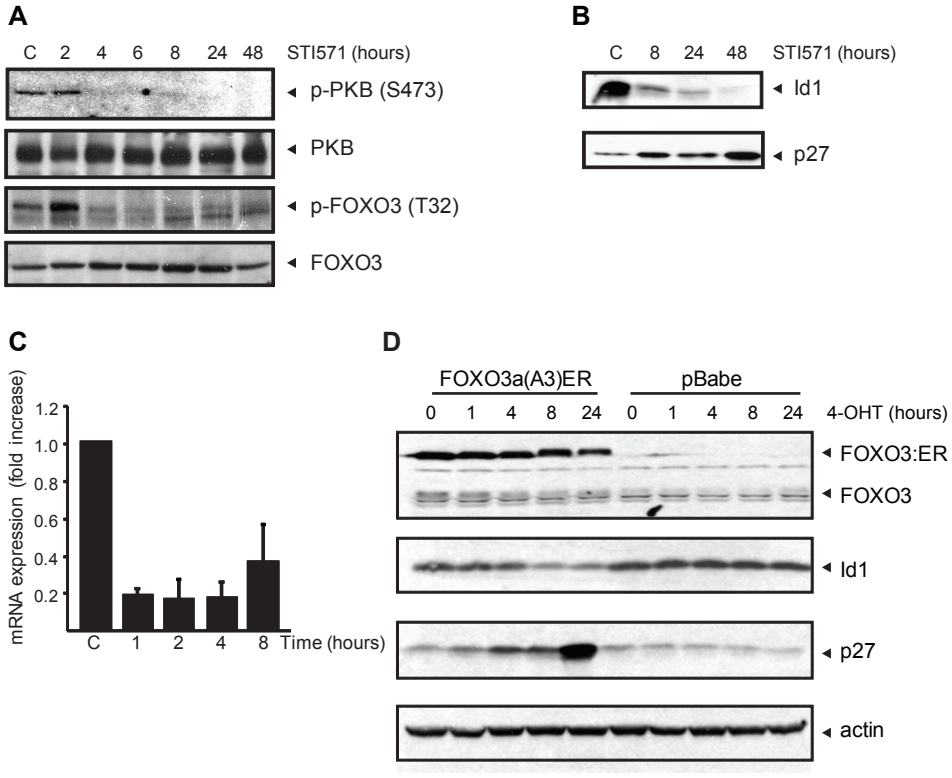
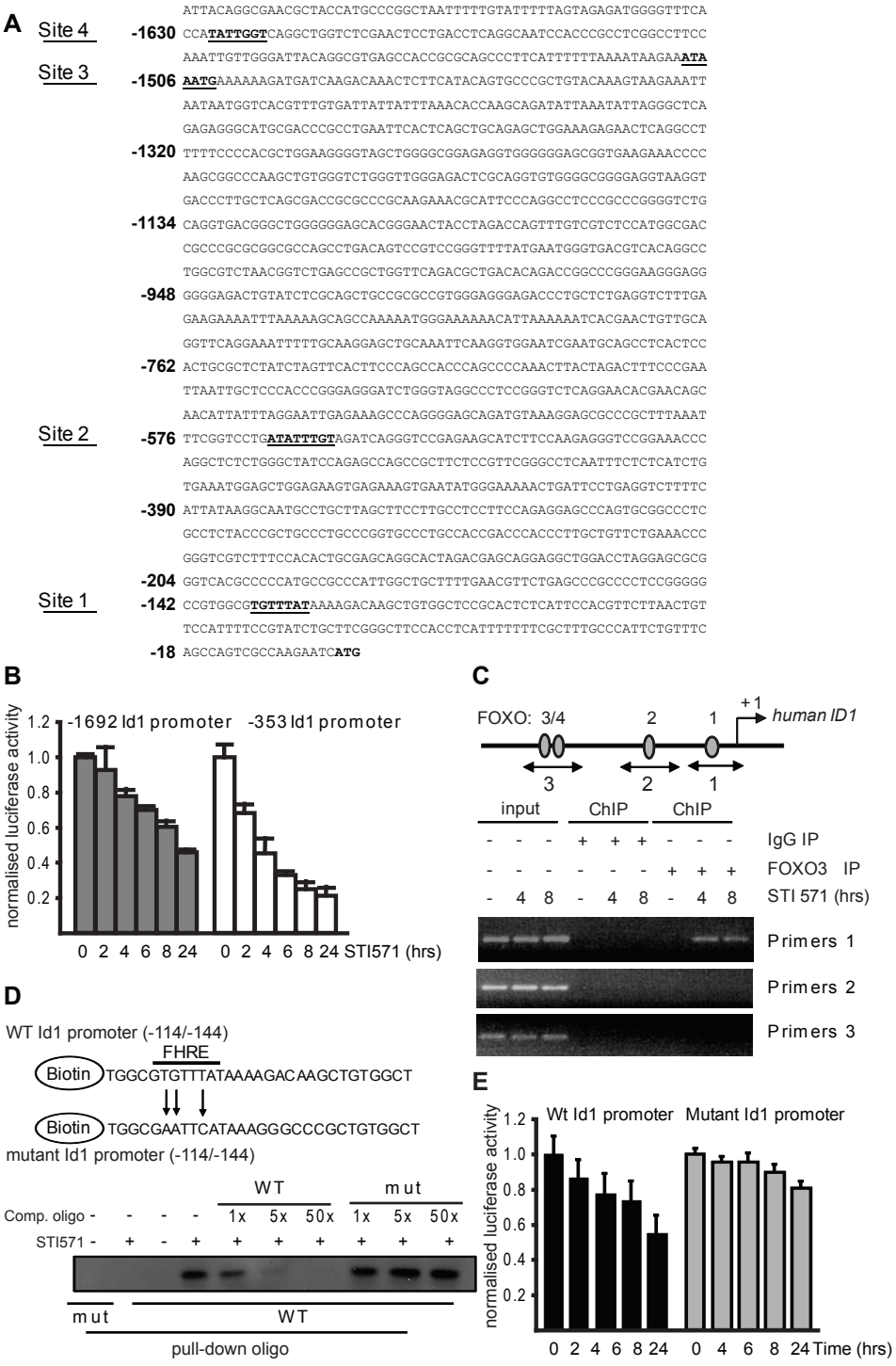


Figure 4. Id1 expression is down-regulated upon STI571 treatment

(A,B) K562 cells were cultured with or without STI571 (5μM) for the indicated times. Cells were lysed and equal amounts of protein were analyzed for levels of phospho-PKB(Ser473), total PKB, phospho-FOXO3(Thr32), total FOXO3, Id1 or p27kip1. **(C)** K562 cells were cultured with or without STI571 (5μM) for the indicated times, RNA was isolated, cDNA synthesized and real time PCR was performed using specific primers for Id1. Results were normalized for the housekeeping genes βactin and GAPDH. The results reflect the relative change of Id1 from three independent experiments ± S.E.M. **(D)** K562 cells were generated expressing FOXO3(A3):ER or control vector (pBABE). Cells were treated with 4-OHT (100nM) for the times indicated and cell lysates prepared. Samples were analyzed for FOXO3, Id1 and p27 expression by SDS-PAGE and Western botting.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FHRE
Biotin
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TGGCGAATTCATAAAGGGCCCGCTGTGGCT
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site for FOXO3-mediated Id1 repression. In order to examine whether FOXO3 directly associates with the Id1 promoter we investigated whether STI571 treatment of K562 cells influences occupation of the four potential FOXO3 binding sites of the Id1 promoter (Fig. 5A) by Chromatin Immunoprecipitation (ChIP) assay. Protein-DNA complexes were formaldehyde-cross-linked, and sites bound by FOXO3 were immunoprecipitated with the appropriate antibodies. PCR primer pairs were designed to detect selectively the four different potential FOXO3 binding sites in the human Id1 gene. In untreated cells, no FOXO3 binding to the Id1 promoter was detected (Fig. 5C). STI571 treatment resulted in a strong association of FOXO3 only to the binding site most proximal to the ATG of the Id1 promoter (site 1). In contrast, the other three sites did not demonstrate any association with FOXO3 (Fig. 5B; Primers 2,3). This is in support of the promoter deletion analysis (Fig. 5B).

While the ChIP analysis confirms that FOXO3 is associated with the Id1 promoter *in vivo*, it does not distinguish between direct DNA binding or indirect association with promoter-bound complexes. To confirm that direct FOXO3 DNA-binding to the Id1 promoter (site 1) occurs, we performed an oligonucleotide “pull-down assay”. As shown in Figure 5D, FOXO3 only associated with the Id1 promoter site 1 when the FHRE binding site was intact. Mutation of the FHRE resulted in abrogation of FOXO3 binding.

In order to demonstrate that binding of FOXO3 to site 1 (-134bp to -128bp) is required for down-regulation of the Id1 promoter, the TTT core sequence was mutated to CGT in the Id1 promoter sequence. As shown in Figure 5e, STI571 treatment of K562 cells resulted in down-regulation of Id1 promoter activity, while no significant effect was observed on the mutated Id1 promoter.

Taken together, these experiments demonstrate that upon STI571-induced FOXO3 activation, FOXO3 directly associates with the Id1 promoter resulting in inhibition of Id1 expression.

FOXO3 is required for STI571 mediated inhibition of Id1 expression

To prove that FOXO3 activity is required for STI571-induced down-regulation of Id1, K562 cells were stably transfected with a vector expressing FOXO3 RNAi duplexes and the effect on Id1 expression was examined. As shown in Figure 6, transfection of cells with FOXO3 RNAi resulted in complete abrogation of FOXO3 protein expression. Interestingly, STI571 treatment had no effect on Id1 protein expression in these K562 FOXO3 knock-down cells. This in contrast to the control cells where Id1 protein expression was strongly

Figure 5. Id1 is a direct transcriptional target of FOXO3

(A) Sequence of the Id1 promoter region. The potential FOXO binding sites are indicated in bold and underlined. **(B)** K562 cells were electroporated with 16µg pGL3-Id1 -1692 or pGL3-Id1 -353 together with 500ng of pRL-TK plasmid as an internal transfection control. Cells were cultured with or without STI571 (5µM) for the indicated times before analyzing luciferase activity as described in material and methods. The results reflect the relative luciferase activity from three independent experiments ± S.E.M. **(C)** Chromatin IP’s were performed for the different FOXO binding sites. K562 cells were treated with STI571 for four or eight hours. Protein·DNA complexes were formaldehyde-cross-linked *in vivo*. Chromatin fragments from these cells were subjected to immunoprecipitation with a control antibody or antibodies to FOXO3 as indicated. After cross-link reversal, the co-immunoprecipitated DNA was amplified by PCR (resolved in 2% agarose gel). **(D)** Nuclear extracts were prepared from K562 cells treated with STI571 and analysed by “pull-down assay” using either wild-type or mutated Id1 FHRE oligonucleotide probes as described in Materials and Methods. Samples were analysed for FOXO3 binding by western blot. **(E)** K562 cells were electroporated with 16µg of either the wild-type or the mutated Id1 promoter. Cells were cultured with or without STI571(5µM) for the indicated times, before analyzing luciferase activity as described in Materials and Methods. The results reflect the relative luciferase activity from three independent experiments ± S.E.M.

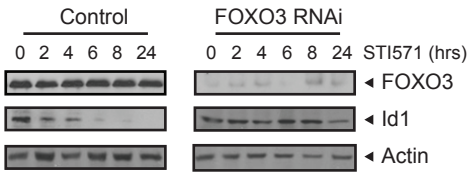

Figure 6. FOXO3 is required for STI571-mediated inhibition of Id1 expression.
Wild-type or FOXO3 knock-down K562 cells were treated with STI571 (5μM) as indicated. Protein lysates were made and analyzed for expression of FOXO3 and Id1 as described in Materials and Methods.

down-regulated after STI571 treatment. These data clearly demonstrate that Id1 is indeed a direct target of FOXO3 and that FOXO3 activity is required for STI571-mediated Id1 down-regulation.

FOXO3 activation induces differentiation of Bcr-Abl transformed cells

Next we questioned whether the Bcr-Abl-mediated inhibition of FOXO3 maintains cells in an undifferentiated state and therefore contributes to the leukemic phenotype. Conversely, whether the activation of FOXO3 and the subsequent down-regulation of Id1 can induce differentiation of K562 cells. Treatment with STI571 resulted in FOXO3 activation and Id1 down-regulation (Fig. 4). K562 cells can be differentiated into the erythrocytic lineage by treatment with STI571, characterized by the expression of hemoglobin. Using K562 cells expressing a 4-OHT inducible FOXO3(A3):ER mutant, addition of 4-OHT resulted in a dramatic increase in the number of cells expressing hemoglobin (Fig. 7A). Furthermore, activation of FOXO3 also resulted in down-regulation of Id1 levels in K562 cells (Fig. 7B).

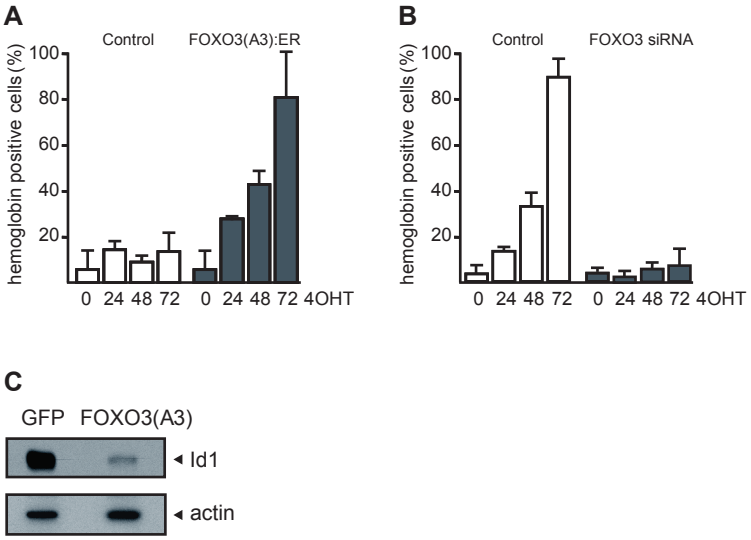

Figure 7. FOXO3 activation induces differentiation of Bcr-Abl transformed cells
(A) K562 cells expressing either eGFP or FOXO3(A3):ER were treated with 4-OHT as indicated and cells analyzed for hemoglobin expression as described in Materials and Methods. The results reflect the amount of hemoglobin from three independent experiments ± S.E.M. **(B)** K562 cells were transduced with either pLZRS-eGFP or pLZRS-FOXO3(A3) and 48 hours after transduction the GFP-positive cells were selected by FACS and analysed for Id1 protein expression. **(C)** K562 cells expressing control vector or FOXO3 siRNA were treated with STI571 (5μM) for the times indicated. Cells were subsequently harvested and the percentage of cells expressing hemoglobin was determined. The results reflect the amount of hemoglobin from three independent experiments ± S.E.M.

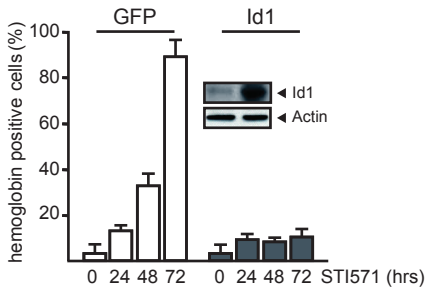

Figure 8. Constitutive Id1 expression inhibits differentiation
K562 cells were transduced with either pLZRS-eGFP or pLZRS-Id1 and 48 hours after transduction the GFP-positive cells were selected by FACS. Subsequently, cells were treated with or without STI571 (5μM) for the indicated times. Cells were subsequently harvested and the percentage of cells expressing hemoglobin was determined. The results reflect the amount of hemoglobin from three independent experiments ± S.E.M. The inset shows increased expression of Id1 in Id1-transduced cells (right lane), relative to control cells (left lane).

To determine whether FOXO3 activity is required for STI571-induced K562 differentiation, we utilized the K562 cells stably transfected with a vector expressing FOXO3 RNAi duplexes (Fig. 6). As shown in Figure 7C, while addition of STI571 lead to a dramatic increase in hemoglobin expression, this was abrogated in the absence of FOXO3.
Taken together these data demonstrate that FOXO3 is both necessary and sufficient for STI571 induced differentiation of K562 cells.

Constitutive Id1 expression maintains Bcr-Abl transformed cells in an undifferentiated state
To determine whether expression of Id1 indeed plays a critical role in maintaining the undifferentiated state of K562 cells, a bicistronic retroviral DNA construct co-expressing eGFP and Id1 was utilized to generate retrovirus and subsequently to infect K562 cells. Two days after infection the eGFP positive cells were selected by FACS and subsequently incubated with or without STI571. As shown in Figure 8, treatment with STI571 resulted in induction of hemoglobin expression. However, in K562 cells transduced with Id1, hemoglobin expression was reduced. These data demonstrate that the expression of Id1 indeed plays a critical role in maintaining the undifferentiated state of K562 cells.

DISCUSSION

Myeloid malignancies are often associated with mutations in upstream signaling components, as for instance Bcr-Abl in CML, which result in constitutive activation of the PI3K/PKB pathway (7-9;15). Leukemic cells are in addition to uncontrolled proliferation and resistance to apoptosis, also characterized by a block in differentiation. The PI3K/PKB signaling pathway plays a crucial role in proliferation and apoptosis and mediates these effects through modulation of the activity of FOXO transcription factors (22;25). Hyperactivation of PKB results in inhibition of FOXO, which we suggest is critical in mediating the transforming activity of PI3K/PKB. We suggest that PKB maintains the leukemic phenotype through FOXO3-mediated regulation of differentiation specific genes. In the present study we aimed to identify novel transcriptional targets of the FOXO3 transcription factor that could play a role in differentiation of hematopoietic cells. Our hypothesis was that inhibition of such genes are likely to play a critical role in the maintenance of the leukemic phenotype. We identified the inhibitor of differentiation Id1 to be a direct target of FOXO3, and could demonstrate that upon activation of FOXO3, Id1 expression is strongly reduced, both at the transcriptional and protein level. The fact that in FOXO3 knockdown cells Id1 was not down-regulated anymore clearly demonstrates that FOXO3 is indeed required for

Id1 down-regulation. Furthermore, by performing ChIP assays and promoter mutations we also provide evidence that FOXO3 directly binds to the Id1 promoter. Our findings are in contrast to the hypothesis of Ramaswamy *et al*.(44), which suggested that FOXOs down-regulate genes not through direct binding to the DNA, but through modulation of co-factors. However, we provide evidence that the FOXO3-mediated down-regulation of Id1 is indeed through direct binding to the promoter. Of course, we cannot rule out the possibility that FOXO3 in addition also regulates the activity of additional transcription factors or co-factors. In accordance with our data are the results of Shi *et al*. who demonstrated that the forkhead transcription factor Foxp1 directly binds to the *c-fms* promoter and functions as transcriptional repressor, thereby controlling monocyte differentiation (45). Treatment of K562 cells with the Bcr-Abl inhibitor STI571 resulted in dephosphorylation and therefore activation of FOXO3. This correlated with down-regulation of Id1 and induction of erythroid differentiation (Fig. 4 and 7). Specific activation of a constitutively active FOXO3 was sufficient to reverse the leukemic phenotype and to induce differentiation (Fig. 7a). We provide evidence that Id1 expression is a critical factor in maintaining the undifferentiated state of K562 cells. Ectopic expression of Id1 resulted in inhibition of STI571-induced differentiation, which suggests that the down-regulation of Id1 is a pre-requisite for STI-571-induced differentiation (Fig. 8).

While involvement of the PI3K/PKB signaling module in Bcr-Abl mediated transformation has been demonstrated, this has previously been placed in the context of apoptosis and proliferation, but a direct link with differentiation has not been demonstrated (23;25;47;48). Skorski *et al* demonstrated that inhibition of PI3K signaling by ectopic expression of dominant-negative PKB suppressed Bcr-Abl-dependent colony formation *in vitro*. Similarly, dominant negative PKB was shown to inhibit leukemia development in SCID mice that were injected with bone-marrow cells expressing Bcr-Abl. Our data strongly suggest that constitutive expression of Id1 is critical for maintenance of the myeloid phenotype and ectopic expression of FOXO3 can reverse this phenotype.
Recently it has been shown that FOXO3 can play a role in erythroid differentiation through up-regulation of the B cell translocation gene 1 (BTG1) protein (38). Bakker *et al*. demonstrated that BTG1 can modulate protein arginine methylation activity, which they propose is a novel mechanism regulating erythroid differentiation. The authors suggest that BGT1-mediated activation of enzymes which induce methylation could contribute to epigenetic gene regulation including condensation of the nucleus and enucleation late in erythroid differentiation. However, we demonstrate regulation of differentiation through FOXO3-mediated modulation of Id1 expression. This directly regulates the activity of basic helix-loop-helix (bHLH) transcription factors that specifically induce expression of differentiation-linked genes (28;41;42). The Id transcriptional repressors have been suggested to act at the checkpoint at which undifferentiated progenitor cells make the commitment to terminal differentiation. Id1 expression is high in proliferating, undifferentiated cells, whereas its expression is down-regulated as cells differentiate (38;49;50). For example, ectopic expression of Id1 inhibits B-cell development and differentiation of muscle and mammary epithelial cells (42). More recently *in vivo* studies using targeted expression of Id1 to thymocytes (51), intestinal epithelia (52) and B-lymphocytes (53) of mice have demonstrated inhibition of cellular differentiation in these systems (41;42). In addition, very recently it was demonstrated that Id1 plays a role in myelopoiesis (30;49). Down-regulation of Id1 was required for normal myelopoiesis and the current study suggests a model whereby high expression of Id1 maintains leukemic cells in an undifferentiated state.
Id proteins do not possess a DNA binding domain and thereby function as dominant-

negative regulators of bHLH proteins (28;41;42). They can dimerize with bHLH transcription factors and inhibit bHLH-dependent expression of differentiation-linked genes. However, relatively little is known concerning the specific molecular mechanisms by which Id1 regulates hematopoiesis. In other cell types bHLH proteins have been identified that can bind to Id1. Id1 inhibits Ets-mediated transcription of p16INK4a, a tumor suppressor (50). In addition, the Id1 target MyoD activates $p21^{Waf1/Cip1}$ gene expression in myoblasts and its partner E2A positively regulates $p21^{Waf1/Cip1}$ transcription in fibroblasts (54). This regulation of p21 by E2A is antagonized by Id1, suggesting that Id1 may stimulate proliferation through antagonism of E2A-dependent p21 expression. The bHLH that is primarily expressed in hematopoietic cells is SCL/TAL1 (38). However, ectopic expression of SCL/TAL1 in the HL-60 granulocytic cell line resulted in enhanced proliferation, not differentiation (55). It remains to be seen which bHLH Id1 binding partners are inhibited in Bcr-Abl transformed cells.

Although it has recently been shown by Kuzelova *et al*. that STI571 can induce, in addition to cell cycle arrest and apoptosis, erythroid differentiation of K562 cells, they do not provide evidence concerning which signal transduction pathways are mediating this effect (56). Here we clearly show that STI571-induced erythroid differentiation is mediated through the FOXO3-mediated down-regulation of Id1. Treatment of CML patients with STI571 is now a common treatment strategy. Although complete remissions are observed upon treatment with STI571 in patients with CML blast crisis, most patients enjoy only a short duration of response, with eventual emergence of STI571-resistant leukemic cells and a clinical relapse (43;57). Therefore, new treatment strategies are required. Since the choice of drug targets must take into account the adverse effects resulting from the inhibition of other general PI3K/PKB-dependent cellular processes, it would be desirable to target down-stream components of this signaling module, such as Id1. In conclusion, our data demonstrate that high expression of Id1, through PI3K/PKB-mediated inhibition of FOXO3 is critical for maintenance of the leukemic phenotype.

EXPERIMENTAL PROCEDURES

Cell culture

Ba/F3 cells were cultured in RPMI 1640 medium supplemented with 8% Hyclone serum (Gibco, Paisley, UK) and recombinant mouse IL-3 produced in COS cells (29). BaF3-FOXO3(A3):ER* cells were previously described (29).

For the generation of clonal Ba/F3 cells stably expressing FOXO3(A3):ER*, the pcDNA3-FOXO3(A3):ER* construct was electroporated into Ba/F3 cells and maintained in the presence of 500µg/ml G418 (Gibco, Paisley, UK). Clonal cell lines were generated by limited dilution. K562 cells were cultured in RPMI 1640 medium supplemented with 8% Hyclone serum (Gibco, Paisley, UK). COS cells were cultured in Dulbecco's modified Eagle's Medium DMEM (Gibco, Paisley, UK) supplemented with 8% heat-inactivated FCS. For cytokine withdrawal experiments, cells were washed twice with PBS and resuspended in AimV medium.

For the generation of clonal K562 cells stably expressing FOXO3(A3):ER*, 10µg of pBabe-puromycin-FOXO3(A3)ER vector or empty vector was electroporated into K562 cells at 0.35V 950uF using BioRad Gene Pulser. Cells were selected and maintained in the presence of 1µg/ml Puromycin (InvivoGen, UK) and clonal cell lines were generated. Single cell clones were obtained with serial dilution and tested by western blotting after addition of 200nM tamoxifen (Sigma) at $5x10^5$ cells/ml for 24 hours (Gibco, Paisley, UK).

Transfection of psiRNA-h7Skzeo:FOXO3 into K562

In order to obtain stable cells lines, the mammalian expression vector psiRNA-h7Skzeo (InvivoGen, California USA) was used for gene silencing of FOXO3 in K562 cells. The psiRNA plasmid (InvivoGen) is specifically designed for the cloning of small synthetic oligonucleotides that encode

two complementary sequences of 19 nucleotides, separated by the used hairpin sequence: TCACTGCATAGTCGATTCA.
The cells were split 24 hours prior to the transfection and were around 0.8 to 1x10^6 cells/ml. 10 □g of the construct was transiently transfected into K562 cells by electroporation using the BioRad Gene Pulser (950mF; 0.350KV). The transfected cells were carefully resuspended in conditioned medium, and left to grow overnight. 24hrs post transfection, cells were checked and carefully washed, then selection was started according to the siRNA manufacturer recommendation. During the first week, the cells were selected in growth medium supplemented with 5ug/ml zeocin, followed by single cell cloning and expansion for another two weeks. The cells were tested for the efficiency of siRNA silencing by western blotting.

