Posttranslational modifications of Forkhead box O transcription factors

Posttranslationele modificaties van Forkhead box O transcriptiefactoren

(met een samenvatting in het Nederlands)

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Abbreviations

4-OHT	4-hydroxytamoxifen
Ac	acetyl-moiety
AMP	adenosine monophosphate
AMPK	AMP kinase
ATP	adenosine trisphosphate
cAMP	cyclic AMP
CBP	cAMP-responsive element binding protein-binding protein
Cdk	cyclin-dependent kinase
CEF	chicken embryonic fibroblast
C.elegans	Caenorhabditis elegans
CH	cysteine and histidine rich
CHX	cycloheximide
CK1	casein kinase 1
ctrl	control
DAF	abnormal dauer phenotype
DBD	DNA binding domain
DUB	deubiquitinating enzyme
DYRK1	dual-specificity tyrosine-phosphorylated and regulated kinase 1
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
FasL	Fas ligand
FOXO	Forkhead box O
GAPDH	glyceraldehyde phosphate dehydrogenase
GCN5	general control non-derepressible 5
GFP	green fluorescent protein
H_2O_2	hydrogen peroxide
HAT	histone acetyltransferase
HAUSP	herpesvirus-associated ubiquitin-specific protease
HDAC	histone deacetylase
HECT	homologous to E6AP C-terminus
HOXD9/10	homeobox D9/D10
IBMX	isobutylmethylxanthine
ICSBP	interferon consensus sequence binding protein
IFN	interferon
IGF	insulin-like growth factor
Ig HC	immunoglobulin heavy chain
IKKβ	IκB kinase β
IL-12	interleukin 12
IP	immunoprecipitation
IRF	interferon regulatory factor
JNK	Jun N-terminal kinase
KIX	kinase inducible interaction
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MnSOD	manganese superoxide dismutase
MYST	MOZ, Ybf2/Sas3, Sas2, Tip60
NAD	nicotinamide adenine dinucleotide
Nam	nicotinamide
NES	nuclear exclusion signal
NFATc	nuclear factor activated in T cells
NFκB	nuclear factor κB
NLS	nuclear localization signal
NRF1	nuclear respiratory factor 1
OTU	Otubain/ovarian tumour protease

P	phosphate-moiety
PCAF	p300/CBP associated factor
PDK1	phopshoinositide-dependent kinase 1
PHD	plant homeodomain
PI3K	phosphatidyl inositol 3-kinase
PKB	protein kinase B
PQ	paraquat
PTEN	phosphatase and tensin homologue deleted from chromosome 10
RING	really interesting new gene
RNAi	interfering RNA
SCF	Skp1, cullin-1, F-box protein
s.d.	standard deviation
SEM	standard error of the mean
SGK	serum and glucocorticoid dependent kinase
Sir	silent information regulator
siRNA	small interfering RNA
SIRT	sirtuin
TAP	tandem affinity purification
TD	transactivation domain
T_H1	T helper 1
TIF1	Transcriptional Intermediary Factor 1
TL	total (cell) lysate
TSA	trichostatin A
Ub	ubiquitin-moiety
USP	ubiquitin-specific protease
WB	Western blot
wt	wild-type
YFP	yellow fluorescent protein

CHAPTER 1

General introduction

The FOXO family of transcription factors has important functions in processes like differentiation, proliferation, apoptosis, DNA repair, metabolism and stress resistance. The transcriptional activity of FOXOs is regulated by a number of pathways. The PI3K/PKB (also named c-Akt) signalling pathway is the most well-studied, but also pathways impinging on other kinases as well as cofactors and interacting transcription factors control FOXO activity. Both the importance of the processes regulated by FOXOs and the many pathways controlling FOXO function imply that FOXOs are central players in determining cell fate.

The FOXO family of forkhead transcription factors

In central processes of life, such as development, differentiation, proliferation, apoptosis and metabolism, forkhead transcription factors play a major role (reviewed in Kaufmann and Knochel, 1996[1]). Forkhead box O (FOXO) transcription factors comprise an important subfamily within this superfamily of forkhead transcription factors, and have been implicated in all processes mentioned above (reviewed in Burgering and Kops, 2002[2]). In mammals, the FOXO family contains 4 members, FOXO1, FOXO3, FOXO4 and the recently cloned FOXO6[3]. FOXOs are classical transcription factors in that they contain a DNA-binding domain, i.e. the forkhead box, and a transactivation domain, located in the N-terminal and C-terminal parts of the protein respectively. In addition, they contain a nuclear localization signal (NLS) and a nuclear exclusion signal (NES). Figure 1 shows a schematic overview of the domains present in FOXOs. The winged-helix DNA-binding domain, consisting of three α-helices, three β-sheets and two loop regions, has a fork-like structure, hence the name forkhead transcription factors[4,5]. This domain, in particular helix 3, is responsible for binding to promoters containing the FOXO consensus motif 5'-TTGTTTAC-3'[6]. A clear function for FOXO transcription factors was first described in Caenorhabditis elegans (C.elegans). In this small nematode, DAF-16, the worm homologue of the mammalian FOXO transcription factors, is required for the more than two-fold extension of lifespan caused by eliminating the insulin receptor DAF-2 (abnormal DAuer Formation-2)[7]. Since the discovery that the pathway from insulin receptor to FOXO is conserved in evolution (see below and Figure 2), numerous genes were found to be regulated by mammalian FOXO transcription factors. Table 1 depicts these genes, categorized by the process that is regulated. It is generally accepted that expression of these genes can be controlled by either of the FOXO transcription factors, but that specificity is obtained by their expression pattern. Expression of one of the FOXO target genes in Table 1, cyclin D, is inhibited by FOXOs. This seems not to require direct DNA-binding of FOXO to the cyclin D promoter as cyclin D expression is also downregulated by a mutant of FOXO (FOXO1-H215R) that is unable to bind to DNA[17]. Of particular interest for the scope of this thesis are $p27^{kip1}$ and MnSOD (manganese superoxide dismutase) as they are used as target genes indicative of FOXO transcriptional activity. The cyclin-dependent kinase inhibitor $p27^{kip1}$ is a negative regulator of the G1 to S phase transition by binding to and inactivating the cyclin E-Cdk2 complex. MnSOD is a superoxide dismutase and as such catalyses the conversion of superoxide radicals into hydrogen peroxide. By inducing the expression of this protein, FOXO transcription factors enhance cellular stress resistance. Importantly, this increase in oxidative stress resistance has been claimed to be responsible for the lifespan increase of C.elegans DAF-2 mutants.