Antibodies and reagents
Polyclonal antibodies against PKB and phospho-Ser473 PKB were from Cell Signaling Technologies (Hitchin, UK). anti-$p27^{Kip1}$ was purchased from Transduction Laboratories (Lexington, Kentucky, USA). Polyclonal antibodies against Id1 and actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Total and Phospho-Thr32 FOXO3 were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). STI571 was a kind gift from Dr. S.Ebeling (Dept. Hematology, UMC Utrecht, The Netherlands).
The pLZRS-FOXO3(A3) construct was generated as followed. First, the Xba/HindIII FOXO3(A3) fragment from the pECE-FOXO3(A3) vector was ligated into Xba/HindIII cut dephosphorylated pBluescript. Subsequently, pLZRS-FOXO3(A3) was created by ligating a Xho/NotI fragment from pBluescript into Xho/NotI cut pLZRS.
The pGL3-Id1 promoter construct was obtained by amplifying a 1692 bp fragment of the Id1 promoter from human chromosomal DNA which was ligated into pGL3 (pGL3-basic from Promega) cut with SmaI and XhoI. The FOXO3 binding site1 was mutated using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to manufacturer's protocol with primer 5'-GGGGGCCGTGGCGTGCGTATAAAAGACAAGC -3' and its complementary sequence.

Viral transduction
A bicistronic retroviral DNA construct was utilized, expressing Id1 (kindly provided by Dr. H. Spits, Amsterdam, The Netherlands) and an Internal Ribosomal Entry Site (IRES) followed by the gene encoding eGFP (30). Retrovirus was produced by transient transfection of 293T cells by FUGENE-6 (Roche, Basel, Switserland). 0.6x10^6 cells were seeded in 9-cm dishes. The following day, twenty minutes before transfection the medium of the cells was refreshed. 12 µl Fugene-6, 2 µg pCL-ampho and 2µg pLZRS-eGFP, pLZRS-Id1 or pLZRS-FOXO3(A3) were added to 184µl DMEM, incubated at room temperature for 15 minutes and subsequently added to the cells. Again after 24 hours the medium was replaced with RRMI 1640 medium (supplemented with 8% Hyclone). After again 24 hours the viral supernatants were collected, filtered through a 0.22 µM acrodisc filter and stored at –80°C.
Before transduction K562 cells were cultured at a density of 2x10^5 cells/ml. The next day K562 cells were transduced by resuspending 10^6 cells in 0.5ml RPMI. After addition of 8µg/ml polybrene (Sigma-Aldrich, Seelze, Germany) and 5 ml viral supernatant, the cells were centrifuged at 2500rpm for 1.5 hours at room temperature. After 24 hours the cells were washed once and transduced for a second time. Two days after transduction, eGFP positive cells were sorted by FACS and treated with or without STI571.

Western blotting
For the detection of Id1, p27, phospho-PKB and phospho-FOXO3, cells were lysed in laemmli sample buffer and the protein concentration was determined. Equal amounts of each protein sample were analyzed by SDS PAGE, electrophoretically transferred to PVDF membrane (Millipore, Bedford, MA) and probed with the respective antibodies. Immunocomplexes were detected using enhanced chemiluminescence (ECL, Amersham).

Luciferase assays
For transient transfections Ba/F3 cells were electroporated (0.28kV, capacitance 950µF) with 16 µg of a luciferase reporter plasmid containing the Id1 promoter. Cells were cotransfected with 50ng of a renilla luciferase plasmid (pRL-TK; Promega) to normalize for transfection efficiency. After transfection cells were cultured with or without IL-3, or in the presence of IL-3 with or without 4-hydroxytamoxifen (100nM) for 24 hours. Cells were then harvested, lysed in commercially available luciferase lysis buffer and luciferase activity was determined treated as previously (29).
COS cells were transiently transfected with the pGL3-Id1 luciferase promoter construct, together with

pECE-FOXO3(A3) (20), or control vectors and the internal transfection control (pRL-TK) by calcium phosphate precipitation. Values were corrected for transfection efficiency and represent the mean of at least three independent experiments (± SEM).

RNA isolation and cDNA synthesis

Cells were stimulated as indicated and at the respective times harvested. $5x10^6$ cells were harvested, washed twice with PBS, lysed in 1ml TRIZOL (Invitrogen, Breda, The Netherlands) and stored at -20°C. Total cellular RNA was isolated and cDNA generated as previously described (31) according to manufacturer's protocol (Invitrogen, Breda, The Netherlands). 10µg of RNA was treated with DNAse according to manufacturer's protocol (DNAfree, Ambion Inc. Austin, TX, USA). cDNA was synthesized using MMLV reverse transcriptase and oligo(dT) primers. Samples containing 1µg of total RNA in a total volume of 12.5 µl were heated for 3 minutes at 65°C and quickly chilled on ice. A mixture of 12.5µl containing 20µg/ml oligo(dT) primers, 2.5µl 5xRT buffer, 20mM DTT, 2mM dNTPs, 0.8U/µl of RNAse inhibitor and 200 units of MMLV reverse transcriptase was added. The total mixture was incubated for 90 minutes at 37°C, followed by inctivation of the reverse transcriptase for 10 minutes at 65°C. cDNA was stored at -20°C before further use. All reagents used for cDNA synthesis were obtained from Life Technologies (Breda, The Netherlands).

Real-time PCR

Id1, β-actin and GAPDH mRNA were analyzed by real time PCR using SYBR green I (Nieuwekerk a/d IJssel, The Netherlands). Primers were designed using Primer 3 software from the Whitehead Institute/ MIT center for genome Research (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). For mouse Id1 a 86 bp fragment was amplified using the FW primer 5'-ACGACATGAACGGCTGCTAC-3' and the reverse primer 5'-CAGGATCTCCACCTTGCTCAC-3'. For human Id1 a 68 bp fragment was amplified using the FW primer 5'-CTGGACGAGCAGCAGGTAAA-3' and the reverse primer 5'-AGCTCCTTGAGGCGTGAGTAA-3'. For mouse GAPDH as a control a 191bp fragment was amplified using the FW primer 5'-AACGACCCCTTCATTGAC-3' and RV primer 5'-TCCACGACATACTCAGCAC-3' were used. For human GAPDH a 135 bp fragment was amplified using the FW primer 5'-AGAAGGCTGGGGCTCATTT-3' and RV primer 5'-GAGGCATTGCTGATGATCTTG-3' and a 174 bp fragment of β-actin was amplified using FW primer 5'-AGCCTCGCCTTTGCCGA-3' and RV primer 5'-CTGGTGCCTGGGGCG-3' (32). The real time PCR was performed as previously described (31). Results were normalized for the housekeeping genes β-actin and GAPDH and results were expressed as fold regulation.

Chromatin immunoprecipitation (ChIP) assay

K562 cells cultured at 10^6 cells/ml were either untreated or stimulated with 5µM STI571 for 4 or 8 hours, and then collected by centrifugation and resuspended in 10ml of cold PBS. ChIP assays were performed as previously described by Fernandez de Mattos *et al* (33). Protein·DNA complexes were formaldehyde-cross-linked, and immunoprecipitated with either the FOXO3 (Upstate, Lake Placid, NY, USA) or an isotype control antibody (Babco). PCRs were then performed on the purified DNA, according to manufacturer's protocol, in the presence of 2.5mM $MgCl_2$, at 55°C, for 28 cycles, using the FOXO3 primers sense (Primer1: 5'CAGAGGAGCCCAGTGCGG; Primer2: 5'CAGCCCC AAACTTACTAGACTTTCC; Primer3:5'CAGGCGAACGCTACC ATGC); anti-sense (Primer1: 5'-AAGTGGAAGCCCGAAGCA; Primer2: 5'-TCTCACTTCTCCAGCTCCATTT; Primer3:5'CCTAATAT TTAATATCTGCTT GGTGTTTAA). Analysis of the PCR products was performed on a standard 2% (w/v) agarose gel, by electrophoresis in Tris-acetate EDTA buffer.

Measurement of hemoglobin expression

The benzidine oxidation test was performed as described (34). In short, cells ($0.2\text{-}3 \times 10^6$ cells/ml) were incubated with STI571 for 24, 48 or 72 hours, then washed twice in PBS at low speeds for 10 minutes, and finally resuspended in 0.9% NaCl-Benzidine reagent solution (to 1 ml of 0.2% tetramethylbenzidine (Sigma) in 0.5 M HAc, 20 ml of 30% H2O2 is added just prior to use) was added to start the reaction. After incubation for 30 min in darkness at room temperature, 200 cells were counted in a Burker chamber. The number of cells containing blue crystals indicative of oxidized tetramethylbenzidine and reflecting hemoglobin production was determined.

Extraction of DNA-binding proteins

Briefly, $25x10^6$ cells were centrifuged at 4000x*g* for 10 min at 4°C, washed with ice-cold PBS, and resuspended in 400µl cold low salt buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM ABSF) by gently flicking the tube. The cells were allowed to swell on ice for 10 min. After a brief vortex, samples were centrifuged for 2 min at 4°C, and the supernatant

fraction was discarded. The pellet was then resuspended in 30-40µl cold high salt buffer C [20 mM HEPES-KOH, pH 7.9, 25% v/v glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 1 mM phenylmethyl sulphonyl fluoride (PMSF)] and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation (13000x*g*, 2 min, 4°C). The supernatant fraction contains the DNA-binding proteins. Protein yield was quantified by Bio-Rad Dc protein assay kit (Bio-Rad).

Oligonucleotide "pull-down" assay
Nuclear extracts were prepared from cultured cells using the high salt buffer as described. After diluting with 2 volumes of low salt lysis buffer, 50µg of the cell extracts were incubated at 30°C for 10 min with either 0.5 nmol of the 5'-biotinylated double stranded wild-type (5'- TGGCGTGTTTATAAAAGACAA GCTGTGGCT-3') or mutant (5'- TGGCGAATTCATAAAGGGCCCGCTGTGGCT-3') oligonucleotides (Invitrogen) previously coupled to streptavidin agarose beads (Sigma, Poole, UK). All the pull-down experiments were performed with exactly equal aliquots from the same sample of either untreated or STI571-treated nuclear extracts. The wild-type oligonucleotide corresponded to the region of –114 to –144 of the human *Id1* promoter. After incubation, the biotinylated oligonucleotide-coupled streptavidin beads were washed at least 6 times with low salt buffer containing 150mM NaCl and denatured in SDS-sample buffer before running on a SDS-acrylamide gel. The separated proteins were then Western blotted for FOXO3 using specific antibodies. For competition experiments, the extracts were incubated with the wild-type oligonucleotide-coupled beads in the presence of 1x, 5x or 50x molar excess of either the wild-type or mutant non-biotinylated oligonucleotide.

FOOTNOTES

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REFERENCES

1. Barreda, D. R., Hanington, P. C., and Belosevic, M. (2004) *Dev. Comp Immunol.* **28,** 509-554
2. Eaves, C. J., Sutherland, H. J., Udomsakdi, C., Lansdorp, P. M., Szilvassy, S. J., Fraser, C.C., Humphries, R. K., Barnett, M. J., Phillips, G. L., and Eaves, A. C. (1992) *Blood Cells* **18,** 301-307
3. Humphries, R. K., Eaves, A. C., and Eaves, C. J. (1981) *Proc. Natl. Acad. Sci. U. S. A* **78,** 3629-3633
4. Metcalf, D. (1989) *Nature* **339,** 27-30
5. Ogawa, M., Porter, P. N., and Nakahata, T. (1983) *Blood* **61,** 823-829
6. Shapiro, L. H. and Look, A. T. (1995) *Curr. Opin. Hematol.* **2,** 3-11
7. Lowenberg, B., Downing, J. R., and Burnett, A. (1999) *N. Engl. J. Med.* **341,** 1051-1062
8. Faderl, S., Talpaz, M., Estrov, Z., O'Brien, S., Kurzrock, R., and Kantarjian, H. M. (1999) *N. Engl. J. Med.* **341,** 164-172
9. Deininger, M. W., Goldman, J. M., and Melo, J. V. (2000) *Blood* **96,** 3343-3356
10. Bennett, J. M., Catovsky, D., Daniel, M. T., Flandrin, G., Galton, D. A., Gralnick, H. R.,

and Sultan, C. (1976) *Br. J. Haematol.* **33,** 451-458

11. Bloomfield, C. D. and de la, C. A. (1987) *Semin. Oncol.* **14,** 372-383
12. Fialkow, P. J., Singer, J. W., Raskind, W. H., Adamson, J. W., Jacobson, R. J., Bernstein, I. D., Dow, L. W., Najfeld, V., and Veith, R. (1987) *N. Engl. J. Med.* **317,** 468-473
13. Ren, R. (2002) *Oncogene* **21,** 8629-8642
14. Salesse, S. and Verfaillie, C. M. (2002) *Oncogene* **21,** 8605-8611
15. Steelman, L. S., Pohnert, S. C., Shelton, J. G., Franklin, R. A., Bertrand, F. E., and McCubrey, J. A. (2004) *Leukemia* **18,** 189-218
16. Sattler, M. and Salgia, R. (1997) *Cytokine Growth Factor Rev.* **8,** 63-79
17. Datta, S. R., Brunet, A., and Greenberg, M. E. (1999) *Genes Dev.* **13,** 2905-2927
18. Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J., and Waterfield, M. D. (2001) *Annu. Rev. Cell Dev. Biol.* **17,** 615-675
19. Scheid, M. P. and Woodgett, J. R. (2001) *Nat. Rev. Mol. Cell Biol.* **2,** 760-768
20. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96,** 857-868
21. Kops, G. J. and Burgering, B. M. (2000) *J. Anat.* **197 Pt 4,** 571-574
22. Birkenkamp, K. U. and Coffer, P. J. (2003) *J. Immunol.* **171,** 1623-1629
23. Kharas, M. G. and Fruman, D. A. (2005) *Cancer Res.* **65,** 2047-2053
24. Birkenkamp, K. U., Geugien, M., Schepers, H., Westra, J., Lemmink, H. H., and Vellenga, E. (2004) *Leukemia* **18,** 103-112
25. Skorski, T., Bellacosa, A., Nieborowska-Skorska, M., Majewski, M., Martinez, R., Choi, J. K., Trotta, R., Wlodarski, P., Perrotti, D., Chan, T. O., Wasik, M. A., Tsichlis, P. N., and Calabretta, B. (1997) *EMBO J.* **16,** 6151-6161
26. Komatsu, N., Watanabe, T., Uchida, M., Mori, M., Kirito, K., Kikuchi, S., Liu, Q., Tauchi, T.,Miyazawa, K., Endo, H., Nagai, T., and Ozawa, K. (2003) *J. Biol. Chem.* **278,** 6411-6419
27. Gesbert, F., Sellers, W. R., Signoretti, S., Loda, M., and Griffin, J. D. (2000) *J. Biol. Chem.* **275,** 39223-39230
28. Norton, J. D. (2000) *J. Cell Sci.* **113 (Pt 22),** 3897-3905
29. Dijkers, P. F., Birkenkamp, K. U., Lam, E. W., Thomas, N. S., Lammers, J. W., Koenderman, L., and Coffer, P. J. (2002) *J. Cell Biol.* **156,** 531-542
30. Buitenhuis, M., van Deutekom, H. W., Verhagen, L. P., Castor, A., Jacobsen, S. E., Lammers, J. W., Koenderman, L., and Coffer, P. J. (2005) *Blood* **105,** 4272-4281
31. Oudijk, E. J., Nijhuis, E. H., Zwank, M. D., van de Graaf, E. A., Mager, H. J., Coffer, P. J., Lammers, J. W., and Koenderman, L. (2005) *Thorax* **60,** 538-544
32. Kreuzer, K. A., Lass, U., Landt, O., Nitsche, A., Laser, J., Ellerbrok, H., Pauli, G., Huhn, D., and Schmidt, C. A. (1999) *Clin. Chem.* **45,** 297-300
33. Fernandez de, M. S., Essafi, A., Soeiro, I., Pietersen, A. M., Birkenkamp, K. U., Edwards, C. S., Martino, A., Nelson, B. H., Francis, J. M., Jones, M. C., Brosens, J. J., Coffer, P. J., and Lam, E. W. (2004) *Mol. Cell Biol.* **24,** 10058-10071
34. Kaiho, S. and Mizuno, K. (1985) *Anal. Biochem.* **149,** 117-120

35. Dijkers, P. F., Medema, R. H., Pals, C., Banerji, L., Thomas, N. S., Lam, E. W., Burgering, B. M., Raaijmakers, J. A., Lammers, J. W., Koenderman, L., and Coffer, P. J. (2000) *Mol. Cell Biol.* **20,** 9138-9148

36. Hribal, M. L., Nakae, J., Kitamura, T., Shutter, J. R., and Accili, D. (2003) *J. Cell Biol.* **162,** 535-541

37. Furuyama, T., Nakazawa, T., Nakano, I., and Mori, N. (2000) *Biochem. J.* **349,** 629-634

38. Bakker, W. J., Blazquez-Domingo, M., Kolbus, A., Besooyen, J., Steinlein, P., Beug, H., Coffer, P. J., Lowenberg, B., von, L. M., and van Dijk, T. B. (2004) *J. Cell Biol.* **164,** 175-184

39. Kruse, J. J., te Poele, J. A., Velds, A., Kerkhoven, R. M., Boersma, L. J., Russell, N. S., and Stewart, F. A. (2004) *Radiat. Res.* **161,** 28-38

40. Trausch-Azar, J. S., Lingbeck, J., Ciechanover, A., and Schwartz, A. L. (2004) *J. Biol. Chem.* **279,** 32614-32619

41. Sikder, H. A., Devlin, M. K., Dunlap, S., Ryu, B., and Alani, R. M. (2003) *Cancer Cell* **3,** 525-530

42. Lasorella, A., Uo, T., and Iavarone, A. (2001) *Oncogene* **20,** 8326-8333

43. Druker, B. J. (2002) *Oncogene* **21,** 8541-8546

44. Ramaswamy, S., Nakamura, N., Sansal, I., Bergeron, L., and Sellers, W. R. (2002) *Cancer Cell* **2,** 81-91

45. Shi, C., Zhang, X., Chen, Z., Sulaiman, K., Feinberg, M. W., Ballantyne, C. M., Jain, M. K., and Simon, D. I. (2004) *J. Clin. Invest* **114,** 408-418

46. Munoz-Alonso, M. J., Acosta, J. C., Richard, C., Delgado, M. D., Sedivy, J., and Leon, J. (2005) *J. Biol. Chem.* **280,** 18120-18129

47. Kharas, M. G., Deane, J. A., Wong, S., O'Bosky, K. R., Rosenberg, N., Witte, O. N., and Fruman, D. A. (2004) *Blood* **103,** 4268-4275

48. Kawauchi, K., Ogasawara, T., Yasuyama, M., and Ohkawa, S. (2003) *Blood Cells Mol. Dis.* **31,** 11-17

49. Nigten, J., Breems-de Ridder, M. C., Erpelinck-Verschueren, C. A., Nikoloski, G., van der Reijden, B. A., van, W. S., van Hennik, P. B., de, W. T., Lowenberg, B., and Jansen, J. H. (2005) *Leukemia* **19,** 799-805

50. Ohtani, N., Zebedee, Z., Huot, T. J., Stinson, J. A., Sugimoto, M., Ohashi, Y., Sharrocks, A. D., Peters, G., and Hara, E. (2001) *Nature* **409,** 1067-1070

51. Kim, D., Peng, X. C., and Sun, X. H. (1999) *Mol. Cell Biol.* **19,** 8240-8253

52. Wice, B. M. and Gordon, J. I. (1998) *J. Biol. Chem.* **273,** 25310-25319

53. Sun, X. H. (1994) *Cell* **79,** 893-900

54. Prabhu, S., Ignatova, A., Park, S. T., and Sun, X. H. (1997) *Mol. Cell Biol.* **17,** 5888-5896

55. Condorelli, G. L., Tocci, A., Botta, R., Facchiano, F., Testa, U., Vitelli, L., Valtieri, M., Croce, C. M., and Peschle, C. (1997) *Mol. Cell Biol.* **17,** 2954-2969

56. Kuzelova, K., Grebenova, D., Marinov, I., and Hrkal, Z. (2005) *J. Cell Biochem.* **95,** 268-280

57. Tauchi, T. and Ohyashiki, K. (2004) *Leuk. Res.* **28 Suppl 1,** S39-S45

CHAPTER 7

Regulation of glutamine metabolism by the PI3K/PKB/FOXO pathway modulates autophagy

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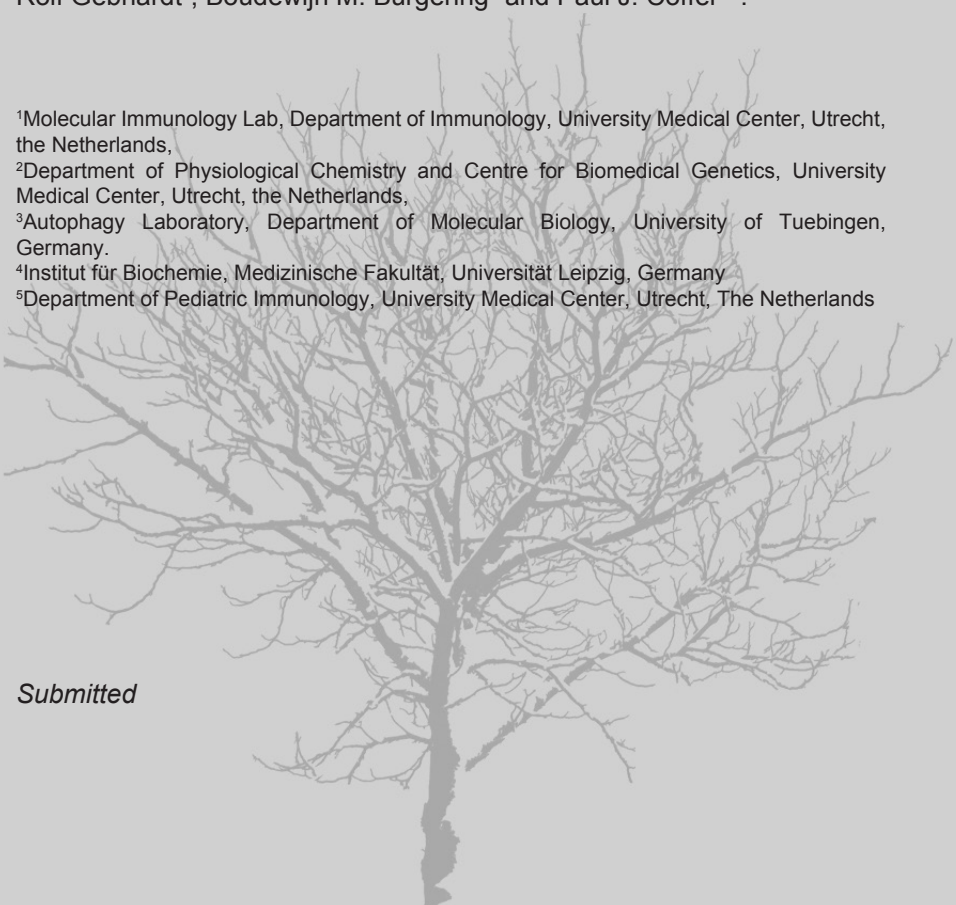


Submitted

ABSTRACT

To identify novel transcriptional targets of the PI3K-PKB-FOXO signalling network involved in survival and stress resistance, we performed microararray analysis after inducible activation of constitutively active PI3K, PKB, FOXO3 and FOXO4. Utilising this unique "pathway" approach in bone marrow-derived Ba/F3 cells, we identified glutamine synthetase (GS) as being transcriptionally regulated by PI3K-PKB-FOXO signalling. GS mRNA and protein expression was inhibited by stimulation with IL-3 or activation of either PI3K or PKB. Inhibition of PI3K or specific activation FOXO3 and FOXO4 resulted in increased mRNA and protein expression, indicating that FOXOs are sufficient for regulating GS expression. Increased GS expression correlated with increased enzyme activity and accumulation of extracellular glutamine. By performing promoter reporter assays we demonstrate that FOXO3 regulates GS expression through a conserved FOXO binding site. In *C elegans*, worms with an inactivating mutation in the insulin receptor showed an increase in GS activity compared to wild type worms. RNAi-mediated knockdown experiments revealed that this increase was dependent on FOXO/DAF-16 expression, demonstrating that the regulation of GS activity by the PI3K-PKB-FOXO pathway is conserved in lower organisms. Activation of FOXO3 in colon carcinoma cells induced the formation of autophagosomes as demonstrated by cleavage and lipidation of LC3 and formation of distinct LC3-positive punctate spots as well as WIPI-positive punctuate spots. Using a specific GS inhibitor it was shown that the induction of autophagy was dependent on GS activity. Abrogation of FOXO3-induced autophagy with chemical inhibitors decreased cell viability, suggesting that the induction of autophagy by FOXO3-mediated upregulation of GS is important for cellular survival. Taken together these results demonstrate that the PI3K-PKB-FOXO pathway modulates autophagy through regulation of glutamine metabolism.

INTRODUCTION

The PI3K-PKB-FOXO signalling module is evolutionary conserved and in the nematode worm *C elegans* activation of an orthologue of the insulin receptor, DAF-2 results in activation of a PI3K orthologue, AGE-1, which induces activation of a PKB (AKT) orthologue (Dorman *et al.*, 1995; Kimura *et al.*, 1997; Paradis and Ruvkun, 1998). This pathway plays an important role in regulation of dauer formation. The normal lifespan of *C elegans* is 2-3 weeks, however in the absence of nutrients the nematode worm can enter a stress-resistant stage in which the worms can survive up to 2-3 months (reviewed in Cassada and Russell, 1975). The DAF-2 pathway negatively regulates the induction of dauer formation and loss of function mutations in the gene encoding DAF-2, the orthologue of the insulin receptor, lead to inactivation of the PI3K-PKB pathway and result in a long-lived phenotype (Kenyon *et al.*, 1993). *daf-2* mutant-induced dauer formation is dependent on the Forkhead Box O (FOXO) transcription factor DAF-16 expression since the long-lived phenotype is reverted in *daf-16* deficient organisms (Lin *et al.*, 1997; Ogg *et al.*, 1997). In the absence of nutrients DAF-16 is activated and induces dauer formation, a larval stage in which worms lower their metabolism and can survive longer. A number of transcriptional targets of DAF-16 have been identified that are required for dauer formation and are involved in stress resistance including superoxide dismutase and heat shock proteins (Partridge and Bruning, 2008; Mukhopadhyay *et al.*, 2006).

In mammals three FOXO homologues are phosphorylated and regulated by PKB: FOXO1, FOXO3 and FOXO4, which act as transcriptional activators as well as repressors (reviewed in Calnan and Brunet, 2008). In mammalian cells, activation of FOXOs can, depending on the cell-type, have influence on a wide range of biological processes including cell cycle regulation, stress resistance, development, reproduction and ageing. Similar to DAF-16, FOXOs can increase survival of cells during growth factor deprivation by increasing stress resistance. FOXO activation can protect cells from damage by upregulating the expression of proteins involved in protection against oxidative stress such as manganese superoxide dismutase (MnSOD) and Growth arrest and DNA damage response gene Gadd45 a protein involved in DNA repair mechanisms (Kops *et al.*, 2002a; Tran *et al.*, 2002). Furthermore, during fasting FOXOs induce autophagy in muscle cells, a process in which the cells induces the formation of vesicles, called autophagosomes, which encapsulate cellular proteins and deliver them to lysosomes (Zhao *et al.*, 2007).

In this study we performed microarray analyses after inducible activation of constitutively active PI3K, PKB, FOXO3 and FOXO4 to identify novel transcriptional targets involved in survival and stress resistance. Using this approach, we have identified glutamine synthetase (GS) as a novel FOXO transcriptional target. Activation of PI3K or PKB results in downregulation of GS expression, while activation of either FOXO3 or FOXO4 induces an upregulation. The increase of GS expression correlates with increased GS activity and increased glutamine levels. After activation of FOXO3 the upregulation of GS induces autophagy, which is important for survival of the cells. These data show that the PI3K modulates glutamine metabolism and reveal a novel mechanism by which the PI3K-PKB-FOXO signalling module regulates autophagy.

RESULTS

Global identification of PI3K-PKB-FOXO pathway transcriptional targets

To identify transcriptional targets regulated by PI3K-PKB-FOXO signalling we generated several cell lines ectopically expressing inducible active variants of the key components in this pathway. By generating individual lines for each pathway component we aimed to better identify true transcriptional targets. The Ba/F3 cell line was chosen as ourselves and others have previously demonstrated that both survival and proliferation are regulated by PI3K signalling (Dijkers *et al.*, 2002). In order to selectively activate PI3K Ba/F3 cell lines were generated stably expressing an inducible active PI3K (myrPI3K-ER) and for PKB, expressing inducible active PKBα (myrPKB-ER)(van Gorp *et al.*, 2006). In addition cell lines expressing inducible constitutively active FOXO3 and FOXO4 constructs were generated by mutating the three PKB phosphorylation sites to alanines (FOXO3(A3)-ER and FOXO4(A3)-ER) and fusion to the hormone binding domain of the estrogen receptor (ER).

To characterise transcriptional targets regulated by the PI3K-PKB-FOXO module we performed microarray analyses after 4-OHT-mediated activation of each pathway component. To identify true "pathway targets" we focussed on changes in mRNA expression (>1.7 fold) that were upregulated by both PI3K and PKB, and down-regulated by FOXO3 and FOXO4 or *vice versa*. Utilising this unique "pathway" approach we aimed to identify novel transcriptional targets with an increased functional relevance. After comparative analysis we identified 35 putative FOXO targets regulated by the PI3K-PKB-FOXO pathway (manuscript in preparation). Among the identified transcriptional targets were Mxi1 and Pink1, which have both been recently described to be regulated by FOXOs (Delpuech *et al.*, 2007; Mei *et al.*, 2009). Transcription of glutamine synthetase (GS) was found to be upregulated by both FOXO3 and FOXO4 activation, while its expression was inhibited by activation of either PI3K or PKB (Fig. 1A).

To further validate the results from the microarray analysis we stimulated Ba/F3 myrPI3K-ER and Ba/F3 myrPKB-ER cells with 4-OHT and evaluated GS mRNA by quantitative RT-PCR (q/RT-PCR). Activation of either PI3K or PKB resulted in a clear downregulation of GS mRNA levels (Fig. 1B, C). In addition, cell lysates were prepared and analyzed for GS expression by western blotting. After activation of either PI3K or PKB with 4-OHT, FOXO3 became phosphorylated correlating with decreased GS expression (Fig. 1D). Ba/F3 cells were also stimulated with IL-3 to determine if cytokine-mediated activation of endogenous PI3K and PKB could also result in regulation of GS expression. As shown in Figure 1E stimulation of Ba/F3 cells with IL-3 resulted in phosphorylation of FOXO3 and

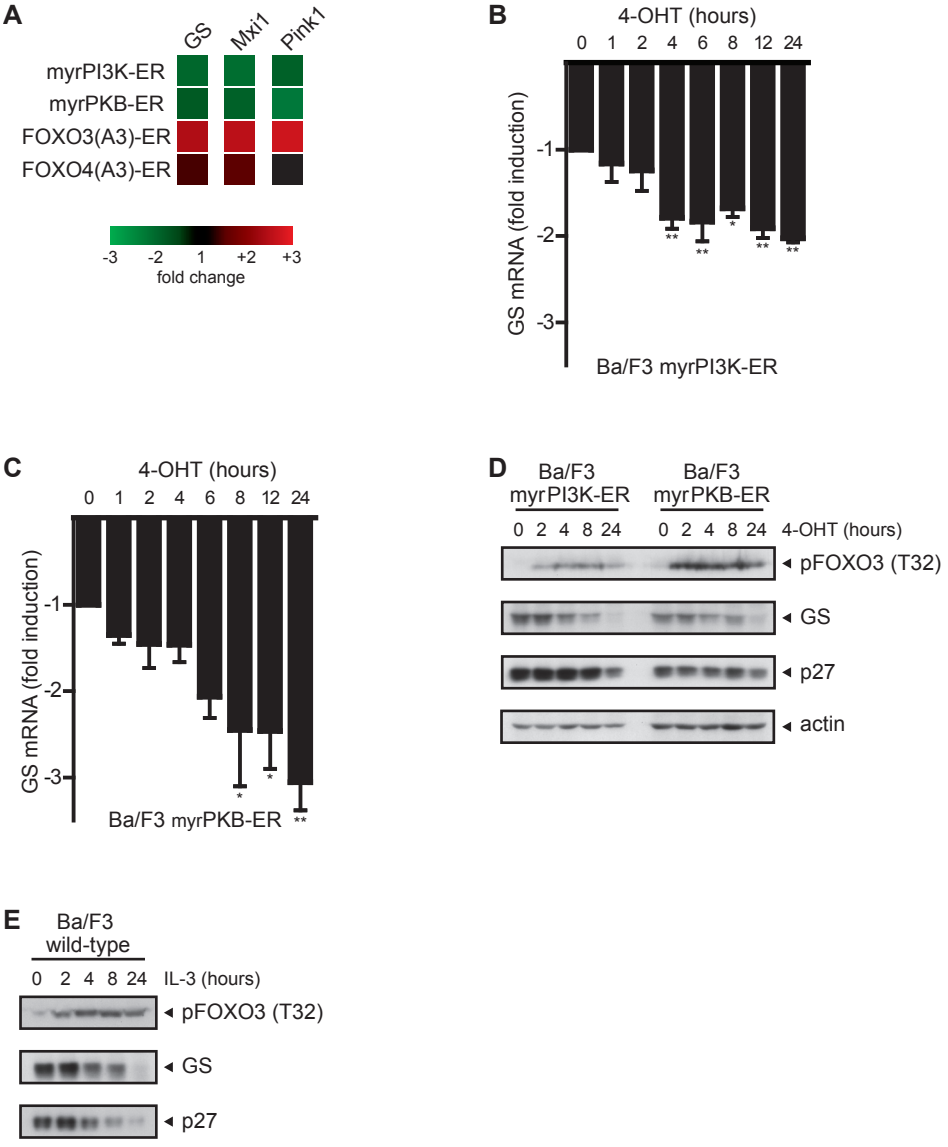
Figure 1. Activation of the PI3K-PKB pathway results in down-regulation of glutamine synthetase mRNA and protein levels

(A) Ba/F3 cells expressing myrPI3K-ER, myrPKB-ER, FOXO3(A3)-ER or FOXO4(A3)-ER were stimulated with 4-OHT, RNA was isolated and microarray analyses were performed. Shown are the fold changes relative to control cells for glutamine syntheatse (GS), Mxi1 and Pink1. Data are represented as mean values of one experiment performed in quadruplicate. **(B), (C)** Ba/F3 cells expressing either myrPI3K-ER or myrPKB-ER were cytokine starved overnight and stimulated with 100 nM 4-OHT for the indicated times. RNA was isolated and relative mRNA levels of GS were analyzed using quantitative RT-PCR. Data are represented as mean ± SEM values normalized for B_2M (n=4). **(D)** Ba/F3 cells expressing either myrPI3K-ER or myrPKB-ER were cytokine starved overnight and stimulated with 4-OHT (100 nM) for the indicated times. Cells were lysed and equal amounts of protein were analyzed by western blotting for levels of phospho-FOXO3 (T32), GS, p27 and actin. Shown are representative blots (n=4). **(E)** Wildtype Ba/F3 cells were cytokine starved overnight and stimulated with mIL-3 (10 ng/ml) for the indicated times. Cells were lysed and equal amounts of protein were analyzed by western blotting for levels of phospho-FOXO3 (T32), GS, p27 and actin. Shown are representative blots (n=4).

down-regulation of the FOXO target p27 with similar kinetics to that observed after 4-OHT-induced PI3K-PKB activation.

FOXOs are both required and sufficient for regulation of GS expression

To confirm that GS is indeed a direct transcriptional target of FOXOs we stimulated Ba/F3 cells expressing either FOXO3(A3)-ER or FOXO4(A3)-ER with 4-OHT and examined GS mRNA levels by q/RT-PCR. Activation of either FOXO3 or FOXO4 resulted in an upregulation of GS mRNA (Fig. 2A). Additionally, western blot analysis of protein lysates after FOXO activation showed a clear upregulation of GS levels with kinetics similar to that



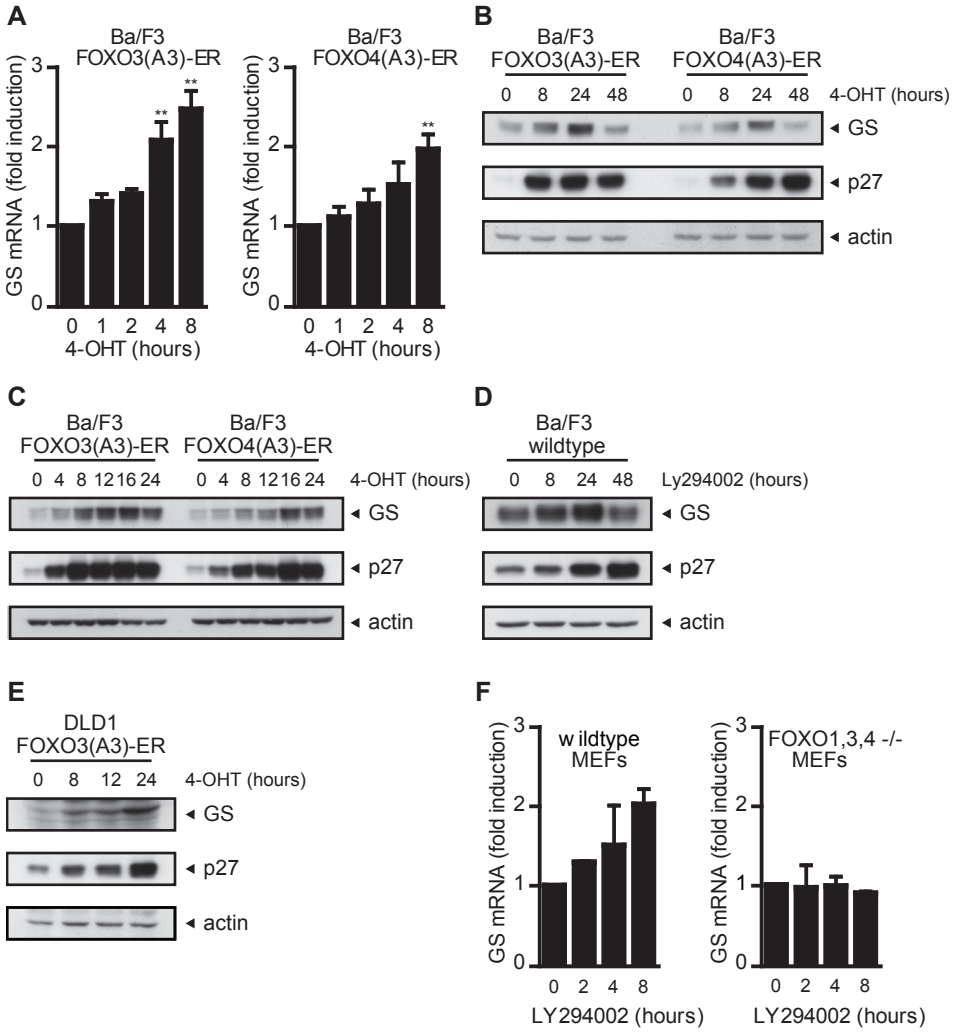


Figure 2. FOXO transcription factors upregulate glutamine synthetase mRNA and protein levels

(A) Ba/F3 cells expressing either FOXO3(A3)-ER or FOXO4(A3)-ER were stimulated with 4-OHT (100 nM) in the presence of mIL-3 (5 ng/ml) for the indicated times. RNA was isolated and relative mRNA levels of GS were analyzed using quantitative RT-PCR. Data are represented as mean ± SEM values normalized for B_2M (n=3). **(B), (C)** Ba/F3 cells expressing either FOXO3(A3)-ER or FOXO4(A3)-ER were stimulated with 4-OHT (100 nM) in the presence of mIL-3 (5 ng/ml) for the indicated times. Cells were lysed and equal amounts of protein were analyzed by western blotting for levels of GS, p27 and actin. Shown are representative blots (n=3). **(D)** Wildtype Ba/F3 cells were incubated with LY294002 (25 μM) in the presence of mIL-3 for the indicated times. Cells were lysed and equal amounts of protein were analyzed by western blotting for levels of GS, p27 and actin. Shown are representative blots (n=3). **(E)** DLD1 cells expressing FOXO3(A3)-ER were stimulated with 4-OHT (500 nM) for the indicated times. Cells were lysed and equal amounts of protein were analyzed by western blotting for levels of GS, p27 and actin. Shown are representative blots (n=4). **(F)** FOXO1,3,4 -/- MEFs and wild-type MEFS were incubated with LY294002 (10 μM) for the indicated times. Cells RNA was isolated and relative mRNA levels of GS were analyzed using quantitative PCR. Data are represented as mean ± SEM values normalized for B_2M (n=2).

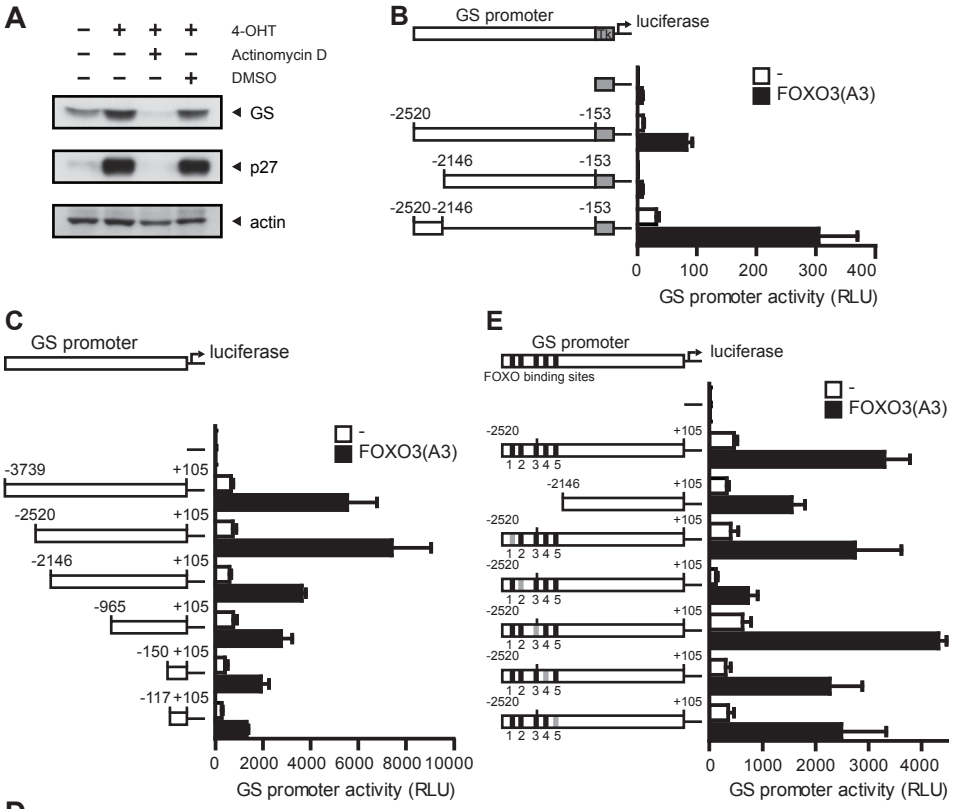
of p27 (Fig. 2B, C). Inhibition of PI3K activity utilising the pharmalogical inhibitor LY294002 was able to recapitulate the effects observed after 4-OHT-induced FOXO activation (Fig. 2D). In order to determine whether regulation of GS expression by FOXO transcription factors was a more general phenomenom, we examined GS expression in the human colon carcinoma cell line DLD1. Stimulation of DLD1 cells expressing FOXO3(A3)-ER with 4-OHT again resulted in upregulation of p27 as well as GS protein levels (Fig. 2E). This suggests that regulation of GS expression by FOXOs is conserved across species and cell type. To confirm that FOXOs are required for GS upregulation we treated both wild-type mouse embryonic fibroblasts (MEFs) and FOXO1,3,4 triple knockout MEFs with LY294002 and evaluated GS mRNA levels. As shown in Figure 2F treatment of wildtype MEFs with the PI3K inhibitor resulted in an upregulation of GS mRNA, which was not observed in the FOXO1,3,4 knockout MEFs. These results show that FOXOs are both required and sufficient for regulating GS expression.

GS expression is upregulated by FOXO3 through a conserved FOXO binding site in the promoter

To investigate whether GS expression is regulated directly by FOXO-mediated transcription, Ba/F3 FOXO3(A3)-ER cells were stimulated with 4-OHT in the presence of the general transcriptional inhibitor actinomycin D. Addition of actinomycin D completely abrogated FOXO-induced GS upregulation as well as the upregulation of p27, indicating that GS expression is indeed regulated at the level of transcription (Fig. 3A). To further elucidate the mechanism of transcriptional regulation we analyzed FOXO-mediated activation of the GS promoter. Co-transfection of FOXO3(A3) increased reporter activity of a thymidine kinase-GS promoter (-2520/-153 bp) reporter by 80-fold (Fig. 3B). FOXO3(A3) failed to increase the luciferase activity of a reporter containing the GS promoter from -2146/-153 bp, while the reporter containing the GS promoter from -2520/-2146 bp showed a 300-fold increase in luciferase activity in the presence of FOXO3(A3). These data suggest that FOXOs increases GS expression through regulation of an enhancer region present in the -2520/-2146 bp region in the promoter. In addition, we analyzed regulation of the GS promoter utilising reporter plasmids containing the GS promoter including the native start codon. Co-transfection of these reporter plasmids with FOXO3(A3) again resulted in increased reporter activity (Fig. 3C). Reporter constructs containing the 3739 bp and 2520 bp fragments of the GS promoter showed the largest FOXO3(A3)-dependent increase in luciferase signal, while deletion of a 384 bp fragment from the -2520 bp reporter resulted in a 2-fold drop in FOXO3(A3)-dependent reporter activity (Fig. 3C). These results suggest that the -2520/-2146 region of the GS promoter contains a region critical for regulation of its expression by FOXOs. FOXOs can bind to promoters that contain FOXO binding sites of which the consensus is TTGTTTAC (Furuyama *et al.*, 2000). Alignment of the 384 bp fragment identified in the previous experiments from human, mouse and rat GS promoters shows the presence of five conserved potential FOXO binding sites (Fig. 3D). To identify which of these sites is critical for FOXO3-induced GS transcription we mutated each site individually and examined the effect on reporter activity. Mutating either site 1, 3, 4 or 5 had no effect, while mutation of site 2 showed a large decrease in FOXO3-induced luciferase levels (Fig. 3E). These results suggest that FOXO3 regulates the expression of GS by directly binding to a conserved FOXO binding site at position -2478 bp.

Regulation of GS expression by FOXO is conserved in *C elegans*

The PI3K-PKB-FOXO pathway is conserved in the nematode worm *C elegans* and plays an important role in the regulation of lifespan. Activation of an orthologue of the insulin receptor, DAF-2 results in activation of a PI3K orthologue, AGE-1, which induces activation



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Hs AGGCCTCTTGTTCACTCCTGAAAGAGTCAGTGGTCATGTATTATGTTCCTAACCCTTACAAAGCGTGCCCCTGGT
Mm AAGCTGCCTGTTTACCCCTGAAAG--TCAGTGGTCACATGAGATGTTCCT----------------------GGT
Rn AAGCTTCTTGTTTACCCCTGAAAG--TCAGTGGTCACATGAGATGTTCCT----------------------GGT

Hs CACATGAATGGATCAAAGCAAATCCATTTCCATTCTTGGAAAAGCAGCTCAAAGCACATGTTCTCTTGATGGTGC
Mm CACATGAAAGGATCAAAGCAAATCCGCTTCTATTCTTGGAAA-------CAGAGCAAATGTTCTCTTGATGCTGC
Rn CACATGGAAGGATCAAAGCAAGCCTGCTTCTATTCTTGGAAA-------CAGAGCAAATGTTCTCTTGATGCTGC

Hs TGCTGTCACTTCTGTGTGGTTAACAAATAT-CATTTATTGAACACTCACCGTGGACACTAC---------T-GGG
Mm CGCTGATGGTACTGTGG----GGTCAATACGTGTTTATTGAA----CAGTCTGGACACTAT---------TCGAG
Rn CGCTGTT---TCTGTGT----GGTCAATTTGTGTTTATCGAACACTCAGTCTGGAAACTGTTTGGGGGCGGGGTG

Hs GGATATGACAATGAATAAACTGCTCAATGAGGTCAAGACATCTAGGAAGGGAAGCAGGCACTCAAATGGCAGAAA
Mm GGCTACGGGA--GAATAAGCAGC-CAAGGAGGTTAACATGTCTAGGAAGGGAAGCGGGCACTCACATGGCAGAAA
Rn GGGTATAGGA--GAATAAGCAGC-AAAAGAGGTTAACGTGTCTAGGAAGGGAAGCCAGCACTCCCGTGGCGGAAA

Hs TACCATAAGCC--TGCAGAACCGCACTCACGCCTT--ACCATTCCGTTCTCTGTTATGTTTGTC--ATCCCCACC
Mm CACAAGAAACCCATGGAAAACAGCACATGTGCTCTGGAATATTGCCCTTTCTATCGTGTTCACT--GCTCCCATT
Rn TGCAAGAAACCCAGGAAAAACAACACATTTGCTCAGGGTTTTCACCTTTTCTACTGAGATTCCCCTGGCCTCATT

Hs TCAGGGGCTGGTGGTGTGTGCAGCAGA-GGCTCTGGCTTA
Mm GTGGAGCCTGGTGAGGAGTGCTGAAGGCTGCCCTGGCTGA
Rn CTGGACCCTGGTGAAGACTGCTGAAGGCTACTCTGTTTGA
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Figure 3. FOXO3 upregulates glutamine synthetase expression through a conserved FOXO binding site in the glutamine synthetase promoter

(A) Ba/F3 cells expressing FOXO3(A3)-ER were stimulated with 4-OHT (100 nM) and actinomycin D (1 µg/ml) or DMSO as a control in the presence of mIL-3 (5 ng/ml) for 16 hours. Cells were lysed and equal amounts of protein were analyzed by western blotting for levels of GS, p27 and actin. Shown are representative blots (n=3). **(B), (C)**, GS reporter plasmids expressing different lengths of the glutamine synthetase promoter in front of a minimal Tk promoter and a luciferase gene **(B)** or expressing different lengths of the glutamine synthetase promoter in front of a luciferase gene **(C)** were transfected in HEK 293 cells together with Renilla and FOXO3(A3) as indicated. Luciferase

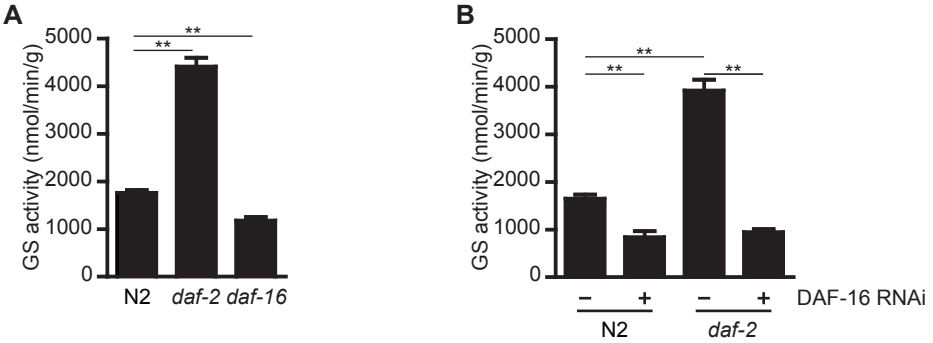


Figure 4. Regulation of GS by FOXOs is conserved in C elegans
(A) Wild-type, *daf-2* and *daf-16* mutant worms were synchronized to L1 by hypochlorite treatment and were placed on NGM agar plates. After 5 days worms were lysed in imidazole and analysed for GS activity. **(B)** Wild-type N2 worms or *daf-2* mutants were synchronized to L1 by hypochlorite treatment and placed on NGM plates with or without bacteria expressing DAF-16 ds RNA. After 5 days worms were lysed in imidazole and analysed for GS activity. **(A,B)** Shown are the mean ± SEM of one experiment with five samples for each condition using technical duplo's.

of a PKB (AKT) orthologue and thereby inactivation of the FOXO orthologue DAF-16. To determine whether GS regulation is evolutionary conserved we examined GS expression in wild-type, *daf-2* and *daf-16* mutant worms. Since *C elegans* expresses five GS genes; gln-1, gln-2, gln-3, gln-5 and gln-6, we analysed total GS activity in adult worms by a GS activity assay. GS activity was significantly increased in *daf-2* mutant worms compared to wild type worms, while *daf-16* mutants showed a lower GS activity compared to wild type (Fig. 4A). In order to define whether the increased GS activity in *daf-2* mutant worms is regulated by the FOXO orthologue DAF-16 we measured GS activity in *daf-2* mutant worms after knockdown of DAF-16 expression by RNA interference (RNAi). As shown in Figure 4B reduction of DAF-16 expression completely blocked the *daf-2*-mediated increase in GS activity. These results show that regulation of GS expression by the PI3K-PKB-FOXO pathway is indeed evolutionary conserved.