The regulation of FOXO activity by insulin signalling may imply that FOXOs have a role in diabetes. Indeed, haploinsufficiency of the FOXO1 gene restores insulin sensitivity and rescues the diabetic phenotype in insulin-resistant mice[47]. FOXOs also might be tumour-suppressor proteins, as they regulate processes such as cell cycle arrest and apoptosis via genes that have been implicated in the development of cancer. The fact that in many tumours PKB is hyperactive, because of overexpression of upstream kinases or inactivation of PTEN (phosphatase and tensin homologue deleted from chromosome ten) by mutations, may reinforce this notion, albeit that PKB has many other substrates as well that could be involved in cancer development. Another reason to ascribe to FOXOs a tumour-suppressor function may be the fact that in alveolar rhabdomyosarcomas, the gene encoding FOXO1 is commonly translocated yielding a PAX3-FOXO1 or PAX7-FOXO1 fusion protein[48,49]. Also in acute leukaemias the FOXO4 gene is found translocated, resulting in an MLL-FOXO4 chimera[50]. However, it is currently unknown whether inactivation of FOXO function is responsible for tumour development as these translocations seem to affect the function of both FOXO and its fusion partner[51]. Additionally, mutations in FOXO transcription factors have not been found in malignancies yet. So, whether FOXOs are genuine tumour-suppressor proteins can be disputed.

Regulation of FOXO transcriptional activity by posttranslational modifications

Phosphorylation

The modification of proteins by phosphorylation has been studied for more than half a century now. The process of phosphorylation is carried out by kinases and phosphatases can in turn remove the phospho group. The majority (98%) of protein phosphorylation occurs at serine and threonine residues, whereas only 2% occurs at tyrosines. There are several hundreds of kinases, but only a few dozens of phosphatases. As about one third of all proteins in any organism can be phosphorylated, it is not surprising that phosphorylation is involved in every aspect of cell biology (reviewed in Berndt, 2003[52]).

The finding that the C.elegans protein DAF-16 is required for lifespan extension by DAF-2 mutations led to the discovery that also in higher eukaryotes FOXO transcriptional activity is regulated by insulin/insulin-like growth factor (IGF) signalling and similar receptor tyrosine kinase signalling pathways. These receptor tyrosine kinase pathways act through phosphatidyl inositol 3-kinase (PI3K), a membrane lipid kinase, phosphoinositide-dependent kinase 1 (PDK1), and protein kinase B (PKB), an important serine/threonine kinase, and is negatively regulated by the lipid phosphatase PTEN. The pathway from tyrosine kinase receptor to FOXO is completely conserved throughout evolution both regarding structure and function of the individual components (Figure 2; reviewed in Burgering and Kops, 2002[2]).

PKB regulates FOXO activity by phosphorylation on one threonine and two serine residues in case of FOXO1, 3 and 4 (FOXO1: T24, S256, S319[53,54]; FOXO3: T32, S253, S315[11]; FOXO4: T28, S193, S258[55]) and on one threonine and one serine residue in case of FOXO6 (T26, S184)[56] (Figure 1). Phosphorylation on these sites leads to inhibition of FOXO transcriptional activity (Figure 3). In case of FOXO1, 3 and 4, this is mediated both by reduced DNA binding and by exclusion of FOXO from the nucleus through binding to 14-3-3 proteins. The activity of FOXO6, in contrast, seems to be inhibited by PKB only

through reduced DNA binding. Nuclear import and export are tightly regulated processes mediated by the small GTPase Ran in concert with importins and exportins respectively (reviewed in Fried, 2003[57]). The first of the two serine residues (S193 in FOXO4) is located in the atypical, basic NLS and phosphorylation on this site blocks recognition by an as yet unknown importin[58]. Export of FOXOs from the nucleus has long been thought to be mediated by the leptomycin B sensitive export receptor Crm1, but recent data argue against this and suggest involvement of exportin 7[59]. Exportin 7 mediates nuclear export of 14-3-3 proteins and therefore may also export FOXO proteins from the nucleus. In this respect, it would be interesting to know whether FOXO6, which is mainly nuclear even in the presence of growth factors, can bind to 14-3-3 proteins.

Besides PKB, several other kinases were found to phosphorylate FOXO transcription factors. One is the serum- and glucocorticoid-dependent kinase (SGK), which is closely related to PKB[60]. Phosphorylation of FOXOs by SGK also results in nuclear exclusion

Table 1 FOXO target genes categorized by the process that is regulated. + or – indicates whether the respective gene is up- or downregulated.

Target gene	Regulation	Process	Reference
Bcl6	+	apoptosis	8
Bim	+	apoptosis	9,10
FasL	+	apoptosis	11
TRADD	+	apoptosis	12
TRAIL	+	apoptosis	13
BTG1	+	cell cycle	14
Cyclin B	+	cell cycle	15
Cyclin D	-	cell cycle	16,17
Cyclin G2	+	cell cycle	18
p130	+	cell cycle	19
$p21^{cip1}$	+	cell cycle	20
$p27^{kip1}$	+	cell cycle	21
Plk	+	cell cycle	15
4EBP1	+	cell growth	22,23
GADD45	+	DNA repair	24,25
collagenase	+	extracellular matrix degradation	26
LPL	+	lipid metabolism	27
Sterol carrier protein	+	lipid metabolism	28
OLD	+	longevity	29
Scl-1	+	longevity	30
G6Pase	+	metabolism	31,32
HMGCS2	+	metabolism	33
IGFBP1	+	metabolism	34
PDK4	+	metabolism	35
PEPCK	+	metabolism	32
PGC1	+	metabolism	36
Atrogin-1	+	muscle atrophy	37-39
MuRF1	+	muscle atrophy	39
catalase	+	oxidative stress	40
Glutathione transferase	+	oxidative stress	41
MafA	+	oxidative stress	42
MnSOD	+	oxidative stress	43
NeuroD	+	oxidative stress	42
caveolin-1	+	signalling	44
InsR	+	signalling	23,45
SMURF2	+	ubiquitin-mediated degradation	46