Upregulation of GS expression is accompanied by an upregulation of GS activity and an increase in glutamine levels

Glutamine synthetase converts glutamate into glutamine, a process which requires ammonia and ATP (Eisenberg *et al.*, 2000). To determine whether the observed FOXO-induced GS upregulation also results in higher GS activity, Ba/F3 cells expressing FOXO3(A3)-ER were stimulated with 4-OHT and GS activity in cell lysates was determined. Activation of FOXO3 resulted in increased GS activity, with a maximum increase of 4-fold being reached after 16 hours of stimulation (Fig. 5A).. Lysates were also analyzed for GS expression by Western blot showing a clear correlation between expression levels and enzyme activity. Methylsulfoximine (MSO) is a potent inhibitor of GS activity and MSO completely abrogated the FOXO3-induced increased GS activity, while GS expression

activity was measured 40 hours after transfection. Data are depicted as relative luciferase units (RLU) compared to control. Shown are mean ± SEM values (n=3). **(D)** Allignement of part of the GS promoter from human, mouse and rat showing the presence of five conserved FOXO binding sites (in boxes). **(E)** GS reporter plasmids carrying mutations in the putative FOXO binding sites were transfected in HEK 293 cells together with Renilla and FOXO3(A3) as indicated. Luciferase activity was measured 40 hours after transfection. Data are depicted as relative luciferase units (RLU) compared to control. Shown are mean ± SEM values (n=3).

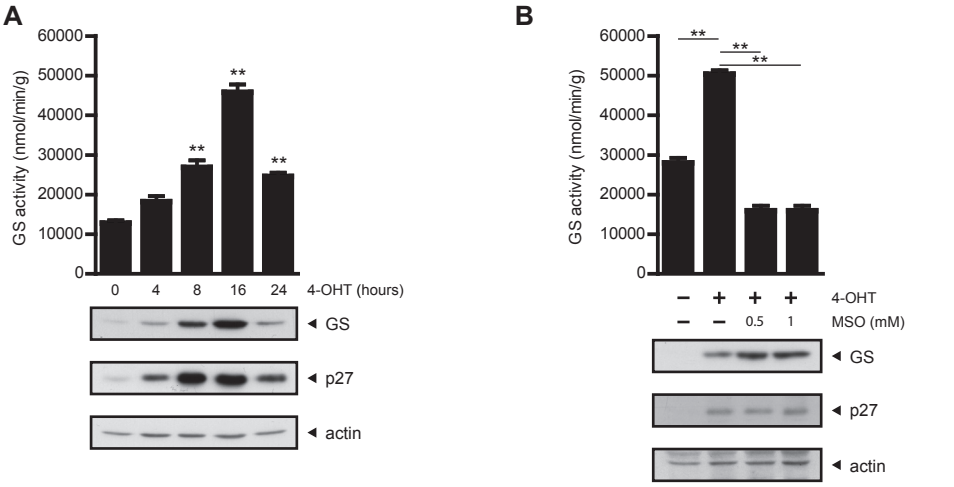
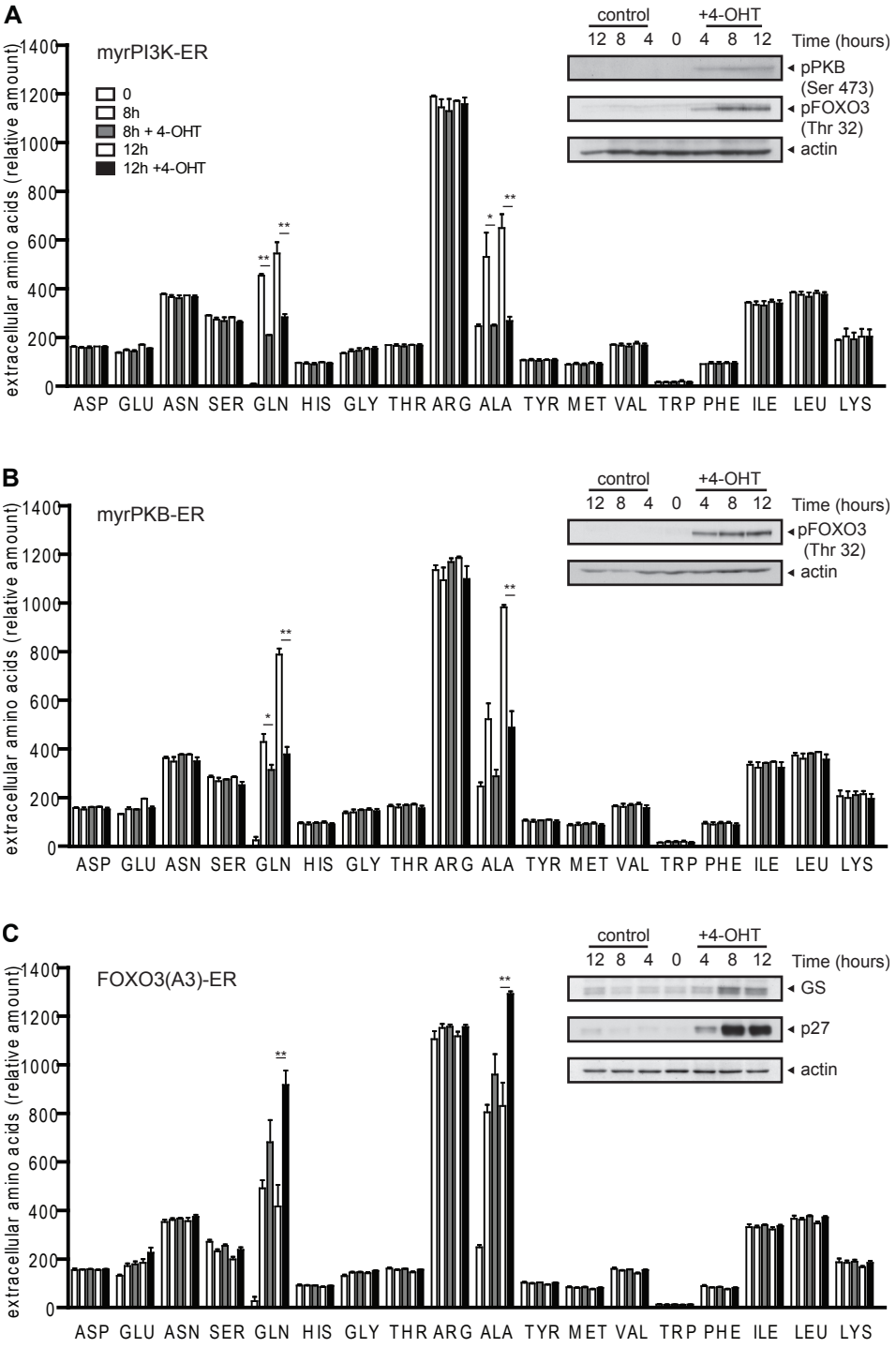


Figure 5. FOXO3-induced GS upregulation results in higher GS enzyme activity
(A) Ba/F3 cells expressing FOXO3(A3)-ER were stimulated with 100 nM 4-OHT in the presence of mIL-3 for the indicated times. Cells were lysed and equal amounts of proteins were analysed for GS activity in an enzyme assay. In addition the expression of GS, p27 and actin was determined using western blot. Shown are the mean ± SEM (n=3) and representative blots of these experiments. **(B)** Ba/F3 cells expressing FOXO3(A3)-ER were stimulated with 100 nM 4-OHT in the presence of mIL-3 together with MSO as indicated. Cells were lysed and equal amounts of proteins were analysed for GS activity in an enzyme assay. In addition the expression of GS, p27 and actin was determined using western blot. Shown are the mean ± SEM (n=3) and representative blots of these experiments. * $p < 0.05$ and ** $p < 0.01$

levels were unaffected (Fig. 5B). In conclusion these results suggest that the PI3K-PKB-FOXO pathway is able to regulate GS expression thereby increasing intracellular activity. To further examine the functional consequences of FOXO-mediated GS regulation we examined the levels of amino acids in the medium of Ba/F3 cells in which either PI3K, PKB or FOXO3 was activated. In cells in which PI3K or PKB were inducibly activated, glutamine levels were decreased compared to control cells, while concentrations of all other amino acids were unaffected (Fig. 6A,B). Moreover, in cells expressing FOXO3(A3)-ER addition of 4-OHT resulted in an increase in glutamine levels, which correlated with expression of GS (Fig. 6C). Alanine levels were also increased, which could be the result of increased glycolysis and subsequent rise in pyruvate as a result of the high levels of glutamine. Taken together, these results show that the PI3K-PKB-FOXO signalling module can specifically regulate glutamine metabolism through control of GS expression.

Figure 6. Modulation of the PI3K-PKB-FOXO pathway results in regulation of glutamine levels
(A), (B) Ba/F3 cells expressing either myrPI3K-ER, myrPKB-ER were cytokine starved overnight. The next day cells were washed in PBS and put in medium without serum with or without 100 nM 4-OHT. After indicated times medium samples were taken and analyzed for amino acid levels by HPLC. In addition cells were lyzed and equal amounts of protein were analyzed by western blotting for levels of phospho-PKB (S473), phospho-FOXO3 (T32), and actin. Shown are the mean ± SEM of relative amino acid levels compared to t=0 (n=2) and representative blots of these experiments. **(C)** Ba/F3 cells expressing FOXO3(A3)-ER were grown overnight in medium containing mIL-3 (5ng/ml). The next day cells were washed in PBS and put in medium containing mIL-3 (5 ng/ml) without serum with or without 100 nM 4-OHT. After indicated times medium samples were taken and analyzed for amino acid levels by HPLC. In addition cells were lyzed and equal amounts of protein were analyzed by western blotting for levels of GS, p27 and actin. Shown are the mean ± SEM of relative amino acid levels compared to t=0 (n=4) and representative blots of these experiments.

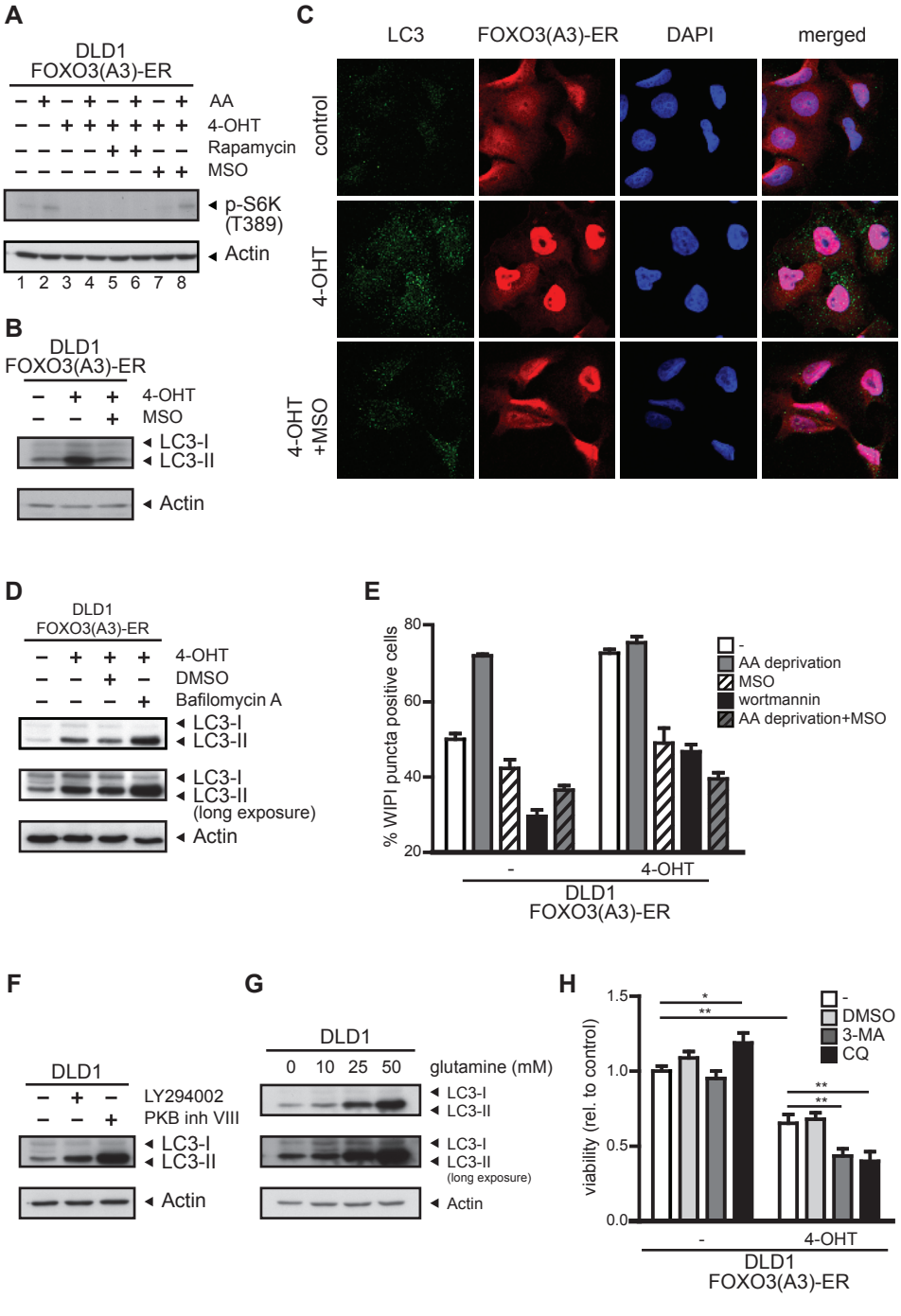

A
myrPI3K-ER
0
8h
8h + 4-OHT
12h
12h +4-OHT
extracellular amino acids (relative amount)
control
+4-OHT
12 8 4 0 4 8 12
Time (hours)
pPKB (Ser 473)
pFOXO3 (Thr 32)
actin
ASP GLU ASN SER GLN HIS GLY THR ARG ALA TYR MET VAL TRP PHE ILE LEU LYS
B
myrPKB-ER
pFOXO3 (Thr 32)
actin
C
FOXO3(A3)-ER
GS
p27
actin

FOXO3 induces autophagosome formation through increased GS activity

Amino acids have been show to play an important role in regulation of a variety of cellular processes including proliferation and cell growth through control of mammalian target of rapamaycin (mTOR) (reviewed in Wullschleger *et al.*, 2006). To determine the functional consequences of PI3K-PKB-FOXO mediated regulation of glutamine metabolism, DLD1 FOXO3(A3)-ER cells were stimulated with 4-OHT for 24 hours, subsequently starved and restimulated with essential amino acids. As shown in Figure 7A stimulation with essential amino acids induced phosphorylation of the mTOR substrate S6 kinase (S6K) (lane 2). This was dependent on mTOR signalling since pre-treatment of the cells with rapamycin completely blocked this induction (lane 6). In cells which had first been stimulated with 4-OHT for 24 hours, amino acid refeeding resulted in decreased mTOR activity as indicated by reduced S6K phosphorylation (lane 4). This decrease in phosphorylation status was dependent on the FOXO3-induced GS expression since incubating the cells with 4-OHT in the presence of MSO reversed the inhibition on S6K phosphorylation (lane 8). Recent studies have implicated mTOR activity as playing a central role in the regulation of autophagy and it has been well documented that inhibition of mTOR signalling can induce autophagosome formation and increase protein turnover (reviewed in Wullschleger *et al.*, 2006). Autophagosome formation can be measured by examining LC3 cleavage and lipidation; the full-length protein, LC3-I, and the truncated protein, LC3-II, can both be detected according to their size (Kabeya *et al.*, 2000). After treatment of DLD1 FOXO3(A3)-ER cells with 4-OHT the level of LC3-II was increased, suggesting increased autophagosome formation. In addition, MSO treatment blocked the increase in LC3-II demonstrating that LC3 cleavage is dependent on GS activity (Fig. 7B). Autophagosomes can be visualized by immunohistochemical staining of LC3; appearing as distinct LC3-positive punctate spots in the cytoplasm. To determine whether FOXO activation indeed resulted in increased autophagosome formation, DLD1 FOXO3(A3)-ER cells were treated with 4-OHT in the presence or absence of MSO and LC3 expression was visualized by confocal imaging. As shown in Figure 7C, FOXO3(A3)-ER is mainly present in the cytoplasm, while addition of 4-OHT results in translocation to the nucleus.

Figure 7. FOXO3-induced GS expression leads to inhibition of mTOR signalling and increased autophagy

(A) DLD1 cells expressing FOXO3(A3)-ER were stimulated with 4-OHT (500 nM) with or without MSO (1mM). After 24 hours, cells were starved in D-PBS containing indicated inhibitors and stimulated with amino acids for 10 minutes. Cells were lysed and analyzed by western blotting for levels of phospho-S6K (T389) and actin. Shown are representative blots of three independent experiments. **(B)** DLD1 cells expressing FOXO3(A3)-ER were stimulated with 4-OHT (500 nM) and MSO (1mM) for 24 hours. Cells were lysed and analyzed by western blotting for levels of LC3 and actin. Shown are representative blots (n=4). **(C)** DLD1 cells expressing FOXO3(A3)-ER were stimulated with 4-OHT (500 nM) and MSO (1mM) for 24 hours in DMEM without glutamine containing 0.1% FCS. Cells were stained for LC3 and ER and analyzed by confocal microscopy. Shown are representative pictures (n=3). **(D)** DLD1 cells expressing FOXO3(A3)-ER were stimulated with 4-OHT (500 nM) and Bafilomycin A (10 nM). After 24 hours cells were lysed and analyzed by western blotting for expression of LC3 and actin. Shown are representative blots (n=4). **(E)** DLD1 cells expressing FOXO3(A3)-ER were transiently transfected with WIPI-GFP and subsequently stimulated with 4-OHT (500 nM), MSO (1mM) and wortmannin (233nM) in DMEM without glutamine containing 0.1% FCS or medium without amino acids for 24 hours. Cells were stained for WIPI and analyzed by confocal microscopy. Depicted are the percentages of WIPI puncta positive cells. Shown are the mean ± SEM (n=4). **(F,G)** DLD1 cells were stimulated with LY294002 (20 μM) or PKB inhibitor VIII (10 μM) (F) or with L-glutamine (G) for 24 hours. Cells were lysed and analyzed by western blotting for levels of LC3 and actin. Shown are representative blots (n=4). **(H)** DLD1 cells expressing FOXO3(A3)-ER were stimulated with 4-OHT (500 nM) and 3-Methyladenine (1mM), chloroquine (10 μM). After 24 hours cell proliferation was measured using WST-1. Relative absorbance measurements are depicted compared to control. Shown are the mean ± SEM (n=3).g


A
DLD1
FOXO3(A3)-ER
AA
4-OHT
Rapamycin
MSO
p-S6K
(T389)
Actin
1 2 3 4 5 6 7 8
B
DLD1
FOXO3(A3)-ER
4-OHT
MSO
LC3-I
LC3-II
Actin
C
LC3
FOXO3(A3)-ER
DAPI
merged
control
4-OHT
4-OHT
+MSO
D
DLD1
FOXO3(A3)-ER
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Bafilomycin A
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LC3-II
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40
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AA deprivation
MSO
wortmannin
AA deprivation+MSO
-
4-OHT
DLD1
FOXO3(A3)-ER
F
DLD1
LY294002
PKB inh VIII
LC3-I
LC3-II
Actin
G
DLD1
0 10 25 50
glutamine (mM)
LC3-I
LC3-II
LC3-I
LC3-II
(long exposure)
Actin
H
viability (rel. to control)
1.5
1.0
0.5
0.0
*
**
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**
-
DMSO
3-MA
CQ
-
4-OHT
DLD1
FOXO3(A3)-ER

In control cells LC3 showed a diffuse cytoplasmic distribution, while after FOXO3 activation distinct LC3-positive punctate spots are clearly visible. Importantly, treating cells with 4-OHT in the presence of MSO almost completely abrogated the formation of autophagosomes (Fig. 7C; lower panels). Next we determined whether FOXO3 not only induces autophagosome formation, but also increases autophagic flux and protein lysis. FOXO3 was activated in DLD1 cells by addition of 4-OHT in the presence of Bafilomycin A. Bafilomycin A inhibits lysosomal protein degradation and if autophagy is increased as a result of FOXO activation the levels of LC3 protein (LC3-II) should be further increased (Rubinsztein *et al.*, 2009). Addition of Bafilomycin A further increased FOXO3-induced LC3 cleavage supporting the previous observation that FOXO activation results in higher levels of autophagy (Fig. 7D).To further validate these results we transiently transfected DLD1 cells expressing FOXO3(A3)-ER with WIPI-1-GFP and looked at the formation of WIPI-1 puncta. WIPI-1 puncta formation has recently beeen described as a novel quantifiable assay to asses autophagy (Proikas-Cezanne *et al.*, 2007). As shown in Figure 7E, the percentage of WIPI-1 puncta positive cells was increased after activation of FOXO3, which was inhibited by MSO. In addition, AA deprivation-induced autophagy was also inhibited by MSO. As a control cells were also incubated with the PI3K inhibitor wortmannin, which blocks the formation of autophagosomes through inhibition of Vps34. Treatment with wortmannin completely abrogated the FOXO3-increased number of WIPI puncta positive cells, indicating that the increase in spots is mediated through increased formation of Autophagosomes. To determine whether inhibition of PI3K-PKB also resulted in increased LC3-II formation, DLD1 cells were treated with LY294002 or PKB inhibitor VIII for 24 hours. LC3-II levels were indeed increased, supporting our data that the PI3K-PKB-FOXO pathway regulates autophagy through modulation of glutamine levels (Fig. 7F). Since FOXO3-mediated induction of autophagy is dependent on GS expression we wanted to determine whether glutamine alone was sufficient to induce autophagy. To this end, DLDL1 cells were incubated with increasing concentrations of glutamine and LC3 levels were again evaluated. Addition of glutamine alone to cells resulted increased LC3-II levels (Fig. 7G). It has been previously shown that induction of autophagy is important for survival of cells and that inhibition of autophagy can result in increased cell death (Lum *et al.*, 2005). To investigate the functional effect of FOXO-mediated induction of autophagy, DLDL1 cells expressing FOXO3(A3)-ER were stimulated with 4-OHT in the presence of 3-methlyladenine (3-MA) and chloroquine (CQ), which inhibit induction of autophagy and after 24 hours the viability was determined. In the absence of 4-OHT both autophagy inhibitors had no effect on viability of the cells. However, activation of FOXO3 resulted in reduced cell viability, which was even further diminished in the presence of 3-methyladenine and chloroquine (Fig. 7H). In conclusion, these results suggest that the induction of autophagy by FOXO3-mediated upregulation of GS is important for cellular survival.

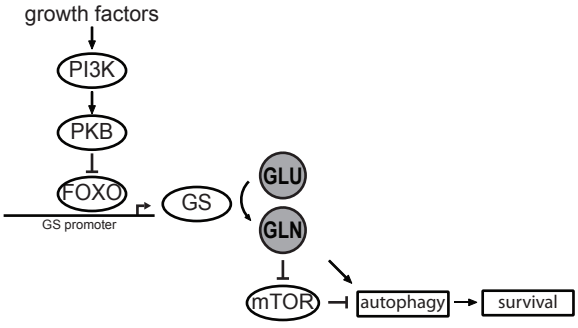


Figure 8. Regulation of GS expression modulates autophagy
In the absence of growth factors, FOXO becomes active resulting in upregulation of GS expression. The subsequent increase in glutamine levels inhibits mTOR signalling and induces autophagy. In addition DAF-16-induced GS expression might be important for the DAF-16 induced increased life span in C elegans.

DISCUSSION

By comparative microarray analysis GS was identified as a novel downstream effector of the PI3K-PKB-FOXO signalling module. We have demonstrated that activation of FOXO3 and FOXO4 induces a rapid upregulation of GS expression and activity resulting in increased glutamine levels. Importantly our data reveals that these increases glutamine are required for FOXO3-mediated induction of autophagy. This is apparently an important survival mechanism for the cells, since blockade of autophagy with chemical inhibitors reduced the viability of FOXO3 activated cells. Analysis of the PI3K-PKB-FOXO in *C elegans* demonstrated that the regulation of GS by this pathway is also evolutionary conserved. Taken together, these data indicate that the PI3K pathway regulates autophagic flux through modulation of GS expression. In the absence of growth factors activation of FOXOs results in a rise in glutamine levels, inducing autophagosome formation and allowing cells to survive during periods of nutrient deprivation (Fig. 8).

Our experiments showed increased GS expression after FOXO activation. Furthermore, by using promoter reporter assays we have shown that the presence of a conserved FOXO binding site in the GS promoter is critical for regulation of GS transcription by FOXO3 (Fig. 3). It has been described that GS expression in the liver is upregulated after activation of β-catenin (Loeppen *et al.*, 2005). β-Catenin has been described to physically interact with FOXO and co-expression of these proteins increases transcription from several FOXO promoter reporters (Essers *et al.*, 2005). To test whether FOXO3 controls GS transcription in conjunction with β-catenin cells were transfected with a GS promoter reporter construct together with constitutively active FOXO3 and TCF4, a binding partner of β-catenin, which cant inhibit FOXO3 activity by competing for β-catenin. Cotransfection of TCF4 inhibited the FOXO3-induced GS promoter activity (unpublisehed data), suggesting that indeed β-catenin is required for FOXO3-mediated GS expression.

FOXO3 has recently been reported to be an important regulator of autophagy during muscle atrophy by increasing the expression of several autophagy-related genes, including Gabarapl1, atg12l, Bnip3 and Beclin 1 (Mammucari *et al.*, 2007; Zhao *et al.*, 2007). However, the precise molecular mechanisms underlying FOXO3-induced autophagy remain unclear. It has been hypothesised that upregulation of autophagy-related genes may in fact act to replace components which are consumed during autophagic flux since their increase was not itself sufficient to induce autophagy (Mammucari *et al.*, 2007; Zhao *et al.*, 2007). Here we demonstrate that glutamine metabolism links FOXO transcription factor activity to autophagy. We have shown that the FOXO3-induced GS activity is required for induction of autophagy, while glutamine treatment alone is sufficient.
Our results indicate that FOXO3 triggers autophagosome formation by increasing GS expression and subsequently inhibiting mTORC1 activity. We have demonstrated that GS activity decreased phosphorylation of S6K and resulted in formation of autophagosomes, suggesting that FOXO3 induces autophagy by glutamine-mediated inhibition of mTOR (Fig. 7). These findings reveal a novel mechanism by which FOXOs induce autophagy and provide furhter insight into the regulation of autophagy by amino acids. Previous studies have demonstrated that the level of autophagy is regulated by the nutrient-sensing mTOR pathway (reviewed in Pattingre *et al.*, 2008). Upon activation by growth factors and nutrients mTORC1 increases protein synthesis and cell growth, while inhibition of autophagy blocks protein degradation mTORC1 has been proposed to phosphorylate and thereby inhibit the Atg1 complex, which is required for the early steps of autophagosome formation (Pattingre *et al.*, 2008). Amino acids can activate mTORC1 through the class III PI3K hVps34, but

the mechanism is poorly understood (Wullschleger *et al.*, 2006). Recently it has been reported that activation of mTORC1 by amino acids was dependent on a bidirectional transporter, which regulates the simultaneous import of essential amino acids into the cell and the efflux of glutamine (Nicklin *et al.*, 2009). In this study pre-loading cells with glutamine for one hour was required for amino acid-stimulated phosphorylation of S6K. In contrast, we have shown that FOXO3 activation for 24 hours decreased amino acid-induced phosphorylation of S6K in a GS-dependent manner (Fig. 7A). This suggests that the effect of glutamine on mTOR signalling may have a differential outcome depending on the duration of the glutamine treatment, however the mechanism of glutamine-mediated inhibition of mTOR signalling remains to be investigated.