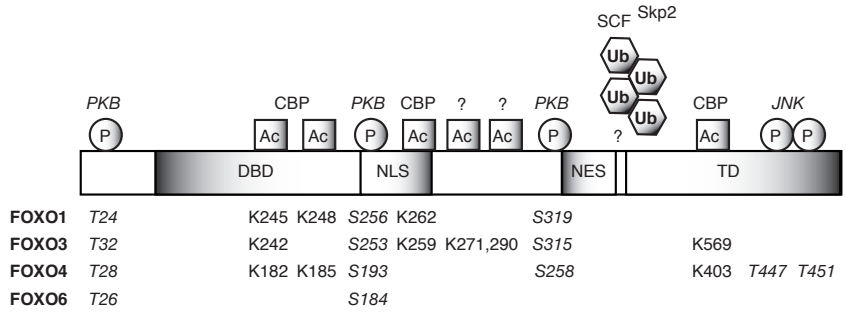

Figure 1 Schematic representation of the domain structure of FOXO transcription factors. FOXOs comprise of a DNA-binding domain (DBD) and a transactivation domain (TD). Furthermore, they contain an NLS (nuclear localisation signal) and a NES (nuclear exclusion signal). For reasons of clarity, only those posttranslational modifications (and the proteins responsible for them) that are central in this thesis are indicated. The enzyme(s) for acetylation of K271 and K290 in FOXO3 is unknown. Also the site(s) of polyubiquitination is not known. Ub=ubiquitin-moiety, Ac=acetyl-moiety, P=phosphate-moiety.

and together with PKB, SGK may complete FOXO phosphorylation on the so-called PKB consensus sites. It has even been suggested that SGK and PKB form a complex, but whether both proteins are active at the same time is unclear as they seem to have different functions, at least in C.elegans, where PKB regulates dauer formation and SGK is involved in stress resistance and lifespan regulation[61].

Phosphorylation of Ser319 in FOXO1 by PKB acts as a signal for subsequent phosphorylation of Ser322 by CK1 (casein kinase 1), which in turn primes the CK1-catalysed phosphorylation of Ser325[62]. The same group identified DYRK1A (dual specificity tyrosine-phosphorylated and regulated kinase 1A) as a kinase phosphorylating the conserved residue Ser329 in FOXO1[63]. Ser329 phosphorylation also decreases the ability of FOXO1 to stimulate gene transactivation by reducing the fraction of FOXO1 present in the nucleus. However, unlike the residues targeted by PKB or CK1, Ser329 phosphorylation is not increased by stimulation with IGF-1 or by transfection with PDK1[63]. DYRK1A in fact seems to be a constitutively active kinase whose activity does not seem to depend on other phosphorylation events. How this function of DYRK1 relates to the co-activator role of DYRK1 that has been suggested towards FOXO-mediated glucose-6-phosphatase expression is at present unclear[64]. The cluster of phosphorylation sites at Ser319, Ser322, Ser325 and Ser329 accelerates nuclear export by controlling the interaction of FOXO with the Ran-containing protein complex that mediates this process[65]. Whether this nuclear export relies on binding of FOXO1 to 14-3-3 proteins is at present unclear.

Other kinases that regulate FOXO activity are IκB kinase β (IKKβ), the Rho-associated kinase ROCK and AMPK (AMP-activated protein kinase). IKKβ, by phosphorylating FOXO3, links the NFκB and FOXO pathways. Functionally, constitutive expression of IKKβ promotes cell proliferation and tumourigenesis, which can be overridden by FOXO3[66]. ROCK inhibits FOXO1 activity in myoblasts through nuclear exclusion, possibly as a result of FOXO1 phosphorylation, thereby preventing myoblast fusion. Similarly, downregulation of ROCK activity during myoblast differentiation induces FOXO1 activity leading to terminal differentiation of these cells to skeletal muscle cells[67-69]. AMPK seems to destabilize FOXO, but whether this depends on direct phosphorylation is not known[70]. Whether the above-mentioned kinases can phosphorylate the different FOXOs is unclear.

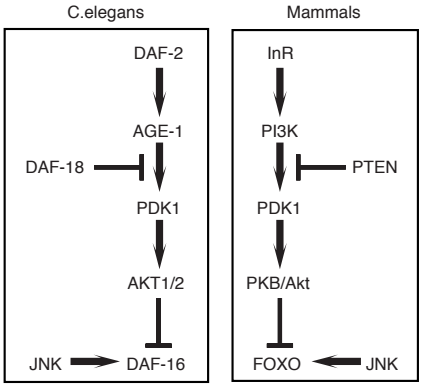

Figure 2 Conservation of the insulin receptor signalling pathway between C.elegans and mammals. Activation of the insulin receptor by insulin results in activation of PI3K, a lipid kinase that phosphorylates inositol lipids in the plasma membrane. These phosphorylated lipids form docking sites for PDK1 and PKB. PDK1 activates PKB and PKB in turn inhibits FOXO transcription factors by phosphorylation. Recently, it was also described that JNK signalling towards FOXOs is conserved.

Finally, apart from PKB probably the most well-studied FOXO kinase is the stress kinase JNK (Jun N-terminal kinase), which directly phosphorylates FOXO4 on Thr447 and Thr451 upon oxidative stress signalling via the small GTPase Ral[71,72] (Figures 1 and 3). This phosphorylation stimulates FOXO4 translocation to the nucleus and concomitantly activates FOXO4. Similar results were obtained in C.elegans and Drosophila, where in addition an increase in lifespan mediated by JNK via FOXO was observed[73,74]. Thr447 and Thr451 do not seem to be conserved between the different FOXO proteins and whether JNK also phosphorylates FOXOs other than FOXO4 is at present not known. Interestingly, oxidative stress signalling also induces binding of β-catenin to FOXO and the β-catenin/FOXO complex is found in the nucleus. It is unlikely, however, that this interaction depends on phosphorylation of FOXO4 by JNK, because mutating the JNK phosphorylation sites to alanines does not impair binding (Marieke Essers, Thesis, 2004[189]). These findings and the regulation of MnSOD expression by FOXO imply that FOXO is a central player in oxidative stress signalling.