We have shown that the induction of autophagy by activation of FOXO3 is important for cellular survival. Blockade of autophagy with 3-MA and CQ decreased the viability of DLD1 cells after FOXO activation, while these inhibitors did not decrease the viability of DLD1 cells in the absence of FOXO activity (Fig. 7H). These result demonstrate the importance of FOXO-induced autophagy for cellular survival. Although increased levels of autophagy may ultimately lead to cell death, at lower levels this process is considered to be a survival mechanism. During starvation low levels of autophagy can increase survival through suppressing oxidative damage by scavenging of mitochondria (Eisenberg-Lerner *et al.*, 2009). In addition, the induction of autophagy after growth factor withdrawal and the subsequent breakdown of proteins can provide a source of energy and nutrients, which are required for survival (Lum *et al.*, 2005). Knockdown of autophagy-related genes in *C elegans* and *Drosophila* decreases lifespan, while long-lived *daf-2* loss of function worms showed increased levels of autophagy (Hansen *et al.*, 2008; Melendez *et al.*, 2003). Knockdown of the autophagy-related gene *beclin 1* blocked dauer formation in *daf-2* mutants and completely inhibited the increase in life span (Hansen *et al.*, 2008). These results indicate that autophagy induction may act as a regulatory mechanism of animal aging. We have demonstrated that the regulation of GS expression by FOXOs is conserved in the nematode worm *C elegans* (Fig. 4A/B). This regulation might be responsible for the increased levels of autophagy observed in *daf-2* mutants and play an essential role in daf-16 mediated longevity.

Our data suggest that increased PKB activity results in decreased levels of autophagy through inhibiting the induction of GS expression. While the PI3K-PKB pathway can increase survival of cells by inhibiting programmed cell death, recent evidence indicates that under certain circumstances activation of this signalling cascade can increase cell death by blocking autophagy (Degtyarev *et al.*, 2008; Wu *et al.*, 2009). Growth factor-mediated activation of PKB has been shown to indirectly increase mTORC1 activity through inhibition of the TSC1/TSC2 complex (Inoki *et al.*, 2002; Manning *et al.*, 2002). In L929 cells treated with a general caspase inhibitor and in H_2O_2-treated $Bax^{-/-}$ $Bak^{-/-}$ mouse embryonic fibroblasts activation of PI3K-PKB pathway promoted cell death by suppression of autophagy (Wu *et al.*, 2009). In addition, it has been demonstrated that knockdown of PKB in PTEN deficient cancer cells induced an increase in autophagy. Preventing autophagy accelerated apoptotic cell death, indicating that the induction of autophagy after PKB inhibition is an important determinant of cellular survival (Degtyarev *et al.*, 2008). It remains to be determined in these experimental systems whether FOXO-mediated GS expression is responsible for the induction of autophagy and survival.

FOXOs have been indicated to function as tumour suppressors by blocking cell cycle progression and actively inducing apoptosis. Furthermore, inducible *Foxo1,3,4*$^{-/-}$ mice

develop spontaneous lymphomas and hemangiomas, proving that FOXOs act as redundant tumour suppressors. Autophagy has been proposed to be tumour suppressive, by protecting cells from metabolic stress and oxidative damage. Activation of oncogenic signalling pathways, such as PI3K and mTOR suppresses autophagy, while many tumour suppressors, including PTEN and p53, lead to an induction through mTOR inhibition. Here we have demonstrated that GS is a novel transcriptional target of FOXO signalling and its upregulation triggers suppression of mTOR signalling and consequently autophagy. The induction of autophagy by FOXO-mediated GS expression might contribute to the tumour suppressive function of FOXOs and provide a novel target for cancer therapies.

ACKOWLEDGEMENT

We thank M. Putker for generating the *Foxo1,3,4*$^{-/-}$ MEFs.

MATERIALS AND METHODS:

Constructs
SRα-myrp110α-ER, SRα-myrPKB-ER and pcDNA3-HA-FOXO3(A3)-ER have been described previously (Dijkers *et al.*, 2000a; Dijkers *et al.*, 2000b) pcDNA3-HA-FOXO4(A3)-ER was generated by cloning FOXO4(A3) without the stopcodon into pcDNA3 containing the hormone-binding domain of the estrogen receptor (pcDNA3-ER). The pT81 and pXP2 construct expressing the GS reporter have been described previously (Fahrner *et al.*, 1993; Gaunitz *et al.*, 2001). In the pXP2 construct expressing -2520/+105 bp from the GS reporter FOXO binding sites were mutated by quickchange mutagenesis: the core TGT sequence from FOXO motif TTGTTTAC was mutated into ACA.

Primers used for quickchange mutagenesis in GS reporter (putative FOXO binding sites in bold and mutations in bold and small):
FOXO binding site 1:
Forward primer: AAGCTTCTacaTTACCCCTGAAAGTCAGTGGTCAC
Reverse primer: GTGACCACTGACTTTCAGGGGTAATGTAGAAGCTT
FOXO binding site 2:
Forward primer: AGTGGTCACATGAGAacaTCCTGGTCACATGGAAGG
Reverse primer: CCTTCCATGTGACCAGGATGTTCTCATGTGACCACT
FOXO binding site 3:
Forward primer: CTTGGAAACAGAGCAAAacaTCTCTTGATGCTGCCG
Reverse primer: CGGCAGCATCAAGTGATGTTTTGCTCTGTTTCCAAG
FOXO binding site 4:
Forward primer: TTG ATG CTG CCG CacaTT CTG TGT GGT CAA TTT GTG
Reverse primer: CACAAATTGACCACACAGAATGTGCGGCAGCATCAA
FOXO binding site 5:
Forward primer: GTGGTCAATTTGacaTTATCGAACACTCAGTCTGG
Reverse primer: CCAGACTGAGTGTTCGATAATGTCAAATTGACCAC
FOXO binding site 6:
Forward primer: CACTCAGTCTGGAAACacaTTGGGGGCGGGGTG
Reverse primer: CACCCCGCCCCCAATGTGTTTCCAGACTGAGTG

Cell culture
Ba/F3 cells were cultured in RPMI-1640 containing glutamax (Invitrogen), 10% fetal bovine serum (Hyclone, Logan, UT), penicillin and streptomycin (Invitrogen), 0.5 mM β-mercaptoethanol (Merck, Darmstadt, Germany) and recombinant mouse IL-3 produced in Cos cells (Caldenhoven *et al.*, 1995). For the generation of clonal Ba/F3 cells stably expressing myrPI3K-ER or myrPKB-ER, the SRα-myrp110α-ER construct and the SRα-myrPKB-ER construct were electroporated into Ba/F3 cells

together with pSG5 conferring neomycin resistance. For the generation of clonal Ba/F3 cells stably expressing FOXO3(A3)-ER or FOXO4(A3)-ER, the pcDNA3-HA-FOXO3(A3)-ER construct and the pcDNA3-HA-FOXO3(A3)-ER construct were electroporated into Ba/F3 cells. Cells were maintained in the presence of 1 mg/ml G418 (Gibco, Paisley, UK) and clonal lines were generated by limited dilution.
MEFs derived from triple conditonal FoxO knockout mice (FoxO1L/L::FoxO3L/L::FOXO4L/L , Paik *et al* Cell 2007, a generous gift from Ji-Hye Paik and Ronald DePinho) were immortalized by viral transduction with pBabe-Hygro-SV40-LargeT. To ensure equal levels of large T in all cells, clonal cell lines were made by seeding single cells in the wells of 96 wells plates. Successful immortalization was evident from outgrowth of colonies. Immortalized FoxO1L/L::FoxO3L/L::FOXO4L/L MEFs were infected with 100 MOI Cre recombinase expressing adenovirus (Vector Biolabs). subsequently clonal cell lines derived from the Cre infected FoxO1L/L::FoxO3L/L::FOXO4L/L MEFs were screened for recombination of the LoxP sites (resulting in a knockout allele) for all three FoxOs by PCR with primer sets discriminating between wt, floxed and recombined alleles as described in Paik *et al.,* Cell 2007. MEFs, DLD1 cells, and 293 cells were cultured in DMEM containing glutamax (Invitrogen), 10% fetal bovine serum and penicillin and streptomycin (Invitrogen). DLD1 cells expressing FOXO3(A3)-ER have been described previously(Kops *et al.*, 2002b).

Antibodies and reagents
Antibodies against actin and ER were from Santa Cruz (Palo Alto, CA). Antibodies against phospho-PKB (S473) and Phospho-S6K (T389) were from Cell Signaling Technologies (Danvers, MA). Antibodies against glutamine synthetase and p27 were from BD Biosciences (San Diego, CA). Antibody against phospho-FOXO3 (T32) was from Upstate (Billerica, MA) and the antibody against LC3 from Nanotools (Teningen, Germany). Secondary antibodies donkey-anti-mouse-DyLight488 and donkey-anti-rabbit-DyLight549 were obtained from Jackson (West Grove, PA). Glutamine, 4-OHT, L-Methionine sulfoximine (MSO), 3-Methyladenine, Chloroquine and Bafilomycin A were obtained from Sigma-Aldrich (Missouri, USA). Murine IL-3 was obtained from Peprotech (New Jersey, USA). LY294002 was obtained from Cayman Chemical (Ann Arbor, MI), PKB inhibitor VIII from Calbiochem (Darmstadt, Germany) and rapamycin was from Biomol Research (Plymouth Meeting, PA).

Western blotting
Western blot analysis was performed using standard techniques. In brief, Ba/F3 cells were lysed in Laemmli buffer (0.12 M Tris HCl pH 6.8, 4% SDS and 20% glycerol, 35 mM β-mercaptoethanol and bromophenol blue) and boiled for 5 min. DLD1 cells were lysed in 1x sample buffer (2% SDS, 10%glycerol, 2% β-mercaptoethanol, 60 mM Tris pH 6.8 and bromophenolblue) Equal amounts of total lysate were analyzed by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to Immobilon-P and incubated with the appropriate antibodies according to the manufacturer's conditions. Membranes were washed, incubated with appropriate secondary antibodies and developed by ECL (Amersham Pharmacia, Amersham, UK).

RNA isolation and quantitative RT-PCR
Cells were stimulated as indicated an harvested at the respective times, washed twice with PBS, lysed in 1 ml Trizol (Invitrogen, Breda, The Netherlands). For isolation of total RNA samples were incubated at room temperature for 3 minutes, 0.2 ml chloroform was added, vortexed and centrifuged for 15 minutes at 8000 rpm at 4°C. Subsequently, the aqueous phase was transferred to new tubes, 0.5 ml isopropanol was added and incubated for minimal 30 minutes at -20°C. Samples were centrifuged for 10 minutes at 14000 rpm at 4°C. The pellet was washed with 70% ethanol and dissolved in water. DNAse treatment and purification was performed using Qiagen's RNeasy kit (Qiagen Inc., Valencia, CA). Equal amounts of total RNA were reversed transcribed with SuperScript III reverse transcriptase (Invitrogen, Breda, the Netherlands). The resulting cDNA was amplified using an Biorad Icycler with the primer pairs for mouse GS and B2M, a nonregulated housekeeping gene, was used as an internal control to normalize input RNAMouse GS: forward primer: TGAACAAAGGCATCAAGCAAATG, reverse primer: CAGTCCAGGGTACGGGTCTT.

Reporter assays
For transient reporter assays HEK293 cells were transfected with GS reporter constructs together with pECE-HA-FKHRL1(A3) and renilla to normalize for transfection efficiency using PEI (Polyethylenimine). After 40 hours, cells were washed twice with PBS, lysed in 100 µL passive lysis buffer and assayed for luciferase activity using Dual-Luciferase Reporter Assay System (Promega).

Glutamine Synthetase activity assay
Ba/F3 cells were stimulated as indicated, washed with PBS, lysed in 50 mM Imidazole and incubated at -80°C for at least 4 hours. Protein concentrations were determined using Bradford protein assay (Biorad). Equal amounts of proteins were analyzed for GS activity as previously described [REF]. In brief, samples were mixed with 2x GS activity buffer (50 mM imidazole, 25 mM arsenic acid, 0.16 mM ADP, 50 mM L-glutamine, 25 mM hydroxylamine and 2mM MnCl2) and incubated at in a 96-wells plate at 37°C for 30 minutes. Next, an equal volume of GS stop solution (2.42% ferric chloride, 1.45% TCA, 1.82% HCL) was added and the absorbance at 560 nm was determined. Absorbance values of the formed product glutamyl-γ-hydroxamate are converted to nanomoles of product by a callibration curve using commercially available glutamyl-γ-hydroxamate.

Determination of extracellular amino acid concentrations
Ba/F3 cells expressing myrPI3K-ER, myrPKB-ER, FOXO3(A3)-ER or FOXO4(A3)-ER were stimulated as indicated. After indicated times medium samples were taken and stored at -80°C. Concentrations of free amino acids were determined by an automated reversed phase high performance liquid chromatography system (RP-HPLC) with precolumn derivazation using the opthaldialdehyde method (Guthke *et al.*, 2006).

Proliferation assay
DLD1 cells expressing FOXO3(A3)-ER were plated in 96 wells-plates. The next day, cells were washed twice with PBS and put in medium without glutamine containing 0,1 % FCS with 4-OHT (500 nM) and inhibitors. After 24 hours medium was removed, 45 µl DMEM + 5 µl WST-1 (Roche, Mannheim, Germany) was added and cells were incubated at 37°C. After 30 minutes absorbance (450 nm) was measured.

Confocal studies
DLD1 cells were adhered to microscope glasses and the next day cells were stimulated with 4-OHT and MSO. After 24 hours cells were fixed in PBS containing 3% paraformaldehyde (Merck) for 15 minutes at room temperature and subsequently in 100% methanol (Merck) for 30 min at -20°C. Cells were blocked in PBS containing 10% normal donkey serum (Jackson, Westgrove, PA) for 30 minutes at room temperature. Next, cells were incubated with mouse anti LC3 antibody and rabbit anti ER antibody for 1 hour at room temperature, followed by PBS washes and incubation with donkey-anti-mouse-DyLight488 and donkey-anti-rabbit-DyLight549 (all antibody stainings were in PBS with 10% donkey serum). Slides were then washed extensively, and cells were mounted in mowiol containing 3%DABCO followed by a glass cover. Slides were examined with a 63x objective on a Zeiss LSM 710 fluorescence microscope (Oberkochen, Germany).
For the analysis of WIPI-1 puncta, DLD1 cells expressing FOXO3(A3)-ER were transiently transfected with WIPI-1-GFP. The percentage of WIPI-1 puncta positive cells was analysed by quantitave confocal microscopy as previously described(Proikas-Cezanne *et al.*, 2007).

C elegans assays
Nematode strains were cultured at 20°C using standard conditions as described(Brenner, 1974; Lewis and Fleming, 1995). Mutant strains used in this study were *daf-16*(mu86)(Lin *et al.*, 1997) and *daf-2*. In order to measure GS activity, strains were synchronized to L1 by hypochlorite treatment and were placed on NGM agar plates. After 5 days worms were lysed in 50 mM Imidazole and incubated at -80°C for at least 4 hours.
Statistical analysis
Data are expressed as mean ± SEM. Significant differences between mean values were evaluated using 1-way ANOVA followed by Dunnet test (for serial analysis of one variable) or 2-way ANOVA followed by Bonferoni post-tests (for serial analysis of two variables). * $p < 0.05$ and ** $p < 0.01$

REFERENCES

Brenner,S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94.

Caldenhoven,E., van,D.T., Raaijmakers,J.A., Lammers,J.W., Koenderman,L., and De Groot,R.P. (1995). Activation of the STAT3/acute phase response factor transcription factor by interleukin-5. J. Biol. Chem. *270*, 25778-25784.

Calnan,D.R. and Brunet,A. (2008). The FoxO code. Oncogene *27*, 2276-2288.

Cassada,R.C. and Russell,R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Dev. Biol. *46*, 326-342.

Degtyarev,M., De,M.A., Orr,C., Lin,J., Lee,B.B., Tien,J.Y., Prior,W.W., van,D.S., Wu,H., Gray,D.C., Davis,D.P., Stern,H.M., Murray,L.J., Hoeflich,K.P., Klumperman,J., Friedman,L.S., and Lin,K. (2008). Akt inhibition promotes autophagy and sensitizes PTEN-null tumors to lysosomotropic agents. J. Cell Biol. *183*, 101-116.

Delpuech,O., Griffiths,B., East,P., Essafi,A., Lam,E.W., Burgering,B., Downward,J., and Schulze,A. (2007). Induction of Mxi1-SR alpha by FOXO3a contributes to repression of Myc-dependent gene expression. Mol. Cell Biol. *27*, 4917-4930.

Dijkers,P.F., Birkenkamp,K.U., Lam,E.W., Thomas,N.S., Lammers,J.W., Koenderman,L., and Coffer,P.J. (2002). FKHR-L1 can act as a critical effector of cell death induced by cytokine withdrawal: protein kinase B-enhanced cell survival through maintenance of mitochondrial integrity. J. Cell Biol. *156*, 531-542.

Dijkers,P.F., Medema,R.H., Lammers,J.W., Koenderman,L., and Coffer,P.J. (2000a). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. Curr. Biol. *10*, 1201-1204.

Dijkers,P.F., Medema,R.H., Pals,C., Banerji,L., Thomas,N.S., Lam,E.W., Burgering,B.M., Raaijmakers,J.A., Lammers,J.W., Koenderman,L., and Coffer,P.J. (2000b). Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). Mol. Cell Biol. *20*, 9138-9148.

Dorman,J.B., Albinder,B., Shroyer,T., and Kenyon,C. (1995). The age-1 and daf-2 genes function in a common pathway to control the lifespan of Caenorhabditis elegans. Genetics *141*, 1399-1406.

Eisenberg,D., Gill,H.S., Pfluegl,G.M., and Rotstein,S.H. (2000). Structure-function relationships of glutamine synthetases. Biochim. Biophys. Acta *1477*, 122-145.

Eisenberg-Lerner,A., Bialik,S., Simon,H.U., and Kimchi,A. (2009). Life and death partners: apoptosis, autophagy and the cross-talk between them. Cell Death. Differ. *16*, 966-975.

Essers,M.A., de Vries-Smits,L.M., Barker,N., Polderman,P.E., Burgering,B.M., and Korswagen,H.C. (2005). Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science *308*, 1181-1184.

Fahrner,J., Labruyere,W.T., Gaunitz,C., Moorman,A.F., Gebhardt,R., and Lamers,W.H. (1993). Identification and functional characterization of regulatory elements of the glutamine synthetase gene from rat liver. Eur. J. Biochem. *213*, 1067-1073.

Furuyama,T., Nakazawa,T., Nakano,I., and Mori,N. (2000). Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. Biochem. J. *349*, 629-634.

Gaunitz,F., Weber,S., Scheja,L., and Gebhardt,R. (2001). Identification of a cis-acting element and a novel trans-acting factor of the glutamine synthetase gene in liver cells. Biochem. Biophys. Res. Commun. *284*, 377-383.

Guthke,R., Zeilinger,K., Sickinger,S., Schmidt-Heck,W., Buentemeyer,H., Iding,K., Lehmann,J.,

Pfaff,M., Pless,G., and Gerlach,J.C. (2006). Dynamics of amino acid metabolism of primary human liver cells in 3D bioreactors. Bioprocess. Biosyst. Eng *28*, 331-340.

Hansen,M., Chandra,A., Mitic,L.L., Onken,B., Driscoll,M., and Kenyon,C. (2008). A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. PLoS. Genet. *4*, e24.

Inoki,K., Li,Y., Zhu,T., Wu,J., and Guan,K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol. *4*, 648-657.

Kabeya,Y., Mizushima,N., Ueno,T., Yamamoto,A., Kirisako,T., Noda,T., Kominami,E., Ohsumi,Y., and Yoshimori,T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. *19*, 5720-5728.

Kenyon,C., Chang,J., Gensch,E., Rudner,A., and Tabtiang,R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461-464.

Kimura,K.D., Tissenbaum,H.A., Liu,Y., and Ruvkun,G. (1997). daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science *277*, 942-946.

Kops,G.J., Dansen,T.B., Polderman,P.E., Saarloos,I., Wirtz,K.W., Coffer,P.J., Huang,T.T., Bos,J.L., Medema,R.H., and Burgering,B.M. (2002a). Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature *419*, 316-321.

Kops,G.J., Medema,R.H., Glassford,J., Essers,M.A., Dijkers,P.F., Coffer,P.J., Lam,E.W., and Burgering,B.M. (2002b). Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. Mol. Cell Biol. *22*, 2025-2036.

Lewis,J.A. and Fleming,J.T. (1995). Basic culture methods. Methods Cell Biol. *48*, 3-29.

Lin,K., Dorman,J.B., Rodan,A., and Kenyon,C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science *278*, 1319-1322.

Loeppen,S., Koehle,C., Buchmann,A., and Schwarz,M. (2005). A beta-catenin-dependent pathway regulates expression of cytochrome P450 isoforms in mouse liver tumors. Carcinogenesis *26*, 239-248.

Lum,J.J., Bauer,D.E., Kong,M., Harris,M.H., Li,C., Lindsten,T., and Thompson,C.B. (2005). Growth factor regulation of autophagy and cell survival in the absence of apoptosis. Cell *120*, 237-248.

Mammucari,C., Milan,G., Romanello,V., Masiero,E., Rudolf,R., Del,P.P., Burden,S.J., Di,L.R., Sandri,C., Zhao,J., Goldberg,A.L., Schiaffino,S., and Sandri,M. (2007). FoxO3 controls autophagy in skeletal muscle in vivo. Cell Metab *6*, 458-471.

Manning,B.D., Tee,A.R., Logsdon,M.N., Blenis,J., and Cantley,L.C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol. Cell *10*, 151-162.

Mei,Y., Zhang,Y., Yamamoto,K., Xie,W., Mak,T.W., and You,H. (2009). FOXO3a-dependent regulation of Pink1 (Park6) mediates survival signaling in response to cytokine deprivation. Proc. Natl. Acad. Sci. U. S. A *106*, 5153-5158.

Melendez,A., Talloczy,Z., Seaman,M., Eskelinen,E.L., Hall,D.H., and Levine,B. (2003). Autophagy genes are essential for dauer development and life-span extension in C. elegans. Science *301*, 1387-1391.

Mukhopadhyay,A., Oh,S.W., and Tissenbaum,H.A. (2006). Worming pathways to and from DAF-16/FOXO. Exp. Gerontol. *41*, 928-934.

Nicklin,P., Bergman,P., Zhang,B., Triantafellow,E., Wang,H., Nyfeler,B., Yang,H., Hild,M., Kung,C., Wilson,C., Myer,V.E., MacKeigan,J.P., Porter,J.A., Wang,Y.K., Cantley,L.C., Finan,P.M., and Murphy,L.O. (2009). Bidirectional transport of amino acids regulates mTOR and autophagy. Cell *136*, 521-534.

Ogg,S., Paradis,S., Gottlieb,S., Patterson,G.I., Lee,L., Tissenbaum,H.A., and Ruvkun,G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature *389*, 994-999.

Paradis,S. and Ruvkun,G. (1998). Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes Dev. *12*, 2488-2498.

Partridge,L. and Bruning,J.C. (2008). Forkhead transcription factors and ageing. Oncogene *27*, 2351-2363.

Pattingre,S., Espert,L., Biard-Piechaczyk,M., and Codogno,P. (2008). Regulation of macroautophagy by mTOR and Beclin 1 complexes. Biochimie *90*, 313-323.

Proikas-Cezanne,T., Ruckerbauer,S., Stierhof,Y.D., Berg,C., and Nordheim,A. (2007). Human WIPI-1 puncta-formation: a novel assay to assess mammalian autophagy. FEBS Lett. *581*, 3396-3404.

Rubinsztein,D.C., Cuervo,A.M., Ravikumar,B., Sarkar,S., Korolchuk,V., Kaushik,S., and Klionsky,D. J. (2009). In search of an “autophagomometer”. Autophagy. *5*, 585-589.

Tran,H., Brunet,A., Grenier,J.M., Datta,S.R., Fornace,A.J., Jr., DiStefano,P.S., Chiang,L.W., and Greenberg,M.E. (2002). DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. Science *296*, 530-534.

van Gorp,A.G., Pomeranz,K.M., Birkenkamp,K.U., Hui,R.C., Lam,E.W., and Coffer,P.J. (2006). Chronic protein kinase B (PKB/c-akt) activation leads to apoptosis induced by oxidative stress-mediated Foxo3a transcriptional up-regulation. Cancer Res. *66*, 10760-10769.

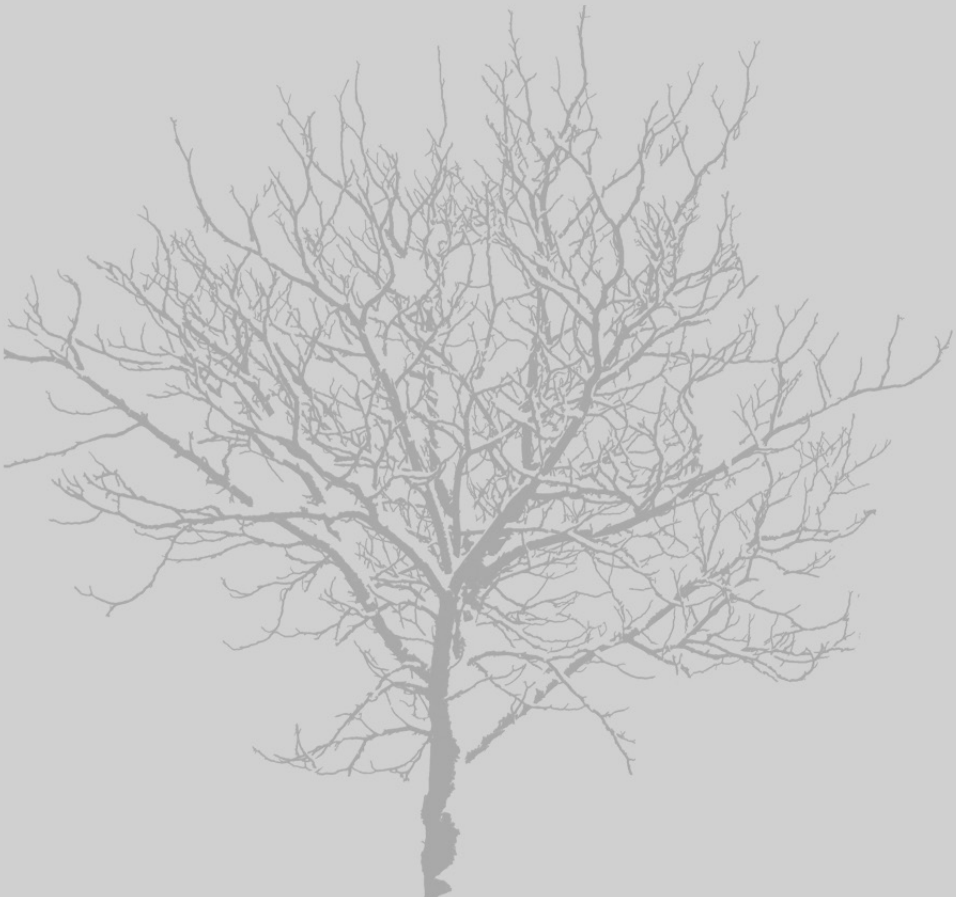
Wu,Y.T., Tan,H.L., Huang,Q., Ong,C.N., and Shen,H.M. (2009). Activation of the PI3K-Akt-mTOR signaling pathway promotes necrotic cell death via suppression of autophagy. Autophagy. *5*, 824-834.

Wullschleger,S., Loewith,R., and Hall,M.N. (2006). TOR signaling in growth and metabolism. Cell *124*, 471-484.

Zhao,J., Brault,J.J., Schild,A., Cao,P., Sandri,M., Schiaffino,S., Lecker,S.H., and Goldberg,A.L. (2007). FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. Cell Metab *6*, 472-483.

CHAPTER 8

General discussion



EIGHT

1. FOXO-PKB SIGNALLING IN ONCOGENESIS

The PI3K-PKB-FOXO signalling module plays a pivotal role in a wide variety of cellular processes and represents one of the most diverse yet significant signalling system in the cell. The identification and characterisation of novel components of this pathway will likely provide novel targets for the development of anti-cancer therapeutics. In this thesis we have identified novel phosphorylation and transcriptional targets of this signal transduction cascade.

Phospho-proteomic analysis after activation of PKB has identified eIF4B as a novel PKB substrate thereby linking PKB signalling to translation regulation (Chapter 4). eIF4B is a component of the pre-initiation complex regulating protein translation and interacts with both mRNA and the 18S portion of the small ribosomal subunit, thereby acting as an anchor (reviewed in Methot *et al.*, 1994). In addition, eIF4B functions as a critical co-factor for eIF4A, through increasing the helicase activity of eIF4A, which is required for unwinding of mRNA and initiation of translation (Methot *et al.*, 1996). We demonstrate that phosphorylation of eIF4B by PKB on Ser 422 activates eIF4B, resulting in increased translation initiation. PKB signalling has been shown to be hyperactivated in a wide variety of neoplasias (reviewed in Engelman, 2009). In addition, the activation of PKB has been reported to alter profiles of mRNA associated to polysomes, with mRNAs mainly encoded for of pathways critical to cell growth, such as growth factors, transcription factors, tyrosine kinases and receptors (Rajasekhar *et al.*, 2003; Ruggero and Pandolfi, 2003). This suggests that activation of eIF4B by deregulated PKB signalling may be critical in the induction of cellular transformation.

Furthermore, we focussed on identification of novel transcriptional targets of the PI3K-PKB-FOXO module through global microarray analyses after activation of the key components in this pathway. We validated Id1, GS and JAK2 as FOXO transcriptional targets, which might be involved in oncogenic transformation.

1.1 Identification of Id1 as an important therapeutic target in leukemia

Cancer cells are characterized by increased proliferation, decreased cell death and a block in differentiation. In Chapter 6 we demonstrate that the leukemic fusion protein Bcr-Abl promotes proliferation through inactivation of FOXO3. Furthermore Id1 was identified as a FOXO3 target and constitutive activation of FOXO3 resulted in repression of Id1 mRNA and protein expression. *Id* genes have been shown to function as oncogenes in various contexts (reviewed in Norton, 2000). Their expression has been found to be elevated in many tumour types, including pancreatic cancer, breast carcinoma, adenocarcinoma and myeloma. Loss of *Id* expression in tumour cell lines leads to suppression of cell growth suggesting that Id proteins play a causal role in tumorigenesis. Recently, comparative gene analysis identified Id1 as a common transcriptional target of oncogenic tyrosine kinases involved in haematopoietic malignancies (Tam *et al.*, 2008). Knockdown of Id1 expression inhibited growth of leukemic cell lines, indicating that Id1 is an important target of constitutively activated tyrosine kinases and could serve as therapeutic target. Furthermore, constitutive active JAK2 signalling, due to a mutation found in patients with the myeloproliferative disorder polycythema vera, also increased Id1 expression. In addition, high Id1 expression was found to be associated with poor prognosis in patients with acute myeloid leukaemia (Tang *et al.*, 2009). Although these studies highlight the importance of Id1 as a oncogene, they do not reveal the mechanism by which Id1 overexpression results in oncogenic transformation. We have shown that treatment of the CML cell line K562 cells with the Bcr-Abl inhibitor STI571 resulted in activation of FOXO3 and down-regulation of Id1. The decrease in expression was associated with the induction

of erythroid differentiation, suggesting that Id1 promotes proliferation through blockade of differentiation. Expression of a constitutively active FOXO3 mutant was sufficient to reverse the leukemic phenotype and to induce differentiation, while ectopic expression of Id1 resulted in inhibition of STI571-induced differentiation. These results indicate that Bcr-Abl-mediated inactivation of FOXO3 and consequent increased Id1 expression is a critical factor in maintaining the undifferentiated state of CML cells. Promoting differentiation of leukemic cells by downregulation of Id1 expression either through RNAi-mediated knockdown, PKB inhibition or FOXO3 activation could serve as a novel therapeutic intervention in the treatment of haematopoietic malignancy.

1.2 A role for GS-mediated autophagy in cancer and ageing

Cancer and ageing are regulated by increased oxidative stress and the accumulation of damaged proteins and organelles is a hallmark of both. This leads to a build-up of cellular damage which results in genomic instability. In mammals, chromosomal instability and the incidence of cancer increases with age (reviewed in Finkel *et al.*, 2007a). FOXOs can counteract both tumorigenesis and ageing by decreasing oxidative stress and inducing senescence, apoptosis and DNA repair (Fig. 1). Autophagy has been reported to inhibit ageing and tumorigenesis by degrading proteins and organelles resulting in lower oxidative stress. The induction of autophagy may thus be an important downstream effect of FOXO activation that mediates the tumour suppressive and lifespan lengthening properties of the FOXO transcription factors.

1.2.1 Inhibition of tumorigenesis by autophagy

The protective role of autophagy in the development of cancer is highlighted by the fact that many oncogenes inhibit autophagy, while tumour suppressors can increase autophagy through modulation of the mTORC1 complex. The PI3K pathway is hyperactivated in many tumours caused by activating mutations in Ras or PKB or by loss of function mutations in PTEN (Coleman *et al.*, 2004; Sansal and Sellers, 2004). This can result in increased mTORC1 activity through PKB-mediated inhibition of the TSC1/TSC2 complex (Inoki *et al.*, 2002). It has been shown that PTEN expression counteracts cytokine-mediated

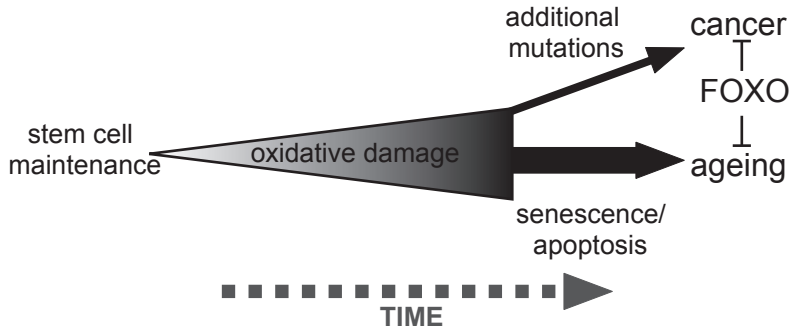


Figure 1. The role of oxidative stress in cancer and ageing

During the lifespan of an organism, stem cells accumulate DNA damage caused by oxidative stress. Damaged cells undergo growth arrest, apoptosis or senescence thereby decreasing the pool of functional stem cells. This decrease impairs tissue homeostasis and contributes to aging and age-related diseases. Occasionally, some cells can escape from this pathway by acquisition of additional mutations allowing them to survive. These proliferating but damaged cells might initiate tumour formation. FOXOs can counteract both aging and tumorigenesis by decreasing oxidative stress, increasing DNA repair and inducing senescence or apoptosis.

downregulation autophagy, while constitutively active PKB inhibited autophagy, indicating that hyperactivation of the PI3K pathway can down-modulate autophagy (Arico *et al.*, 2001). In addition, mutations of the tumour suppressor p53 result in mTOR activation through inhibition of AMP-dependent kinase and decreased autophagy (Morselli *et al.*, 2008). Furthermore, oncogenic alterations in the mTOR pathway also result in increased mTOR signalling. For instance, the expression of RheB, the mTOR activating GTPase, is elevated in many tumours, while the TSC genes, which inhibit RheB, are mutated in tuberous sclerosis (Basso *et al.*, 2005; Kwiatkowski, 2003).

Our results indicate that FOXO3 triggers autophagosome formation by increasing GS expression, which results in inhibition of mTORC1 activity (Chapter 7). We have demonstrated that GS activity decreased phosphorylation of S6K and triggered the formation of autophagosomes, suggesting that FOXO3 induces autophagy by glutamine-mediated inhibition of mTOR. Our findings reveal a novel mechanism by which amino acids modulate mTOR activity. Although many studies have shown that amino acids can activate the mTORC1 complex, the mechanisms through which amino acids signal to mTOR remain unclear. It has been demonstrated that amino acids can activate mTORC1 through the class III PI3K mammalian Vps (vacuolar protein sorting) 34 homologue (hVps34) (Nobukuni *et al.*, 2005b). In addition, RheB activity was found to be required but not sufficient for AA induced mTORC1 activation (Nobukuni *et al.*, 2005a). This suggest that a parallel pathway mediates the amino acid-mediated activation of the mTORC1 complex via hVps34, which is distinct from that controlled by insulin through TSC1/2 and RheB. In addition, it has been demonstrated that amino acids enhance the binding of RheB to mTORC1 (Long *et al.*, 2005). Recently, a Ras-related small GTP-binding protein (RagC) was identified as a binding partner of raptor. In starved cells, mTOR was present throughout the cytoplasm, whereas in cells stimulated with amino acids, mTOR localised to the perinuclear region of the cell (Sancak and Sabatini, 2009). It was proposed that amino acids might control the activity of the mTORC1 pathway by regulating, through the Rag proteins, the movement of mTOR to the same intracellular compartment that contains its upstream activator Rheb. These results provide clues for understanding the regulation of mTORC1 activity by intracellular glutamine levels. We have demonstrated that GS activity is required for the FOXO3-mediated repression of amino acid-induced mTOR activation. We hypothesise that glutamine exerts its inhibitory effect on mTORC1 activity through blocking the mTOR translocation by inhibiting the Rag proteins or through inhibition of the basal RheB activity (Fig. 2).

The identification of Beclin 1 as a tumour suppressor further supports a role of autophagy in tumour progression. Beclin 1 is required for the initiation of autophagosome formation, but the exact mechanism remains unclear. Allelic deletions beclin1 were detected in human breast carcinoma cell line (Aita *et al.*, 1999), suggesting that loss of *beclin 1* expression through decreasing autophagy might contribute to the cancer phenotype. Indeed, restoration of *beclin 1* expression in human breast carcinoma cell inhibited cellular proliferation and tumorigenesis in mice (Liang *et al.*, 1999). Deletion of the *becllin 1* gene in mice has confirmed that Beclin 1 acts as a tumour suppressor. While *beclin1*$^{-/-}$ animals died early in embryogenesis, haploinsufficient mice show an increased frequency of spontaneous malignancies (Qu *et al.*, 2003; Yue *et al.*, 2003b). Examination of the tumours revealed thay they still expressed Beclin 1 protein expression, indicating that reduced autophagy can cause tumour progression (Yue *et al.*, 2003a). In addition, immortalised kidney epithelial cells from *beclin*$^{-/+}$ mice show an increase in DNA double strand breaks, gene amplification and chromosomal number disorder after growth factor deprivation (Mathew *et al.*, 2007). These reports demonstrate that compromised autophagy may promote tumorigenesis through increased genomic instability.

In this thesis we demonstrate that the PI3K-PKB-FOXO pathway modulates autophagy through regulation of glutamine metabolism. FOXO3 or FOXO4 activation resulted in increased GS expression and glutamine levels which resulted in induction of autophagy. Blocking the autophagosome formation resulted in lower viability of the cells after FOXO activation, indicating that the induction of autophagy may be required for cellular survival under stress conditions. The PI3K pathway is frequently hyperactivated in many cancers and FOXOs have been found to function as tumour suppressors. The FOXO-mediated induction of autophagy could contribute to the tumour suppressive function of these transcription factors by lowering oxidative stress and genomic instability thereby protecting the cells from the build-up of cellular damage.

1.2.2 Autophagy and ageing

As already mentioned, damaged proteins and organelles accumulate as an animal ages and it is believed that autophagy acts as a central regulatory mechanism of animal ageing by decreasing cellular damage (reviewed in Finkel *et al.*, 2007b). The model organism *C elegans* has provided the first evidence linking autophagy to the ageing process. In several *C elegans* models for dietary restriction longer lifespan is associated with increased autophagy (Hansen *et al.*, 2008a; Morck and Pilon, 2006). Knockdown of several autophagy related genes in the worm reverted the lifespan extension after dietary restriction (Hansen *et al.*, 2008b). In addition, knockdown of Beclin-1 blocked normal dauer formation and reduced lifespan of *daf-2* loss of function mutants, indicating the importance of autophagy in regulating longevity (Melendez *et al.*, 2003).

A link between autophagy and ageing was also observed in the fruit fly *Drosphila melanogaster*. It has been demonstrated that in response to starvation activation of dFOXO induces autophagy in the fat body (Juhasz *et al.*, 2007). Furthermore, the expression of several autophagy-related genes in neural tissue reduces with age (Simonsen *et al.*, 2008a). This reduction correlated with an increase in insoluble ubiquitinated proteins, suggesting that during ageing decreased autophagy results in an accumulation of damaged proteins. In contrast, enhanced expression of one of the affected autophagy-related genes atg8a in older fly brains extended average lifespan (Simonsen *et al.*, 2008b).

The critical role of FOXOs in regulation of lifespan has been highlighted by studies in *C elegans* and *D melanogaster*. In the nematode worm, nutrient deprivation causes inactivation of an orthologue of the insulin receptor (DAF-2) resulting in activation of the FOXO transcription factor DAF-16 (See Chapter 1, Fig. 4). This induces dauer formation, a stress-resistant stage in which the worms can survive up to 2-3 months (Kenyon *et al.*, 1993). Loss of function mutations in the *daf-2* gene causes an increase in lifespan, which is abrogated in *daf-16* deficient worms, indicating that DAF-16 is required for lifespan extension (Lin *et al.*, 1997; Ogg *et al.*, 1997). Overexpression of dFOXO or inhibition of insulin signalling by mutating the insulin receptor also extends lifespan in the fruit fly *D melanogaster*, indicating that the regulation of lifespan by the insulin pathway is conserved in invertebrates (Giannakou *et al.*, 2004; Hwangbo *et al.*, 2004). Interestingly, recent studies among centenarians in humans revealed that genetic variation within the *FOXO3* gene was also associated with the ability to attain exceptionally long age, suggesting that the FOXO transcription factors also play an import role in human aging (Flachsbart *et al.*, 2009; Willcox *et al.*, 2008). In *C elegans* many transcriptional targets of DAF-16 have been identified that are required for the DAF-16-mediated long-lived phenotype. Many of these targets are involved stress resistance and metabolism, including superoxide dismutatase, catalase, apolipoprotein genes and small heat shock protein genes (reviewed in Mukhopadhyay *et al.*, 2006). Interestingly, our experiments indicate that the regulation of GS activity by the PI3K-PKB-FOXO signalling module is conserved in

C elegans. In Chapter 6 we demonstrate that GS activity is dramatically increased in *daf-2* mutant worms compared to wild-type worms. Importantly, knockdown of DAF-16 expression demonstrated that this increase is dependent on activation of DAF-16. The DAF-16 induced GS activity might be important for FOXO-mediated longevity in the worm by increasing autophagy. The identification of GS as a transcriptional target thus reveals a novel mechanism for the regulation of longevity by DAF-16. Whether DAF-16-mediated increase in glutamine also triggers autophagosomes formation in *C elegans* and whether this is required for the lifespan promoting properties of DAF-16 awaits further research. Performing lifespan assays in the presence or absence of the GS inhibitor MSO in wild-type and *daf-2* mutant worms might reveal whether DAF-16 induced GS activity is indeed required for lifespan extension. In addition, worms expressing the LC3 orthologue LGG1 fused to GFP can provide insights in the regulation of autophagy by DAF-16.

In many organisms a calorie-restricted diet extends lifespan. In addition, calorie restriction reduces spontaneous tumour formation in several mouse models, demonstrating the close link between ageing and cancer (reviewed in Saunders and Verdin, 2007). Studies in rodent livers have revealed that caloric restriction also prevents the decline in autophagic flux observed with age, suggesting a link between ageing and autophagy in mammals (Cavallini *et al.*, 2001; Donati *et al.*, 2001). The sirtuin deacetylases have been found to be required for lifespan extension after dietary restriction in various organisms, including yeast, *C elegans, D melanogaster* (reviewed in Longo and Kennedy, 2006b). Sirtuins have a conserved catalytic domain that functions as a lysine deacetylase and through deacetylation of many targets sirtuins have been reported to protect against DNA damage and oxidative stress (reviewed in Longo and Kennedy, 2006a). SIRT1, for example, deacetylates histones and other nuclear proteins, including p53, Ku70 and FOXOs. In mammalian cells SIRT1 has been reported to promote cellular survival by deacetylating and thereby activating FOXOs (Brunet *et al.*, 2004). Interestingly, in *C elegans* increased expression of the sirtuin sir-2.1, the orthologue of human SIRT1, increases lifespan of the worms up to 50%, which is dependent on DAF-16 (Tissenbaum and Guarente, 2001). Furthermore, SIRT expression increases during calorie restriction in rodents and its expression is required for increased lifespan (Cohen *et al.*, 2004; Boily *et al.*, 2008), demonstrating the importance of SIRT1 in mammalian longevity. Taken together, these results points towards an important role for SIRT in regulation of lifespan in response to caloric restriction. Caloric restriction induces both autophagy and SIRT expression and additionally SIRT can activate FOXO transcriptional activity. Therefore, it is tempting to speculate that FOXO-induced GS expression and subsequent induction of autophagy are required for SIRT-mediated lifespan extension. It would be interesting to examine the involvement of FOXO-mediated GS expression in the lifespan promoting properties of SIRT1 in the model organism *C elegans*.

Taken together, the identification of GS as a novel FOXO transcriptional target has provided new insights in the regulation of autophagy. We hypothesise that this regulation plays a pivotal role in the tumour suppressive and life lengthening properties of FOXOs, which is illustrated in the model depicted in Figure 2. Growth factor deprivation, caloric restriction and oxidative stress all trigger the activation of FOXO resulting in upregulation of GS expression. The subsequent rise in glutamine levels inhibits mTOR and induces the formation of autophagosomes by a sofar uncharacterised mechanism. Induction of autophagy results in removal of damaged proteins and organelles might decrease the level of reactive oxygen species (ROS), which in turn might reduce genomic instability or prevent cellular senescence. Together, these mechanisms reduce the incidence of cancer and prolong lifespan.

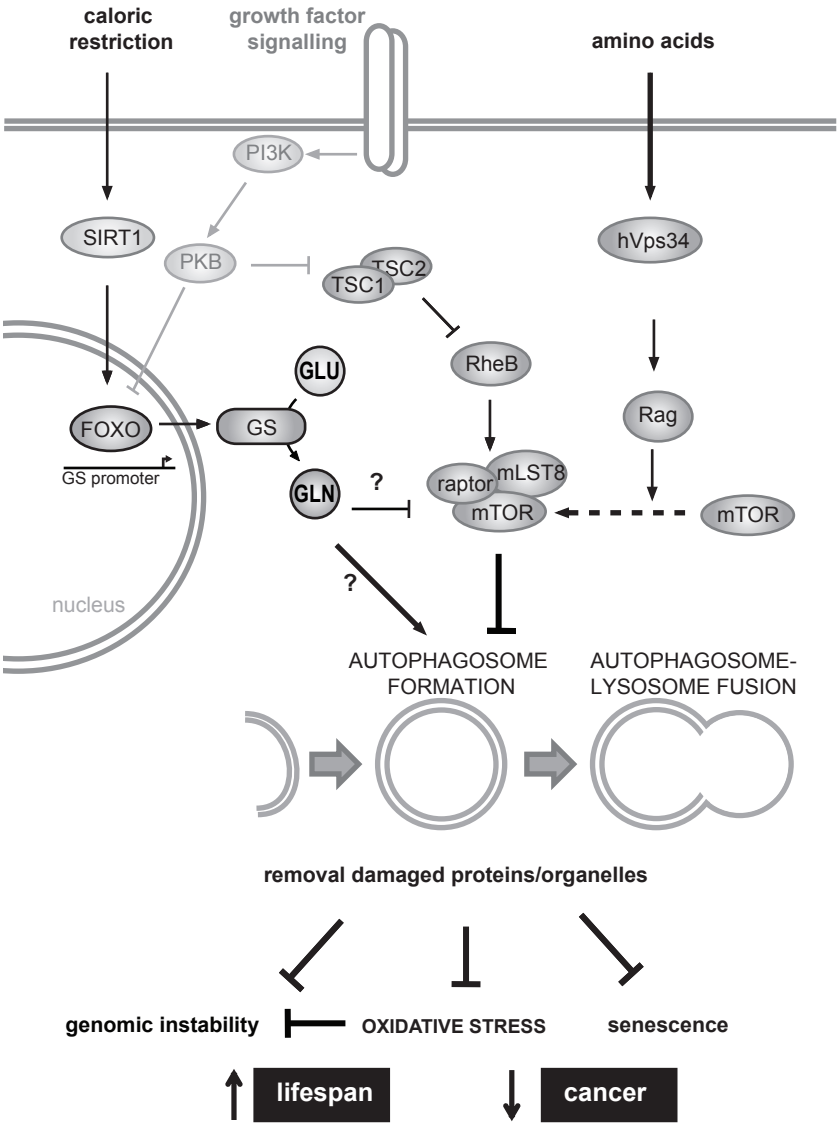


Figure 2. Integrative model showing the links between FOXO-induced GS expression, autophagy, aging and cancer

Growth factor deprivation, caloric restriction and oxidative stress trigger the activation of FOXO resulting in upregulation of GS expression. The subsequent rise in glutamine levels inhibits mTOR and induces the formation of autophagosomes. The removal of damaged proteins and organelles might decrease the level of reactive oxygen species (ROS), which in turn might decrease genomic instability or prevent cellular senescence. These mechanisms might reduce the incidence of cancer and prolong lifespan. (see text for full details)

1.3 Regulation of microRNAs by FOXOs

In 1993 a genetic screen in *C elegans* identified a small regulatory RNA called *lin-4*, which was required for appropriate timing of post-embryonic development (Lee *et al.*, 1993; Wightman *et al.*, 1993). Since this initial discovery, hundreds of microRNAs (miRNAs) have been identified in plants, worms, flies and animals. In humans over 400 miRNAs have been described and predictions indicate that the human genome encode up to 1000 miRNAs. miRNAs are small, noncoding RNAs ~22 nucleotides in length that base-pair with the mRNAs of protein coding genes and negatively regulate their stability and translational efficiency. Unlike short interfering RNAs (siRNAs), miRNAs silence multiple genes because they pair to their target mRNA with imperfect complementarity (Bartel, 2009).

Analysis of microRNA expression profiles in cancer has revealed distinct microRNA profiles in a wide variety of tumours compared to normal tissue. These changes in expression can be used as a diagnostic tool (Dalmay and Edwards, 2006). Importantly, accumulating evidence indicates that the dysregulation of microRNAs plays an important role in cancer pathogenesis and it has been reported that miRNAs itself can function as tumour suppressors or oncogenes (Kent and Mendell, 2006). In addition, regulation of miRNA expression by tumour suppressors like p53 has been found attribute to their tumour suppressive function (Sachdeva *et al.*, 2009).

We wished to determine whether the tumour suppressive function of FOXOs was mediated in part by the transcriptional regulation of miRNAs. To identify FOXO-regulated miRNAs, Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT for 8 hours, miRNAs were isolated and microRNAarray analyses performed. After activation of FOXO3 and FOXO4 we identified five microRNAs whose expression was significantly increased upon 4-OHT stimulation. In Figure 3 the microRNAs are depicted, which were upregulated after FOXO activation compared to untreated cells. Similar to our microarray analysis described in Chapter 5, FOXO3 demonstrated significantly more

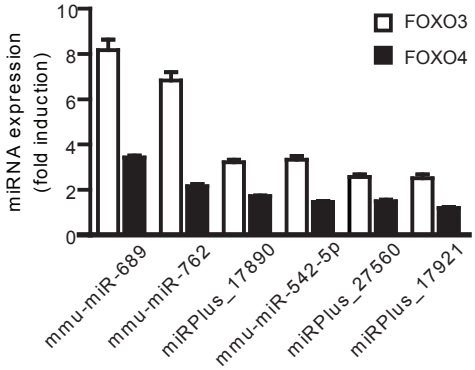


Figure 3. Activation of FOXO3 and FOXO4 results in upregulation of specific microRNAs
Ba/F3 cells expressing FOXO3(A3)-ER and FOXO4(A3)-ER were stimulated with 4-OHT (100 nM) in the presence of mIL-3 (5 ng/ml) for 8 hours and miRNAs were isolated The miRNA expression profile was analysed using a mRCURY LNA array (Exiqon) (Castoldi *et al.*, 2006). Shown are microRNAs, which were significantly changed in response to FOXO3 and FOXO4 activation. Shown are the results of one experiment performed in triplicate. The miRPlus sequences are human sequences of newly discovered microRNAs not yet annotated in miRbase: miRPlus_17890: UGGGGCGGAGCUUCCGGAGGCC; miRPlus_27560:AUGUUGGGAGCGGGCAGGUUGG; miR-Plus_17921: UUGGGGAAACGGCCGCUGAGUGA, miRPlus_17921 corresponds to hsa-mir-2110

effects than FOXO4. Mmu-miR-762 has been reported to be aberrantly expressed in a hamster model for oral squamous cell carcinoma, suggesting a role for this microRNA in oncogenesis (Yu *et al.*, 2009). However, for the other identified microRNAs no functions have been identified yet.
In conclusion, microRNA analysis for the identification of FOXO-regulated microRNAs provides promising putative targets. The identification of the targeted mRNAs and functional research of these microRNAs awaits further research and might reveal novel mechanisms for the protective role of FOXOs in oncogenesis.