Acetylation

Acetylation is an important regulatory posttranslational modification of proteins, whereby the acetyl group from acetyl-CoA is transferred to a substrate. Generally, proteins that are acetylated are involved in regulation of transcription, such as histones (proteins responsible for packing DNA) and transcription factors (reviewed in Legube and Trouche, 2003[75]). As acetylation regulates transcription, it is not surprising that acetylation is involved in many aspects of cell biology. Acetylation can occur either at the α-amino group of the N-terminal residue of a substrate protein or at the ε-amino group of an internal lysine residue. N-terminal acetylation mainly occurs in a co-translational manner and is generally irreversible, whereas lysine acetylation is a reversible modification[76].
Dozens of enzymes that are able to transfer an acetyl moiety to a lysine residue in a substrate, so-called lysine acetyltransferases, were discovered in the past decade (reviewed in Yang, 2004[77]). There a more than 10 families of lysine acetyltransferases of which p300/CBP

[cAMP response element binding protein(CREB)-binding protein], GCN5/PCAF (general control non-derepressible 5 and p300/CBP associated factor) and the MYST (named for members MOZ, Ybf2/Sas3, Sas2, and Tip60) proteins are most well characterised. p300, CBP and PCAF have been misnamed histone acetyltransferases (HATs), as they where first discovered to acetylate histone proteins. Subsequently, however, they were shown to acetylate non-histone proteins, such as transcription factors, as well[78].

Although p300 and CBP show high homology, recent advances demonstrated that they have unique roles, probably most well demonstrated by the fact that genetic (inactivating) aberrations in CBP can cause Rubinstein-Taybi syndrome, whereas there are no known p300 mutations causing congenital disease (reviewed in Kalkhoven, 2004[79]). Rubinstein-Taybi syndrome is an autosomal dominant disorder characterized by mental retardation and increased predisposition to childhood malignancies (reviewed in Iyer *et al*., 2004[80]). p300 and CBP are comprised of several domains. Besides the HAT-domain in which the PHD (plant homeodomain) finger is crucial for its acetylation capacity, they contain three CH (cysteine histidine rich) domains and a KIX (kinase-inducible interaction) domain important for interaction with other proteins. Although these domains of p300 and CBP are highly similar, they seem to interact with different proteins. While the p300 KIX domain interacts with phosphoribosylpyrophosphate synthetase subunit 1[81], the CBP KIX domain preferably binds to CREB, hence the name CBP[82]. Furthermore, p300 and CBP contain a bromodomain that recognizes acetyllysine moieties and as a result they probably mediate

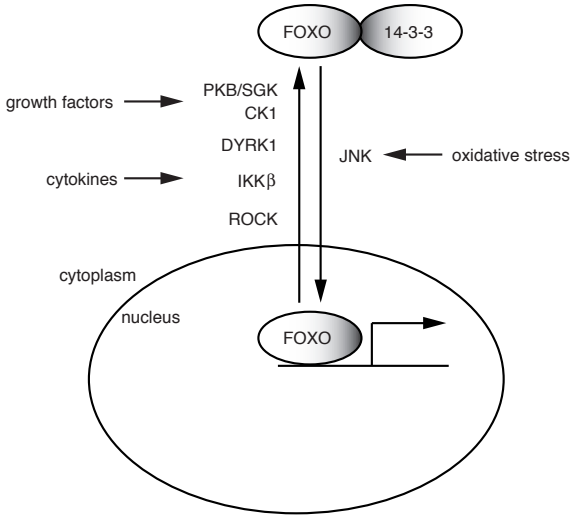

Figure 3 FOXO localization and activity is regulated by a number of kinases. Growth factor stimulation of cells leads to activation of PKB and SGK, which then phosphorylate FOXO transcription factors. One of these phosphorylation sites constitutes a docking site for CK1, which phosphorylates FOXOs at two consecutive sites. Phosphorylation on these PKB/SGF and CK1 sites leads to nuclear exclusion of FOXOs by interacting with 14-3-3 proteins and hence inhibition of FOXO transcriptional activity. In a similar manner, IKKβ is activated by cytokines and inhibits FOXO activity. The activity of ROCK towards FOXOs may be restricted to myoblasts where ROCK prevents myoblast fusion by inhibiting FOXO activity. DYRK1 seems a constitutively active kinase, whose role in the regulation of FOXOs is at present unclear. In contrast to exclusion from the nucleus by the above-mentioned kinases, JNK-mediated phosphorylation of FOXO, which is induced by oxidative stress, may result in FOXO translocation to the nucleus.

Figure 4 Deacetylation reaction mediated by Sir2. Sir2 deacetylates substrates like histones in an NAD-dependent manner. The resulting products are nicotinamide, which can inhibit Sir2 activity, O-acetyl-ADP-ribose, which can be functionally conjugated to other proteins, and the deacetylated substrate.

multiple acetylation events on one substrate[83]. PCAF also contains a HAT domain and a bromodomain. In addition, PCAF has a PCAF-specific domain that interacts with p300 and CBP[84].

Regulation of the activity of acetyltransferases involves various mechanisms. First, acetyl-CoA, an essential cofactor for several acetyltransferases, stabilizes GCN5 and PCAF[85]. Second, formation of multisubunit complexes modulates the substrate specificity and activity of different acetyltransferases. For example, the protein Twist inhibits CBP activity by a direct interaction[86]. Third, posttranslational modifications, such as phosphorylation[87], acetylation[88], ubiquitination[89] and sumoylation[90] affect acetyltransferase activity. And finally, compartmentalization regulates their activity[91].

Generally, acetyltransferases are believed to act as transcriptional coactivators, although few have been shown to do the opposite, i.e. silence gene expression. Coactivation can be a result of loosened DNA packing after histone acetylation, allowing more space for the transcription machinery[92]. Alternatively, increased transcription can be a result of acetylation of transcription factors. Mechanisms include enhanced interaction of acetylated transcription factors with bromodomain-containing coactivators, prevention of inhibitory modifications on the same lysine residue, like ubiquitination, as has been demonstrated for Smad7[93] and enhanced DNA binding, as shown for low stoichiometric acetylation of p53 for example[94]. On the contrary, acetylation of proteins involved in transcription can also lead to reduced transcription. Acetylation of the transcriptional repressor Bcl6 inhibits its DNA binding capacity[95] and acetylation of p53 can disrupt tetramerisation, resulting in p53 being exported from the nucleus[96]. Understandably, for disruption of tetramerisation of p53 the stochiometry of acetylation must be relatively high.

As mentioned above lysine acetylation is a reversible process and the proteins involved in removing acetyl moieties are called histone deacetylases (HDACs). Deacetylation of histones is considered to result in gene silencing and the function of several HDACs seems to be mediating a gene-silencing program resulting in a cell type-specific gene expression pattern. HDACs belong to either of four families: class I, class II, class III and class IV HDACs. HDAC1, 2, 3 and 8 belong to the class I HDACs, whereas HDAC4, 5, 6, 7, 9 and 10 and the Sir2(silent information regulator 2)-like sirtuins (SIRTs) 1 through to 7 belong to the class II and class III HDACs respectively. HDAC11 is at present the only member of the class IV HDACs (reviewed in De Ruijter *et al*., 2003[97] and Blander and Guarente, 2004[98]). Class III HDACs are not related to HDACs from the other classes and therefore will be discussed later in this chapter.

Class I HDACs are expressed in most cell types, whereas HDACs of class II have a more restricted expression pattern, suggesting that they might be involved in cellular differentiation and developmental processes (according to the Human Transcriptome Map, www.amc.uva.nl). In support of this, HDAC4, 5 and 7 are involved in muscle differentiation[99]. The amount of data on the function of specific HDACs is limited, but inhibition of HDAC activity by small molecules can lead to inhibition of proliferation or apoptosis. Hence, several HDAC inhibitors are currently tested in clinical trials as anticancer agents. The mechanism by

which these inhibitors work is displacement of the Zn^{2+} ion that is required for HDAC activity. The most potent small molecule inhibitor of HDACs is TSA (Trichostatin A), with an IC_{50} in low nanomolar range for all HDACs. TSA is a reversible inhibitor containing a hydroxamic acid group and a five carbon atom linker to a phenyl group[100,101] and as such perfectly fits into the catalytic centre.

Class I HDACs are most closely related to the Saccharomyces cerevisiae transcriptional regulator RPD3, whereas class II HDACs share domains with similarity to HDA1, another deacetylase found in yeast[102]. Interestingly, phylogenetic analysis of bacterial HDAC relatives suggests that HDAC classes I, II and IV precede the evolution of histone proteins and raises the possibility that the primary activity of some HDACs is directed against non-histone proteins[103]. Class I HDACs contain more or less exclusively a catalytic domain and generally seem to require other factors to act as active deacetylases. Class II HDACs are larger and consist of protein interaction motifs besides their catalytic domain[97]. Several of the class I and class II HDACs contain an NLS (HDAC1, 3, 4, 5, 7 and 8) and/or a NES (HDAC3, 4, 5). As a result, class I HDACs are almost exclusively found in the nucleus whereas most class II HDACs are able to shuttle in and out of the nucleus, in some cases in a 14-3-3 protein-dependent manner[97]. The fact that some class II HDACs are found in the cytosol reinforces the notion that some HDACs do not make use of histone proteins as their substrate. Taken together, the activity of HDACs depends on their abundance, cellular localization and cofactor binding.

The Sir2 family of histone deacetylases (class III HDACs) is highly conserved in organisms ranging from bacteria to humans[104]. Like the other classes of HDACs, Sir2-like deacetylases appeared in evolution before histone proteins, suggesting that also these deacetylases have substrates other than histones. In humans, seven Sir2-like proteins exist which are named SIRT1 (sirtuin 1) to SIRT7[105,106]. SIRT1 (also named hSir2^{SIRT1} and Sir2) is the most

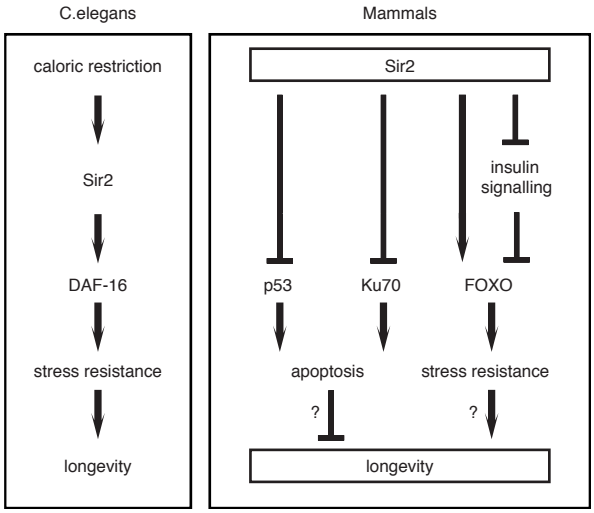

Figure 5 Mechanisms of lifespan regulation by Sir2. In C.elegans Sir2 increases longevity, which requires DAF-16 activity, possibly through increasing cellular stress resistance. In mammals, a similar pathway exists. In addition, Sir2 regulates FOXO activity by modulating insulin signalling. Other proteins downstream of Sir2 that affect lifespan are p53 and Ku, which both have roles in apoptosis. Whether the effects on apoptosis and stress resistance account for increased lifespan is unknown.

close homologue to yeast SIR2 and is ubiquitously expressed. From here on the term Sir2 will be used for the Sir2-like proteins from different organisms that mostly resemble yeast SIR2 (except for Chapter 2 where hSir2^{SIRT1} will be used). Like Sir2, SIRT6 and SIRT7 are localized in the nucleus where they are associated with heterochromatin and nucleoli respectively. SIRT2 is a cytosolic protein involved in tubulin deacetylation and mitotic exit. SIRT3, 4 and 5 are localized to mitochondria[98]. Thus, some mammalian Sir2-like proteins have retained histone deacetylase activity, whereas others use different proteins as a substrate (see below).