2. REGULATION OF GS EXPRESSION IN THE LIVER BY FOXO3

Glutamine synthetase (GS) is a metabolic enzyme, which synthesises glutamine from glutamate and ammonia. GS is ubiquitously expressed with highest expression in brain, liver kidney. In the liver, GS expression plays an important role in nitrogen metabolism and control of body pH by ammonia detoxification (reviewed in Newsholme *et al.*, 2003). In mice, which were chronically starved, the liver shows an increased number of GS positive hepatocytes, however which factors are responsible for this effect remains unclear (Ueberham *et al.*, 2004). To determine whether FOXOs in response to growth factors regulate GS expression in the liver we stained murine liver sections immunohistochemicaly

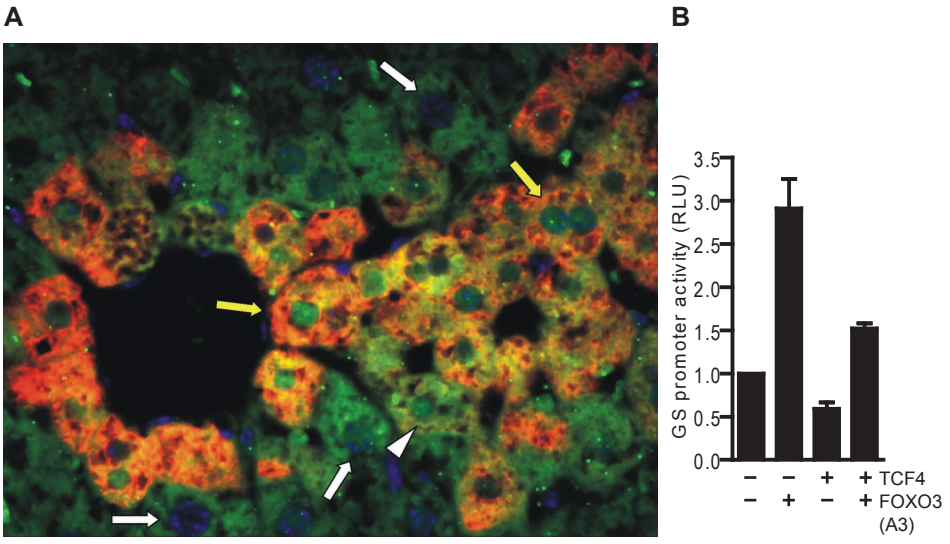


Figure 4. Expression of GS and Foxo3 in liver
(A) Immunohistochemical staining of a mouse liver section (peri central zone). The liver section was stained for FOXO3 (green), GS (red) and nuclei (blue). All GS-positive hepatocytes show nuclear staining of FOXO3 (yellow arrows), while GS negative hepotocytes show a cytoplasmic staining of FOXO3 (white arrows). Very rare exceptions of GS negative cells show nuclear FOXO3 staining (white arrowhead). **(B)** A GS reporter plasmid expressing -2520/+153 of the glutamine synthetase promoter in front of a luciferase gene was transfected in HEK 293 cells together with Renilla, FOXO3(A3) and TCF4 as indicated. Luciferase activity was measured 40 hours after transfection. Data are depicted as relative luciferase units (RLU) compared to control. Shown are mean ± SEM values of three independent experiments performed with triplicate samples.

for FOXO3 and GS expression. This revealed that most GS positive hepatocytes showed a nuclear Foxo3 staining, while in GS negative hepatocytes Foxo3 staining was mainly cytoplasmic, suggesting that FOXO3 also regulates GS expression in liver (Fig. 4A).
Furthermore, the staining revealed that FOXO3 is localised in the nucleus in pericentral hepatocytes, while in the periportal zone its expression is mainly cytoplasmic. The periportal zone is nearest to the blood supply and receives the most oxygenated blood, in contrast, the pericentral zone has the poorest oxygenation. Immunohistochemical studies have revealed that different metabolic pathways are heterogeneously distributed in the liver lobules. Many enzymes show decreasing or increasing gradients of expression over the distance from portal vein to the central vein. The periportal region is specialized for oxidative liver functions such as gluconeogenesis, ß-oxidation of fatty acids and cholesterol synthesis, while pericentral hepatocytes are more important for glycolysis and lipogenesis (reviewed in Gebhardt *et al.*, 2007). GS is expressed in only a subpopulation of pericentral hepatocytes surrounding the central veins (Gebhardt and Mecke, 1983). Wnt signalling has been reported to be required for establishing and maintaining liver metabolic zonation (reviewed in Gebhardt and Hovhannisyan, 2009). It has been hypothesised that a Wnt/β-catenin gradient with the highest activity around the central vein and low activity around the periportal region is responsible for the functional differentiation of the hepatocytes. β-Catenin has been described to physically interact with FOXO and co-expression of these proteins resulted in enhanced transcription from several FOXO promoter reporters (Essers *et al.*, 2005). In addition, it has been described that GS expression in the liver is upregulated after activation of β-catenin (Loeppen *et al.*, 2005), suggesting that FOXO3 might control GS transcription in conjunction with β-catenin. These results suggest that activation of β-catenin in the pericentral region might enhance FOXO3 transcriptional activity and be responsible for the heterogenous distribution of GS expression. To test this hypothesis we transfected cells with a GS promoter reporter construct together with constitutively active FOXO3 and TCF4. TCF is a binding partner of β-catenin and can thereby inhibit FOXO3 activity by competing for β-catenin. As shown in Figure 4B, cotransfection of TCF4 indeed inhibited FOXO3-induced GS promoter activity, suggesting that β-catenin is required for FOXO3-mediated GS expression.
Taken together, these results suggest that FOXO3 together with β-catenin regulates GS expression in the liver. The heterogenous activation of the Wnt cascade and subsequent interaction of FOXO3 and β-catenin might ensure the zonal expression pattern of GS and contribute to liver function.

CONCLUDING REMARKS

The PI3K-PKB-FOXO signalling module plays a pivotal role in regulation of essential biological processes including proliferation, survival, differentiation and metabolism. Inappropriate activation of this signalling module is frequently observed in human cancer and causes uncontrolled proliferation and survival. In this thesis we have identified novel phosphorylation and transcriptional targets of the PI3K-PKB-FOXO signal transduction pathway. We identified eIF4B as a novel PKB substrate and demonstrate that phosphorylation of eIF4B by PKB activates eIF4B, resulting in increased translation initiation. Furthermore, we identified novel transcriptional targets of the PI3K-PKB-FOXO module and validated Id1, GS and JAK2 as FOXO transcriptional targets, which might be involved in oncogenic transformation. We demonstrated that Bcr-Abl-mediated inactivation of FOXO3 and consequent increased Id1 expression is a critical factor in maintaining the undifferentiated state of CML cells. In addition, the FOXO-mediated GS upregulation and subsequent induction of autophagy might contribute to the tumour suppressive function of FOXO transcription factors through protection of cells from the build-up of cellular damage. The characterisation of these targets reveals previously undiscovered signalling pathways and biological functions regulated by PI3K-PKB-FOXO signalling and provides novel targets for the development of anti-cancer therapeutics. Activation of FOXOs through inhibition of PI3K or PKB activation could serve as a therapeutic intervention in the treatment of malignancies. An increasing number of kinase inhibitors have been developed for cancer treatment, including PI3K inhibitors that target specific PI3K isoforms, PKB inhibitors and inhibitors that target oncogenic kinases. The specific Bcr-Abl inhibitor STI571 was the first drug approved and is now a common treatment strategy for the treatment of CML patients. However, since cancer cells are highly genomic instable, resistance is likely to emerge, which has been observed in CML patients treated with STI571. New treatment strategies that complement the current therapies and target multiple downstream components might overcome resistance and combat the ongoing adaptation of cancers.

REFERENCES

Aita,V.M., Liang,X.H., Murty,V.V., Pincus,D.L., Yu,W., Cayanis,E., Kalachikov,S., Gilliam,T.C., and Levine,B. (1999). Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics *59*, 59-65.

Arico,S., Petiot,A., Bauvy,C., Dubbelhuis,P.F., Meijer,A.J., Codogno,P., and Ogier-Denis,E. (2001). The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. J. Biol. Chem. *276*, 35243-35246.

Bartel,D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215-233.

Basso,A.D., Mirza,A., Liu,G., Long,B.J., Bishop,W.R., and Kirschmeier,P. (2005). The farnesyl transferase inhibitor (FTI) SCH66336 (lonafarnib) inhibits Rheb farnesylation and mTOR signaling. Role in FTI enhancement of taxane and tamoxifen anti-tumor activity. J. Biol. Chem. *280*, 31101-31108.

Boily,G., Seifert,E.L., Bevilacqua,L., He,X.H., Sabourin,G., Estey,C., Moffat,C., Crawford,S., Saliba,S., Jardine,K., Xuan,J., Evans,M., Harper,M.E., and McBurney,M.W. (2008). SirT1 regulates energy metabolism and response to caloric restriction in mice. PLoS. One. *3*, e1759.

Brunet,A., Sweeney,L.B., Sturgill,J.F., Chua,K.F., Greer,P.L., Lin,Y., Tran,H., Ross,S.E., Mostoslavsky,R., Cohen,H.Y., Hu,L.S., Cheng,H.L., Jedrychowski,M.P., Gygi,S.P., Sinclair,D.A., Alt,F.W., and Greenberg,M.E. (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.

Castoldi,M., Schmidt,S., Benes,V., Noerholm,M., Kulozik,A.E., Hentze,M.W., and Muckenthaler,M.U. (2006). A sensitive array for microRNA expression profiling (miChip) based on locked nucleic acids (LNA). RNA. *12*, 913-920.

Cavallini,G., Donati,A., Gori,Z., Pollera,M., and Bergamini,E. (2001). The protection of rat liver autophagic proteolysis from the age-related decline co-varies with the duration of anti-ageing food restriction. Exp. Gerontol. *36*, 497-506.

Cohen,H.Y., Miller,C., Bitterman,K.J., Wall,N.R., Hekking,B., Kessler,B., Howitz,K.T., Gorospe,M., de,C.R., and Sinclair,D.A. (2004). Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science *305*, 390-392.

Coleman,M.L., Marshall,C.J., and Olson,M.F. (2004). RAS and RHO GTPases in G1-phase cell-cycle regulation. Nat. Rev. Mol. Cell Biol. *5*, 355-366.

Dalmay,T. and Edwards,D.R. (2006). MicroRNAs and the hallmarks of cancer. Oncogene *25*, 6170-6175.

Donati,A., Cavallini,G., Paradiso,C., Vittorini,S., Pollera,M., Gori,Z., and Bergamini,E. (2001). Age-related changes in the autophagic proteolysis of rat isolated liver cells: effects of antiaging dietary restrictions. J. Gerontol. A Biol. Sci. Med. Sci. *56*, B375-B383.

Engelman,J.A. (2009). Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat. Rev. Cancer *9*, 550-562.

Essers,M.A., de Vries-Smits,L.M., Barker,N., Polderman,P.E., Burgering,B.M., and Korswagen,H.C. (2005). Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science *308*, 1181-1184.

Finkel,T., Serrano,M., and Blasco,M.A. (2007b). The common biology of cancer and ageing. Nature *448*, 767-774.

Finkel,T., Serrano,M., and Blasco,M.A. (2007a). The common biology of cancer and ageing. Nature *448*, 767-774.

Flachsbart,F., Caliebe,A., Kleindorp,R., Blanche,H., von Eller-Eberstein,H., Nikolaus,S., Schreiber,S.,

and Nebel,A. (2009). Association of FOXO3A variation with human longevity confirmed in German centenarians. Proc. Natl. Acad. Sci. U. S. A *106*, 2700-2705.

Gebhardt,R., Baldysiak-Figiel,A., Krugel,V., Ueberham,E., and Gaunitz,F. (2007). Hepatocellular expression of glutamine synthetase: an indicator of morphogen actions as master regulators of zonation in adult liver. Prog. Histochem. Cytochem. *41*, 201-266.

Gebhardt,R. and Hovhannisyan,A. (2009). Organ patterning in the adult stage: The role of Wnt/beta-catenin signaling in liver zonation and beyond. Dev. Dyn.

Gebhardt,R. and Mecke,D. (1983). Heterogeneous distribution of glutamine synthetase among rat liver parenchymal cells in situ and in primary culture. EMBO J. *2*, 567-570.

Giannakou,M.E., Goss,M., Junger,M.A., Hafen,E., Leevers,S.J., and Partridge,L. (2004). Long-lived Drosophila with overexpressed dFOXO in adult fat body. Science *305*, 361.

Hansen,M., Chandra,A., Mitic,L.L., Onken,B., Driscoll,M., and Kenyon,C. (2008a). A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. PLoS. Genet. *4*, e24.

Hansen,M., Chandra,A., Mitic,L.L., Onken,B., Driscoll,M., and Kenyon,C. (2008b). A role for autophagy in the extension of lifespan by dietary restriction in C. elegans. PLoS. Genet. *4*, e24.

Hwangbo,D.S., Gershman,B., Tu,M.P., Palmer,M., and Tatar,M. (2004). Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature *429*, 562-566.

Inoki,K., Li,Y., Zhu,T., Wu,J., and Guan,K.L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat. Cell Biol. *4*, 648-657.

Juhasz,G., Puskas,L.G., Komonyi,O., Erdi,B., Maroy,P., Neufeld,T.P., and Sass,M. (2007). Gene expression profiling identifies FKBP39 as an inhibitor of autophagy in larval Drosophila fat body. Cell Death. Differ. *14*, 1181-1190.

Kent,O.A. and Mendell,J.T. (2006). A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. Oncogene *25*, 6188-6196.

Kenyon,C., Chang,J., Gensch,E., Rudner,A., and Tabtiang,R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461-464.

Kwiatkowski,D.J. (2003). Tuberous sclerosis: from tubers to mTOR. Ann. Hum. Genet. *67*, 87-96.

Lee,R.C., Feinbaum,R.L., and Ambros,V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843-854.

Liang,X.H., Jackson,S., Seaman,M., Brown,K., Kempkes,B., Hibshoosh,H., and Levine,B. (1999). Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature *402*, 672-676.

Lin,K., Dorman,J.B., Rodan,A., and Kenyon,C. (1997). daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Science *278*, 1319-1322.

Loeppen,S., Koehle,C., Buchmann,A., and Schwarz,M. (2005). A beta-catenin-dependent pathway regulates expression of cytochrome P450 isoforms in mouse liver tumors. Carcinogenesis *26*, 239-248.

Long,X., Ortiz-Vega,S., Lin,Y., and Avruch,J. (2005). Rheb binding to mammalian target of rapamycin (mTOR) is regulated by amino acid sufficiency. J. Biol. Chem. *280*, 23433-23436.

Longo,V.D. and Kennedy,B.K. (2006a). Sirtuins in aging and age-related disease. Cell *126*, 257-268.

Longo,V.D. and Kennedy,B.K. (2006b). Sirtuins in aging and age-related disease. Cell *126*, 257-268.

Mathew,R., Kongara,S., Beaudoin,B., Karp,C.M., Bray,K., Degenhardt,K., Chen,G., Jin,S., and White,E. (2007). Autophagy suppresses tumor progression by limiting chromosomal instability. Genes

Dev. *21*, 1367-1381.

Melendez,A., Talloczy,Z., Seaman,M., Eskelinen,E.L., Hall,D.H., and Levine,B. (2003). Autophagy genes are essential for dauer development and life-span extension in C. elegans. Science *301*, 1387-1391.

Methot,N., Pause,A., Hershey,J.W., and Sonenberg,N. (1994). The translation initiation factor eIF-4B contains an RNA-binding region that is distinct and independent from its ribonucleoprotein consensus sequence. Mol. Cell Biol. *14*, 2307-2316.

Methot,N., Song,M.S., and Sonenberg,N. (1996). A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. Mol. Cell Biol. *16*, 5328-5334.

Morck,C. and Pilon,M. (2006). C. elegans feeding defective mutants have shorter body lengths and increased autophagy. BMC. Dev. Biol. *6*, 39.

Morselli,E., Tasdemir,E., Maiuri,M.C., Galluzzi,L., Kepp,O., Criollo,A., Vicencio,J.M., Soussi,T., and Kroemer,G. (2008). Mutant p53 protein localized in the cytoplasm inhibits autophagy. Cell Cycle *7*, 3056-3061.

Mukhopadhyay,A., Oh,S.W., and Tissenbaum,H.A. (2006). Worming pathways to and from DAF-16/FOXO. Exp. Gerontol. *41*, 928-934.

Newsholme,P., Procopio,J., Lima,M.M., Pithon-Curi,T.C., and Curi,R. (2003). Glutamine and glutamate--their central role in cell metabolism and function. Cell Biochem. Funct. *21*, 1-9.

Nobukuni,T., Joaquin,M., Roccio,M., Dann,S.G., Kim,S.Y., Gulati,P., Byfield,M.P., Backer,J.M., Natt,F., Bos,J.L., Zwartkruis,F.J., and Thomas,G. (2005a). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc. Natl. Acad. Sci. U. S. A *102*, 14238-14243.

Nobukuni,T., Joaquin,M., Roccio,M., Dann,S.G., Kim,S.Y., Gulati,P., Byfield,M.P., Backer,J.M., Natt,F., Bos,J.L., Zwartkruis,F.J., and Thomas,G. (2005b). Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc. Natl. Acad. Sci. U. S. A *102*, 14238-14243.

Norton,J.D. (2000). ID helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. J. Cell Sci. *113 (Pt 22)*, 3897-3905.

Ogg,S., Paradis,S., Gottlieb,S., Patterson,G.I., Lee,L., Tissenbaum,H.A., and Ruvkun,G. (1997). The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature *389*, 994-999.

Qu,X., Yu,J., Bhagat,G., Furuya,N., Hibshoosh,H., Troxel,A., Rosen,J., Eskelinen,E.L., Mizushima,N., Ohsumi,Y., Cattoretti,G., and Levine,B. (2003). Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J. Clin. Invest *112*, 1809-1820.

Rajasekhar,V.K., Viale,A., Socci,N.D., Wiedmann,M., Hu,X., and Holland,E.C. (2003). Oncogenic Ras and Akt signaling contribute to glioblastoma formation by differential recruitment of existing mRNAs to polysomes. Mol. Cell *12*, 889-901.

Ruggero,D. and Pandolfi,P.P. (2003). Does the ribosome translate cancer? Nat. Rev. Cancer *3*, 179-192.

Sachdeva,M., Zhu,S., Wu,F., Wu,H., Walia,V., Kumar,S., Elble,R., Watabe,K., and Mo,Y.Y. (2009). p53 represses c-Myc through induction of the tumor suppressor miR-145. Proc. Natl. Acad. Sci. U. S. A *106*, 3207-3212.

Sancak,Y. and Sabatini,D.M. (2009). Rag proteins regulate amino-acid-induced mTORC1 signalling. Biochem. Soc. Trans. *37*, 289-290.

Sansal,I. and Sellers,W.R. (2004). The biology and clinical relevance of the PTEN tumor suppressor

pathway. J. Clin. Oncol. *22*, 2954-2963.

Saunders,L.R. and Verdin,E. (2007). Sirtuins: critical regulators at the crossroads between cancer and aging. Oncogene *26*, 5489-5504.

Simonsen,A., Cumming,R.C., Brech,A., Isakson,P., Schubert,D.R., and Finley,K.D. (2008a). Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. Autophagy. *4*, 176-184.

Simonsen,A., Cumming,R.C., Brech,A., Isakson,P., Schubert,D.R., and Finley,K.D. (2008b). Promoting basal levels of autophagy in the nervous system enhances longevity and oxidant resistance in adult Drosophila. Autophagy. *4*, 176-184.

Tam,W.F., Gu,T.L., Chen,J., Lee,B.H., Bullinger,L., Frohling,S., Wang,A., Monti,S., Golub,T.R., and Gilliland,D.G. (2008). Id1 is a common downstream target of oncogenic tyrosine kinases in leukemic cells. Blood *112*, 1981-1992.

Tang,R., Hirsch,P., Fava,F., Lapusan,S., Marzac,C., Teyssandier,I., Pardo,J., Marie,J.P., and Legrand,O. (2009). High Id1 expression is associated with poor prognosis in 237 patients with acute myeloid leukemia. Blood *114*, 2993-3000.

Tissenbaum,H.A. and Guarente,L. (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature *410*, 227-230.

Ueberham,E., Arendt,E., Starke,M., Bittner,R., and Gebhardt,R. (2004). Reduction and expansion of the glutamine synthetase expressing zone in livers from tetracycline controlled TGF-beta1 transgenic mice and multiple starved mice. J. Hepatol. *41*, 75-81.

Wightman,B., Ha,I., and Ruvkun,G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell *75*, 855-862.

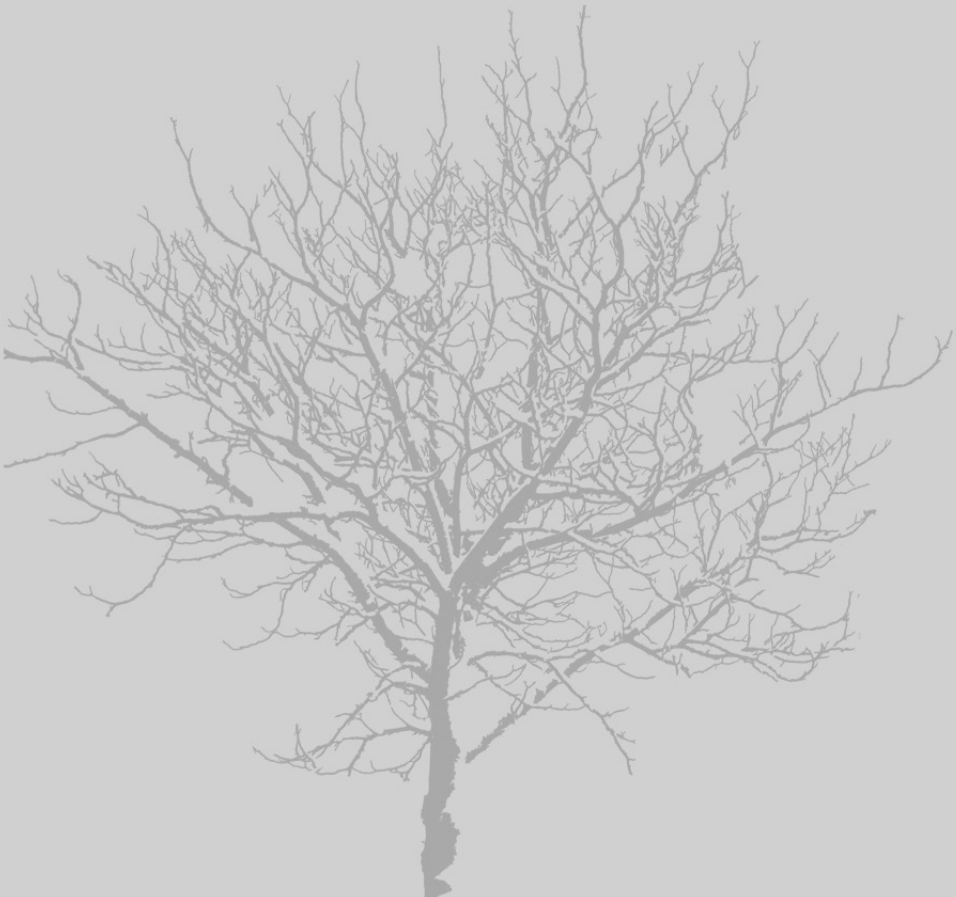
Willcox,B.J., Donlon,T.A., He,Q., Chen,R., Grove,J.S., Yano,K., Masaki,K.H., Willcox,D.C., Rodriguez,B., and Curb,J.D. (2008). FOXO3A genotype is strongly associated with human longevity. Proc. Natl. Acad. Sci. U. S. A *105*, 13987-13992.

Yu,T., Wang,X.Y., Gong,R.G., Li,A., Yang,S., Cao,Y.T., Wen,Y.M., Wang,C.M., and Yi,X.Z. (2009). The expression profile of microRNAs in a model of 7,12-dimethyl-benz[a]anthrance-induced oral carcinogenesis in Syrian hamster. J. Exp. Clin. Cancer Res. *28*, 64.

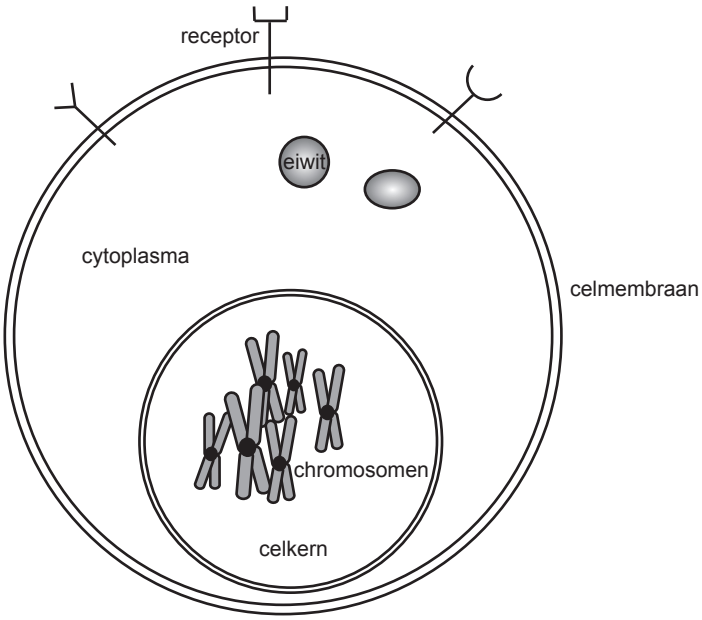
Yue,Z., Jin,S., Yang,C., Levine,A.J., and Heintz,N. (2003a). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc. Natl. Acad. Sci. U. S. A *100*, 15077-15082.

Yue,Z., Jin,S., Yang,C., Levine,A.J., and Heintz,N. (2003b). Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc. Natl. Acad. Sci. U. S. A *100*, 15077-15082.