The reaction mediated by Sir2 is different from the reaction that is catalysed by the other HDACs. Sir2 is unique in the fact that it requires NAD^+ (nicotinamide adenine dinucleotide) for its deacetylase activity. For each acetyl group that is removed from a substrate one NAD^+ is cleaved[107,108]. An explanation why an energetically favourable reaction, like deacetylation, requires energy in the form of NAD^+ could be that in this way Sir2-mediated deacetylation can be regulated by environmental cues, like the metabolic state of the cell. Cleavage of NAD^+ unexpectedly results in nicotinamide and O-acetyl-ADP-ribose, instead of nicotinamide and ADP-ribose[107,108] (Figure 4). Hence, it was proposed that O-acetyl-ADP-ribose has a unique cellular function. Indeed, recent data from yeast showing that O-acetyl-ADP-ribose promotes the association of SIR3 with SIR2 and SIR4 and induces a dramatic structural rearrangement in the SIR complex responsible for chromatin silencing, support this notion[109].

A role for SIR2 (as it is named in Saccharomyces cerevisiae) was unravelled by genetic studies in yeast. Sir2 regulates silencing of three loci containing repeated DNA sequences: (1) the mating-type loci, (2) telomeres and (3) rDNA. For silencing the mating-type loci and telomeres the *SIR3* and *SIR4* genes, which encode proteins that are structurally unrelated to SIR2, are required[110,111]. Subsequent studies revealed a role for Sir2 proteins in the regulation of organismal lifespan in organisms ranging from yeast to Drosophila[112-114]. In yeast, increased silencing of rDNA recombination by Sir2 and hence reduced formation of extrachromosomal rDNA circles enhances replicative lifespan. In contrast, in C.elegans Sir2 affects lifespan by a totally different mechanism. In the worm, Sir2 enhances longevity by stimulating the activity of DAF-16, the worm homologue of FOXO transcription factors (see below). In Drosophila, the mechanism is unknown, but it is tempting to speculate that Sir2 acts via FOXO in the fly as well, as signalling pathways regulating FOXO are conserved in evolution. Likewise, Sir2 may prolong lifespan in mammals and pathways that are regulated by Sir2 in mammals are shown in Figure 5.

The dependence of the Sir2-mediated reaction on NAD^+ and the fact that caloric restriction, i.e. reduced dietary intake, increases the NAD^+/NADH ratio in cells, led to the hypothesis that caloric restriction, which has long been appreciated to increase longevity both in yeast and experimental animals, may act via Sir2 to enhance longevity. Indeed, caloric restriction seems to extend yeast life span by lowering the levels of NADH[115], although another report demonstrated a role for nicotinamide, which inhibits Sir2 activity, in caloric restriction-mediated lifespan extension[116]. These results are not mutually exclusive and future research will yield more details regarding the mechanisms whereby NADH and nicotinamide levels are changed upon caloric restriction. A clue came from the finding that in yeast the level of PNC1, a nicotinamidase that deaminates nicotinamide to nicotinic acid, is increased after caloric restriction[116]. Interestingly, Sir2 levels were also found to be increased upon caloric restriction in mammalian cells[117]. So, it seems that the activity of Sir2 is regulated at multiple levels.

Nicotinamide, which is a product of the Sir2-mediated deacetylation reaction, can inhibit Sir2 activity as mentioned above. Several other compounds that can inhibit Sir2 activity have been discovered and these are potential anticancer drugs[118]. Reversely, activators of Sir2 also have been found and the most interesting substance is resveratrol, a compound that naturally occurs in for example peanuts and red wine. Resveratrol has been associated with a number of health benefits and lifespan extension[119,120]. Activators of Sir2 are of special interest for those who seek the "fountain of youth", i.e. who want to stay young as long as possible. In order to intelligently design more potent inhibitors and activators of Sir2 the protein structure of Sir2 could be of help. The structures of several Sir2 homologues have been elucidated and show that Sir2 proteins have a tunnel-like configuration in between a Rossmann fold and a small domain consisting of a three-stranded antiparallel β-sheet, two α-helices and a long loop[121,122]. In this tunnel the acetyl-lysine residue of the substrate and NAD^+ are brought in close proximity. The fact that only a small part of the substrate fits into this channel has brought up the question what determines the substrate specificity of Sir2 proteins. Although in vivo Sir2 must have substrate specificity, in vitro this does not seem to be the case[123].

The identification of novel substrates of Sir2 extended the number of processes Sir2 was known to be involved in to include metabolism, apoptosis, cell cycle regulation and differentiation. Sir2 substrates and the processes they are involved in are summarized in Table 2. Functions of Sir2 that are mediated via histone modifications are depicted in Table 3.

Like the regulation of FOXO by PI3K/PKB signalling, data suggesting regulation of FOXO by acetylation/deacetylation were first obtained in C.elegans. Tissenbaum and Guarente discovered that when they increased the dosage of the gene encoding Sir2, the lifespan of the worm was extended by 50%[113]. This increase in lifespan was dependent on the presence of the FOXO homologue DAF-16. Two, not mutually exclusive, mechanisms for this dependence have been proposed: (1) Sir2, through its histone deacetylase function, regulates expression of one of the components of the DAF-2 signalling pathway and (2) Sir2, through its transcription factor deacetylase function, directly deacetylates DAF-16. Regarding the first, in mammals Sir2 represses expression of IGFBP1 (IGF binding protein 1), a positive regulator of the IGF signalling pathway, so a negative regulator of FOXO activity[147], as

Table 2 Sir2 substrates from different organisms categorized by the process that is regulated. + or – indicates whether the respective substrate or process (in the case of transcription) is activated or inhibited by Sir2.

Substrate	Regulation	Process	Reference
FOXO	-/+	apoptosis/cell cycle	124-128
Ku70	-	apoptosis	117
NFκB p65	-	apoptosis	129
p53	-	apoptosis	130,131
Bcl6	+	differentiation	95
MEF2	-	differentiation	132
MyoD/PCAF	-	differentiation	133
PPARγ	-	fat mobilisation	134
PGC1α	+	gluconeogenesis	135,136
Acetyl-CoA synthetase	+	metabolism	137
Alba	-	transcription	138
p300	-	transcription	139
TAF(I)68	-	transcription	140

Table 3 Genes from different organisms that are regulated by Sir2. In all cases Sir2 activity results in repression of gene expression. – indicates that the respective gene or protein is unknown

Process	Target (gene)	Partner(s)	Reference
development	Mash1	hHES1, hHEY2	141,142
differentiation	-	CTIP2	143
DNA capping	telomeres	SIR3/SIR4	144
DNA repair	any	Ku/SIR3/SIR4	145
DNA replication	-	-	146
IGF signalling	IGFBP1	-	147
insulin secretion	UCP2	-	148
mating	mating-type loci	SIR3/SIR4	144
ribosome biogenesis	rDNA	SIR4	149

well as insulin secretion[148] (Table 3). In support of the second mechanism, several reports, amongst which the paper in Chapter 2, described that FOXOs can be acetylated by several acetyltransferases (the lysine residues involved are indicated in Figure 1) and that in turn Sir2 deacetylates FOXO[124-128,150-153]. The effects of acetylation and deacetylation on FOXO activity are a matter of debate, which will be discussed in more detail in Chapter 7.