Nederlandse samenvatting



Het menselijk lichaam bestaat uit miljarden cellen. Elke cel maakt deel uit van grotere systemen zoals weefsels en organen. Een cel bestaat uit een celkern, cytoplasma en een celmembraan, een dubbele laag vetmoleculen die de inhoud van een cel omgeeft (Fig. 1). De celkern bevat alle genetische informatie van het organisme en wordt omgeven door een dubbel membraan.
In het menselijk lichaam zijn er heel veel verschillende typen cellen, zoals spiercellen, bloedcellen, zenuwcellen, huidcellen etc. Deze cellen hebben allemaal een andere functie en om deze functies goed uit te kunnen oefenen bevatten deze cellen verschillende soorten eiwitten. Eiwitten kunnen allerlei chemische reacties uitvoeren en vormen met elkaar de fabriekjes van een cel. In een cel spelen zich heel veel processen af. In iedere cel spelen zich basale processen af zoals het omzetten van suiker in energie en het maken en afbreken van bouwstenen. Daarnaast zijn eiwitten belangrijk voor het uitvoeren van de specifieke functies in verschillende typen cellen. Een rode bloedcel bevat bijvoorbeeld het eiwit hemoglobine wat zuurstof kan binden en zo zuurstof vervoert door het hele lichaam. Een huidcel daarentegen heeft geen hemoglobine, maar maakt het eiwit collageen dat zorgt voor stevigheid en elasticiteit van de huid. Een levercel bevat eiwitten die stoffen uit het bloed kan afbreken en een witte bloedcel maakt eiwitten die bacteriën kunnen doden. Dus de functie van een cel wordt bepaald door de specifieke eiwitten die aanwezig zijn in een cel.



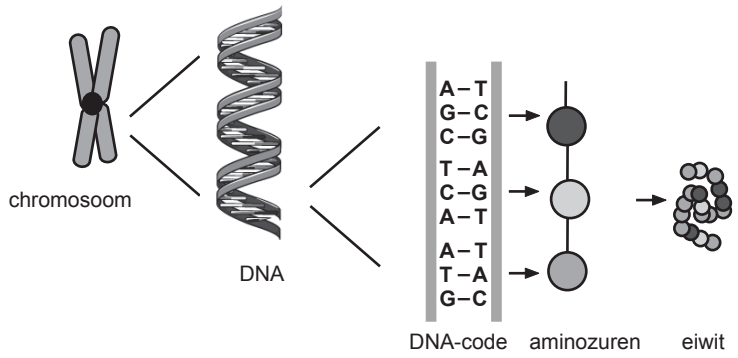
Figuur 1. De opbouw van een cel
Een cel is opgebouw uit een celkern, cytoplasma en een celmembraan. In de kern ligt de erfelijke informatie opgeslagen in de vorm van chromosomen. Speciale signaleringseiwtten in de celmembraan, receptoren, vangen signalen op en geven die door naar binnen

Waarom hebben alle cellen dezelfde genetische informatie, maar hebben ze toch verschillende eiwitten en daardoor verschillende functies?

In de kern van de cel ligt de erfelijke informatie opgeslagen. De mens heeft 46 chromosomen, die bestaat uit DNA. DNA bevat de codes voor het maken van alle eiwitten in het menselijk lichaam. DNA bestaat uit kleine bouwstenen van suikers en eiwitten die in een dubbele keten aan elkaar zitten. De twee ketens die samen de dubbele helix vormen zijn complementair, en binden aan elkaar. DNA bevat vier verschillende bouwstenen die worden aangegeven met een letter: A, T, C en G. De volgorde van deze letters is een code voor het maken van eiwitten. Omdat er heel veel verschillende sequenties mogelijk zijn, kan de volgorde van deze vier bouwstenen alle erfelijke informatie bevatten. Een stuk van het DNA met daarop de code voor één eiwit wordt een gen genoemd. Een paar jaar geleden hebben onderzoekers de sequentie ontrafeld van het menselijk DNA. Hieruit blijkt dat wij ongeveer 23.000 genen hebben. Eiwitten bestaan ook uit kleine bouwstenen, aminozuren, die in een lange streng aan elkaar zitten. We hebben 37 verschillende aminozuren en de welke aminozuren er gebruikt worden voor een eiwit en op welke plaats bepaalt de 3D vorm van een eiwit. De DNA-code van een gen kan worden vertaald in een aminozuurcode in een proces dat transcriptie heet (Fig. 2). Het vertalen van de erfelijke informatie op een gen leidt tot het maken van een eiwit, dat een specifieke functie kan uitoefenen in de cel. Elke cel in ons lichaam heeft alle mogelijke erfelijke informatie, maar de functie van de cel wordt bepaald door welke genen vertaald zijn in eiwitten. Een voorbeeld: elke cel heeft de genetische informatie om hemoglobine, het eiwit dat zuurstof kan vervoeren, te maken, maar alleen een rode bloedcel heeft die informatie omgezet in het eiwit.

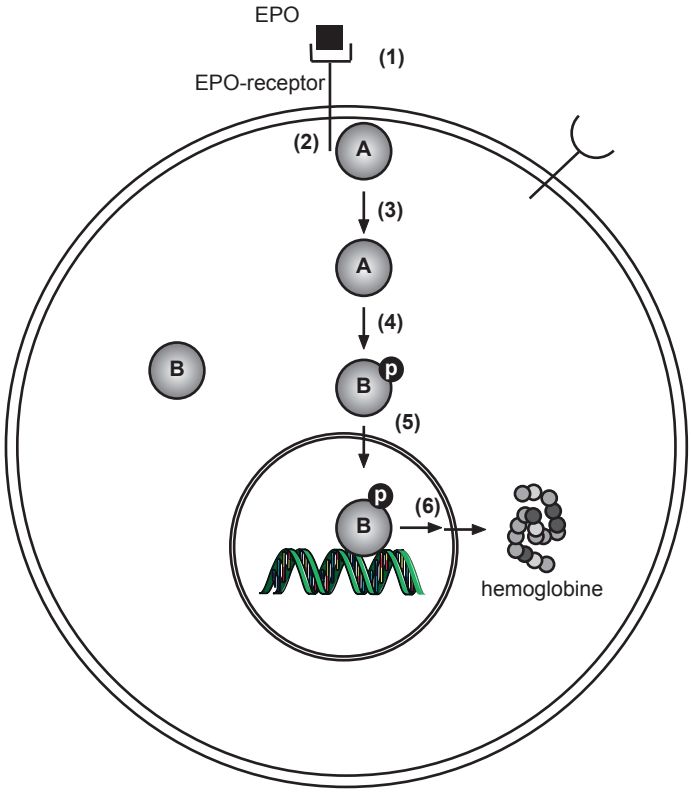
Hoe wordt bepaald welk gen wordt vertaald in een eiwit?

De cellen in ons lichaam moeten goed samenwerken om te zorgen dat alles goed functioneert. Als er cellen zijn beschadigd of doodgegaan moeten cellen aangezet worden om te delen. Om te zorgen dat het aantal cellen in ons lichaam hetzelfde blijft, is de communicatie tussen de cellen erg belangrijk. Cellen beïnvloeden elkaars gedrag door het uitzenden van groeifactoren. Deze groeifactoren activeren andere cellen in de omgeving en geven bijvoorbeeld de boodschap door dat er te weinig cellen zijn en dat dus alle cellen



Figuur 2. Het vertalen van een gen in een eiwit
Chromosomen bestaan uit DNA, een dubbele keten van bouwstenen: A, T, G en C. De volgorde van deze bouwstenen is een code voor het maken van eiwitten. Drie letters samen vormen de code voor een aminozuur en meerdere aminozuren achter elkaar vormen een eiwit. Het vertalen van DNA in een eiwit heet transcriptie

moeten delen. Specifieke eiwitten op de buitenkant van de cel, receptoren, kunnen deze groeifactoren binden en geven het signaal door naar binnen. Omdat elke groeifactor en elke receptor een unieke 3D structuur heeft past elke groeifactor maar op één specifieke receptor. Op deze manier kan een specifieke groeifactor alleen de cellen die de goede receptor hebben activeren.
Een voorbeeld voor deze groeifactoren is EPO. Als wij hoog in de bergen zijn is de zuurstofspanning veel lager dan normaal en heeft ons lichaam moeite om genoeg zuurstof naar onze spieren te transporteren. Omdat er nu eigenlijk te weinig zuurstof wordt vervoerd gaan de niercellen in ons lichaam meer EPO produceren. EPO is een groeifactor die bindt aan de EPO-receptor, deze receptor zit alleen op rode bloedcellen. Hierdoor worden de bloedcellen aangezet om te delen en om hemoglobine te maken. Het resultaat is dat er meer rode bloedcellen worden aangemaakt en er dus meer zuurstof naar de spieren vervoerd kan worden. Als wij nu weer terug naar Nederland gaan hebben wij die grote aantallen rode bloedcellen niet nodig en een verlaging van EPO concentraties in het bloed zorgt dat er minder rode bloedcellen aangemaakt worden.



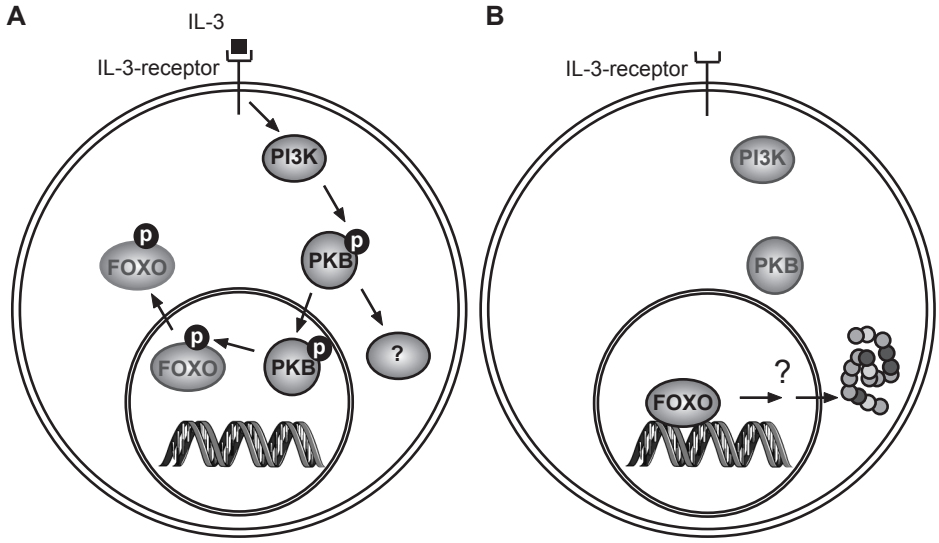
Figuur 3. Een voorbeeld van een signaaltransductie route
Als de groeifactor EPO bindt aan de EPO receptor leidt dat actvatie van signaleringseiwitten. Hierdoor wordt het signaal van buiten naar binnen doorgegeven. Deze signaleringseiwitten kunnen een transcriptie factor activeren. Binding van de transcriptie factor op een specifieke plaats in het DNA leidt dan tot het vertalen van dit stuk DNA in een eiwit.

Hoe wordt een signaal van buiten doorgegeven naar de kern?
Groeifactoren geven belangrijke signalen door aan een cel en zetten bijvoorbeeld een bloedcel aan tot het maken van hemoglobine. Als een groeifactor bindt aan een receptor op de cel wordt het signaal door middel van signaleringseiwitten doorgegeven naar de kern. De overdracht van signalen binnenin de cel wordt signaaltransductie genoemd. De eiwitten communiceren met elkaar door aan elkaar te binden en elkaars activiteit te veranderen. Veel signaleringseiwitten signaleren door via een chemische reactie een fosfaatgroep op een eiwit te zetten. Hierdoor kan 3D structuur van een eiwit veranderen en in veel gevallen werkt een fosfaatgroep als een schakelaar om een eiwit actief te maken. Doordat het ene signaaleiwit een volgend signaaleiwit activeert wordt zo een signaal in de cel doorgegeven. In de kern van de cel zitten speciale eiwitten die binden aan het DNA en zorgen dat de DNA code wordt vertaal in een eiwit.
Als EPO bindt aan de EPO-receptor op een voorloper van een rode bloedcel (Fig. 3, stap1), zorgt dat voor een verandering in de vorm van de receptor (stap 2). Eiwit A wat al aan de receptor zat gebonden laat nu los (stap 3) en botst daardoor tegen eiwit B. Tijdens de botsing zet eiwit A een fosfaat groep op eiwit B (stap 4). Eiwit B is een transcriptiefactor en door de fosfaatgroep kan eiwit B binden aan transporteiwitten die eiwit B de kern binnen loodsen (stap 5). Transcriptiefactoren zijn gespecialiseerde signaleringseiwitten die binden aan het DNA en daardoor zorgen dat de DNA-code wordt vertaald in een eiwit. Als eiwit B naar de kern wordt getransporteerd bindt het op een specifieke plaats in het DNA en zorgt dat de DNA-code wordt vertaald in het eiwit hemoglobine (stap 6). Op deze manier kan een groeifactor zorgen dat de cel andere eiwitten gaat maken en daardoor verandert. Als de signaleringseiwitten zorgen dat er hemoglobine wordt gemaakt zal de cel veranderen in een rode bloed cel. Maar als er eiwitten gemaakt worden die nodig zijn voor celdeling zal de cel delen.

Wat gaat er mis in de communicatie bij kanker?
Als cellen delen wordt het DNA gekopieerd en verdeeld over de 2 dochtercellen, zodat die dezelfde erfelijke informatie bevatten als de moedercel. Tijdens celdeling gaat het DNA als een ritssluiting open en wordt elke keten gekopieerd waardoor er 2 dubbele ketens van DNA ontstaan die netjes verdeeld worden over de 2 dochtercellen. Ondanks ingewikkelde processen die controleren of er tijdens het kopiëren geen fouten zijn gemaakt, ontstaan er tijdens elke celdeling kleine foutjes in het DNA. Hierdoor is soms de code voor een eiwit niet meer goed en wordt een eiwit gemaakt dat niet functioneel is of juist veel actiever dan normaal.
In het gezonde lichaam hebben cellen eigenlijk drie keuzemogelijkheden: ze kunnen delen, zich specialiseren of doodgaan. Cellen die niet meer nodig zijn of die niet goed functioneren kunnen getriggered worden om “netjes” dood te gaan. Dit proces heet gereguleerde celdood en zorgt ervoor dat de cel in kleinere fragmenten uit elkaar valt die dan kunnen worden gerecycled door andere cellen. De beslissing tussen delen, specialiseren en gereguleerde celdood wordt bepaald door de optelsom van de interne signalen die ontstaan na binding van de groeifactoren aan de receptoren van de cel. Op deze manier blijven er precies zoveel cellen leven als er op dat moment nodig zijn. Bij kanker functioneren sommige signaleringseiwitten niet meer of ze zijn juist veel actiever geworden. Bijvoorbeeld een eiwit dat de celdeling blokkeert wordt niet meer gemaakt of een eiwit dat het signaal afgeeft dat er gedeeld moet worden blijft continu actief. Hierdoor worden processen als celdeling, differentiatie en celdood verstoord. De kankercellen zijn onafhankelijk geworden van de invloed van groeifactoren. Hierdoor blijven kankercellen zich delen, specialiseren ze zich niet en gaan ze ook niet dood als er geen groeifactoren aanwezig zijn.

Het onderzoek beschreven in dit proefschrift

Gedurende mijn promotieonderzoek heb ik gekeken naar een specifieke signaalroute: de PI3K-PKB-FOXO route. Onderzoek heeft uitgewezen dat deze signaalroute een sleutelrol speelt in de regulatie van celdeling en dat deze signaalroute verhoogd actief is in kankercellen. Groeifactoren activeren de signaleringseiwitten PI3K en PKB en dit resulteert in celdeling. Om deze signaalroute te onderzoeken heb ik gebruik gemaakt van een cellijn die zich alleen deelt in de aanwezigheid van de groeifactor IL-3. Deze cellijn is een vroege voorlopercel van witte bloedcellen. Als IL-3 bindt aan de IL-3 receptor worden meerdere intracellulaire signaal routes geactiveerd, waaronder de PI3K-PKB-FOXO route. De receptor wordt geactiveerd wat leidt tot activatie van het signaleringseiwit PI3K. PI3K kan als het geactiveerd is een fosfaatgroep zetten op PKB, waardoor PKB ook geactiveerd wordt. PKB kan op zijn beurt op meerdere eiwitten een fosfaatgroep zetten, waaronder de FOXO transcriptiefactor. Als PKB een fosfaatgroep op FOXO zet, wordt deze transcriptiefactor snel uit de kern getransporteerd. Het gevolg is dat de FOXO transcriptiefactor in haar functie, zorgen dat DNA wordt vertaald in nieuwe eiwitten, wordt geremd door PKB (Fig. 4A). Dus als de groeifactor IL-3 aanwezig is zijn de signaleringseiwitten PI3K en PKB actief, maar is de transcriptie factor FOXO inactief. In deze situatie zorgen andere signaalroutes ervoor dat de cellen zich snel vermenigvuldigen. Als er nu geen IL-3 aanwezig is wordt de receptor niet geactiveerd en zijn ook de signaleringseiwitten PI3K en PKB niet actief. Omdat PKB niet actief is wordt de FOXO transcriptiefactor niet meer geremd en kan het nu haar functie uitoefenen. FOXO bindt op specifieke plekken in het DNA en zorgt ervoor dat de DNA-code wordt vertaald in



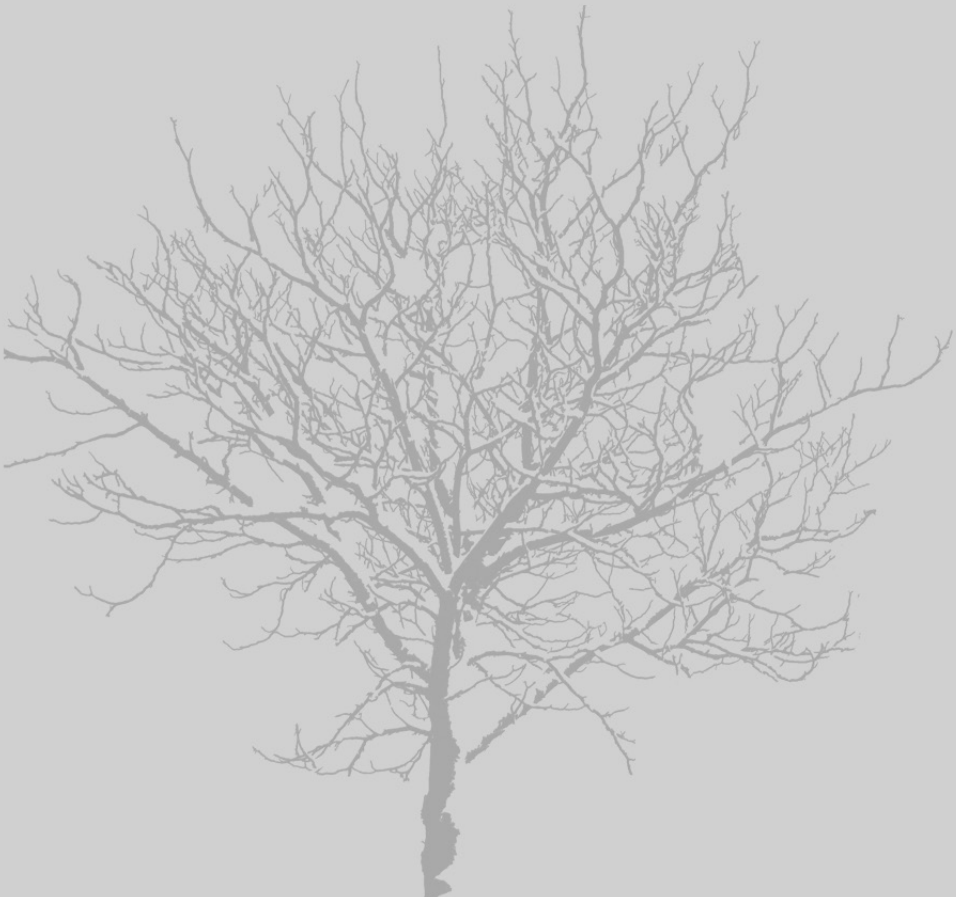
Figuur 4. De PI3K-PKB-FOX signaalroute

(A) Binding van IL-3 aan de IL-3 receptor leidt tot activatie van de IL-3 receptor en hierdoor laat PI3K los. PI3K kan dan zorgen dat PKB een fosfaatgroep krijgt en daardoor actief wordt. PKB kan nu naar de kern gaan en daar op de transcriptiefactor FOXO een fosfaatgroep zetten. FOXO wordt door de fosfaatgroep herkend door transporteiwitten die FOXO uit de kern transporteren. **(B)** In de afwezigheid van IL-3 zijn PI3K en PKB inactief en wordt FOXO niet de kern uit getransporteerd. FOXO kan dan aan het DNA binden en zorgen dat het DNA wordt vertaald in een eiwit.

nieuwe eiwitten (Fig. 4B). Het is bekend dat sommige van deze eiwitten actief de celdeling stoppen. Op deze manier zet FOXO dus een rem op de celdeling als er geen groeifactor aanwezig is. Een verstoring van dit proces kan leiden tot ongecontroleerde celdeling en het ontstaan van kanker. Tijdens mijn promotie heb ik gekeken op welke eiwitten PKB nog meer een fosfaatgroep zet. We hebben ontdekt dat eIF4B een target is van PKB. eIF4B is betrokken bij het vertalen van de DNA-code naar een eiwit en PKB stimuleert dit proces dus direct door de activiteit van eIF4B te beïnvloeden (hoofdstuk 4). Verder heb ik gekeken welke eiwitten er gemaakt worden als FOXO actief is en waarom deze eiwitten gemaakt worden. Om nieuwe targets van de FOXO transcriptiefactor te identificeren hebben we cellijnen gemaakt waarin we selectief FOXO kunnen activeren. Vervolgens hebben we gekeken welke eiwitten er toen gemaakt werden (hoofdstuk 5). Hierdoor hebben we onder andere ontdekt dat Id1, een signaleringseiwit dat betrokken is bij de specialisatie voorlopercellen naar rode bloedcellen, door FOXO gereguleerd wordt. De regulatie van Id1 door FOXO blijkt te zijn verstoord in cellen van patiënten met leukemie. Leukemie is een verzamelnaam voor verschillende soorten van kanker van bloedcellen. In tegenstelling tot gezonde bloedcellen, vermenigvuldigen de leukemische cellen zich ongecontroleerd en specialiseren zich niet. Als we nu in deze cellen FOXO activeren stoppen de cellen met delen en specialiseren zich naar rode bloedcellen (hoofdstuk 6). Behalve Id1 hebben we ook ontdekt dat FOXO zorgt voor het aanmaken van het eiwit GS. Dit eiwit is geen signaleringseiwit maar een metabool enzym dat belangrijk is voor het omzetten van bepaalde aminozuren. Verder onderzoek onthulde dat de aanmaak van GS zorgt voor recycling van eiwitten in de cel. Het recyclen van eiwitten is belangrijk voor de overleving van cellen als er weinig voedingsstoffen zijn. Dit onderzoek staat beschreven in hoofdstuk 7.

Het ontdekken welke eiwitten er gereguleerd worden door de PI3K-PKB-FOXO signaalroute, welke functie deze nieuw gemaakte eiwitten hebben in de cel en wat het gevolg is van de aanmaak van deze eiwitten geeft nieuwe inzichten in de biologische processen die worden gereguleerd door deze signaalroute. Het is belangrijk te begrijpen hoe deze processen verlopen in een gezonde cel en wat er mis gaat in kankercellen. Deze nieuwe ontdekkingen leveren potentiële aangrijppunten voor de ontwikkeling van nieuwe kanker therapieën.

Dankwoord



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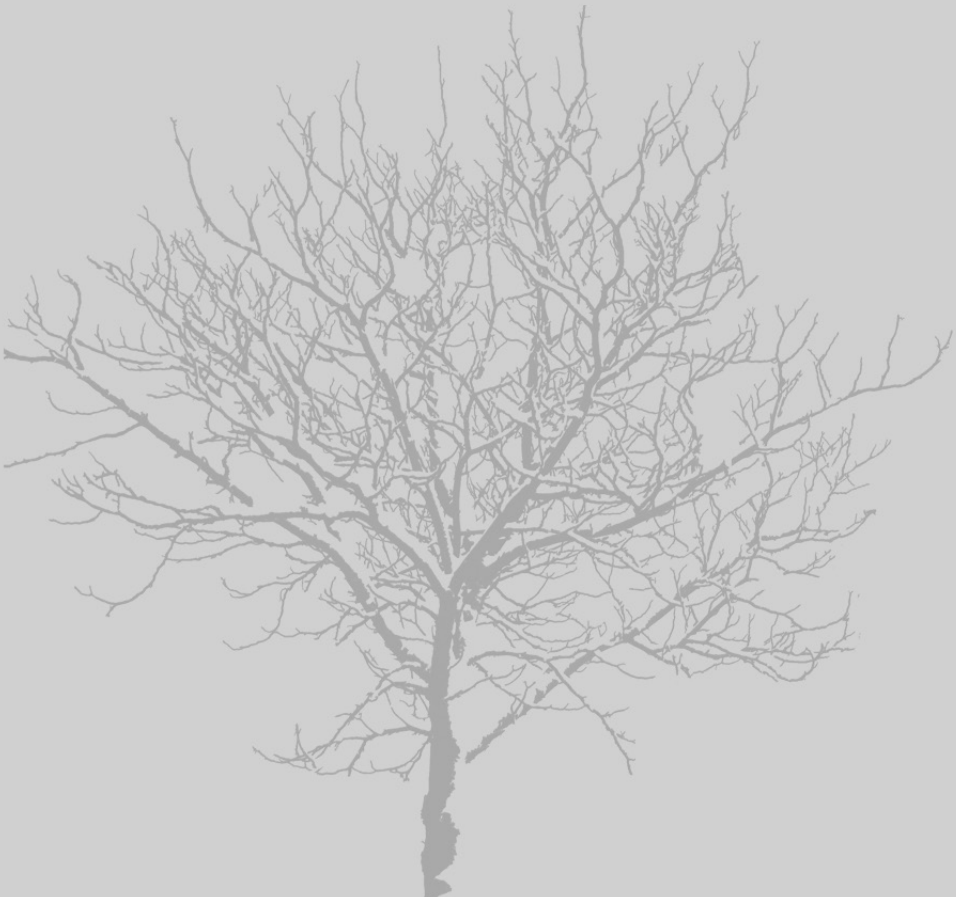
Kristan

Curriculum Vitae



Kristan van der Vos werd geboren op 25 maart 1981 te Breda. In 1999 behaalde zij het VWO-diploma aan het Sint Oelbert Gymnasium in Oosterhout. Datzelfde jaar begon zij met de studie Medische Biologie aan de Universiteit Utrecht. Tijdens haar opvolgende master studie Biology of Disease werden wetenschappelijke stages uitgevoerd in het Universitair Medisch Centrum Utrecht bij achtereenvolgens de afdelingen Immunologie onder begeleiding van drs. N.M. van Sorge en Prof.dr. J.G. van de Winkel en de afdeling Longziekten onder begeleiding van Dr. K.U. Birkenkamp en Dr. P.J. Coffer. In augustus 2004 werd de master titel behaald. Vanaf september 2004 werkte zij als assistent in opleiding in het Molecular Immunology Lab, afdeling Immunology van het Universitair Medisch Centrum Utrecht. Het in dit proefschrift beschreven onderzoek vond plaats onder begeleiding van Prof.dr. P.J. Coffer.