Ubiquitination

Ubiquitination is a multistep posttranslational modification by which free ubiquitin is covalently coupled to a substrate protein (reviewed in Andersen *et al*., 2005[154]). Ubiquitin is a 8.5 kDa protein that is highly conserved throughout evolution from bacteria to humans. Ubiquitination originally was discovered as a modification that targets proteins to degradation by the proteasome, a 2,000 kDa multisubunit protease. Targeting proteins for degradation turned out to be dependent on polyubiquitination (i.e. ubiquitin-conjugation to the ubiquitin moiety of a ubiquitinated protein) via lysine 48 of ubiquitin. More recently, polyubiquitination via other lysine residues of ubiquitin, monoubiquitination and modifications using ubiquitin-like proteins, such as SUMO and Nedd8, were found. Non-lysine 48-linked polyubiquitination and monoubiquitination do not lead to degradation of the substrate but are involved in processes such as endocytosis, DNA repair and transcription. Often, binding of other proteins via a so-called ubiquitin binding domain, of which 6 have been characterised, is involved in mediating the effect of ubiquitination[154].
At least three enzymes are necessary for ubiquitination (reviewed in Pickart, 2001[155]): the ubiquitin-activating enzyme E1, a ubiquitin-conjugating enzyme E2 and a ubiquitin ligase E3 (Figure 6). Recent data suggest that a ubiquitin chain elongation factor E4 is involved in polyubiquitination[156]. It is disputed, however, whether the function of E4 enzymes is not merely substituting for the function of E3s, which means that E4s constitute a novel family of E3 enzymes. The first step in the ubiquitination process is activation of ubiquitin by the conserved E1 enzyme at the expense of one molecule of ATP. In this step a thiolester bond is formed between glycine 76 of ubiquitin and the active site cysteine of E1. Next, ubiquitin is transferred to the active site cysteine of an E2 enzyme. The third and last step involves the formation of an isopeptide bond between glycine 76 of ubiquitin and the ε-amino group of a lysine residue or the α-amino group of the N-terminal residue of a substrate, which is mediated by an E3 enzyme. Generally, E3 ligases can interact with multiple E2s and substrates and, conversely, substrates can be recognized by several E3 ligases. Which

E3 ligase mediates ubiquitination of a particular substrate at a certain moment may be determined by posttranslational modifications and localization of the proteins involved. Moreover, two models have been proposed to explain how the decision between mono- and polyubiquitination is made: (1) multimerisation of the E2 could mediate ubiquitin-chain elongation[157], or (2) the availability of an E4 could be involved in chain elongation[156], which would argue against the model that E4s are a subfamily of E3s. Whether a substrate is polyubiquitinated via lysine 48 or via lysine 63 seems to be determined by the E2 enzyme[158].

Whereas there is only one E1 enzyme, about 30 E2s exist in the mammalian genome. These E2s share a conserved globular catalytic domain of around 150 residues. Some E2s have N- and/or C-terminal extensions, which may determine E3 association, intrinsic E2 activity or substrate recognition (reviewed in Pickart, 2004[159]). However, this substrate recognition does not seem to be specific for a particular lysine residue as most substrates can be modified on several lysines. Therefore it is not surprising that a ubiquitination consensus site has not been defined yet.

There are two, or three if one includes the E4 enzymes, families of E3 ligases in the mammalian genome comprising several hundreds of these enzymes: the HECT (homologous to E6AP C-terminus) domain E3 ligases and the RING (really interesting new gene) finger E3 ligases. Whereas HECT E3 ligases, which themselves have an active-site cysteine, contribute to the ubiquitination reaction, RING finger E3 ligases function as a scaffold, bringing E2 and substrate together.

HECT E3s contain a conserved 350 amino acid HECT domain that has been defined by the founding member of this family, E6AP. E6AP is an interesting E3 in the sense that it

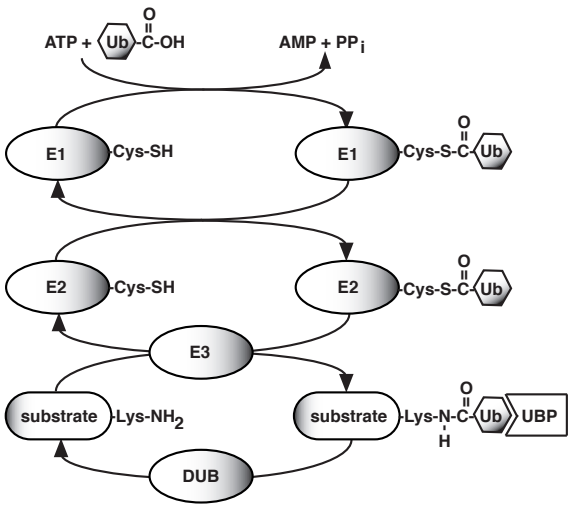

Figure 6 Schematic representation of the ubiquitin-conjugation system. In the first step ubiquitin is activated by the ubiquitin-activating enzyme E1 at the expense of one molecule of ATP. In the second step, activated ubiquitin is transferred to the active-site cysteine of the ubiquitin-conjugating enzyme E2. Finally, the ubiquitin-moiety is conjugated to a lysine residue of the substrate protein. Dependent on the type of ubiquitin ligase (E3), the E3 functions as a scaffold bringing E2 and substrate in close proximity or it functions as a true enzyme conjugating the ubiquitin. Only E3s with an active-site cysteine can conjugate ubiquitin themselves. Ubiquitin can be removed from substrates by so-called DUBs. Generally, UBPs determine the destiny of ubiquitinated proteins.

is both targeted by the oncogenic human papillomavirus protein E6 and that mutations in E6AP can cause Angelman syndrome, characterized by neurological abnormalities such as mental retardation and epilepsy[160,161]. Besides the HECT domain, these E3s contain one or more WW domains important for protein-protein interactions. The RING finger family of E3 ligases is much larger and encompasses both monomeric, for example c-Cbl, and multimeric, e.g. SCF and APC, members. The RING domain contains eight highly conserved Zn^{2+}-coordinating residues and interacts directly with the E2[162,163]. Additionally, monomeric E3s contain a substrate binding domain and as such bring E2 and substrate in close proximity. Multimeric E3s are made up of 4 or more subunits of which one contains the E2-interacting RING finger and one functions as the substrate recognition module. In case of SCF (Skp1, cullin-1, F-box protein) complexes Rbx1 (also called Hrt1 or Roc1) is the RING finger protein and the F-box protein fulfils the role of substrate recognition element. So, by varying the F-box protein, many different E3s, each targeting their specific substrate(s), can be built from a common building block. For example, an SCF complex containing the F-box protein Skp2 ubiquitinates $p27^{kip1}$ [164], whereas a similar complex with β-TRCP as the F-box protein targets IκB for ubiquitination[165].

Taken together, regulation of ubiquitination occurs at multiple levels, including E2-E3 interactions, substrate recognition, chain elongation and binding of ubiquitin to conserved motifs. In addition, removal of ubiquitin by a process called deubiquitination plays a role (reviewed in Fang and Weisman, 2004[166]).

Ubiquitin moieties are cleaved from substrates by deubiquitinating enzymes (DUBs). Removal of the ubiquitin-moiety can fulfil several roles: (1) aberrantly ubiquitinated proteins are prevented from degradation, or (2) the fate of the protein is changed, in case it is monoubiquitinated or polyubiquitinated via a lysine other than lysine 48. Around 80 DUBs are present in the human genome[167]. DUBs belong to the superfamily of proteases. Most DUBs are cysteine proteases, i.e. their activity relies on the thiol group of the active site cysteine, the remainder are metalloproteases. In addition, cysteine proteases require a histidine and an aspartic acid residue for catalysis, i.e. for deprotonating the active-site cysteine[168]. These cysteine proteases can be divided into four subclasses based on their Ub-protease domains[167]: (1) ubiquitin-specific proteases (USPs), (2) ubiquitin C-terminal hydrolases (UCHs), (3) Otubain or ovarian tumour proteases (OTUs) and (4) Machado-Joseph disease proteases (MJDs). The USP subclass represents the bulk of DUBs encoded by the human genome. The catalytic domain of these USPs contains two short, well-conserved motifs, called Cys and His boxes. Due to large unrelated sequences the complete catalytic domain comprises 300 to 800 amino acids. In contrast, the UCH subclass consists of only four members that share close homology in their catalytic domains. The role of these DUBs is poorly understood, but it has been suggested that they take part in processing ubiquitin precursors and recycling ubiquitin when it is inappropriately conjugated to nucleophiles, like glutathione[169]. OTUs, of which about a dozen exist, are unique in the fact that the aspartic acid in the catalytic triad, which is essential in the other DUB families, is not involved in catalysis, but instead a hydrogen bonding network is formed, which takes over the role of the aspartic acid[170]. The founding member, Drosophila OTU, is involved in the development of the fly ovary where it may regulate the localization and translation of certain RNA transcripts[171]. The role of the other OTUs remains to be investigated. The fourth family of DUBs, the MJDs, is a small family of 4 members, of which the catalytic triad is conserved to other DUBs. MJDs appear to be a relatively recent family of DUBs as no homologues exist in yeast. The only member for which a function has been described

is ataxin-3, which regulates transcription of inflammatory genes and is mutated in the spinocerebellar ataxia syndrome SCA-3[172].

The specificity of DUBs may rely on both the ubiquitin moiety and the substrate protein. Furthermore, protein localization and interactions with binding partners may serve a role in specificity. As mentioned above, ubiquitin can be attached to several lysines of ubiquitin to result in polyubiquitin. Several DUBs have been shown to have specificity towards one type of ubiquitin chain. For example, CYLD (Cylindromatosis suppressor-suppressor gene) preferably cleaves ubiquitin chains linked via lysines other than lysine 48[173]. Preference for a particular ubiquitin chain may be determined by domains outside the catalytic domain. The catalytic domain of USP2 prefers lysine 63 fusions, whereas the full-length protein rather cleaves lysine 48-linked ubiquitin[174]. Several mechanisms have been proposed for the regulation of DUBs. Enzymatic activity is only displayed when ubiquitin is bound to the catalytic domain due to a conformational change upon binding, thereby preventing protease activity towards other substrates. In addition, cofactor binding can either inhibit or enhance DUB activity, as shown for GMP synthetase, a cofactor for USP7[175]. Also, ubiquitin-mediated degradation, transcriptional regulation and posttranslational modifications play a role in regulating DUB activity[176,177].

Not surprisingly, like ubiquitination, DUBs have been implicated in regulating numerous cellular processes, like DNA repair, transcription, signalling and endocytosis. Regarding transcription, modification of histone proteins by acetylation alike, monoubiquitination of histone H2B modulates transcription. Apparently, dynamic H2B ubiquitination is required for optimal transcription[178]. Alternatively, USP7 regulates p53-mediated transcription by deubiquitinating and thereby stabilizing this transcription factor itself[179].

Ubiquitination of FOXOs has been observed in several studies. These studies all relate to lysine 48-linked polyubiquitination[180-182]. Initially, it was shown that FOXOs are targeted for polyubiquitination by growth factors through PI3K/PKB signaling[181,182]. However, in these studies the kinetics of polyubiquitination (detectable after ~12 hrs) as well as of protein degradation (half-life of ~9 hrs) are relatively slow compared to proteins like p53 and $p27^{kip1}$ that are regulated primarily by degradation (half-lives of less than one hour[183,184]). In chicken embryonic fibroblasts (CEFs) transformed through overexpression of activated forms of PI3K and/or PKB, platelet-derived growth factor treatment resulted in a strong reduction in FOXO half-life[185]. However, this is probably due to increased expression of the F-box protein Skp2 in these transformed CEFs since it has been shown that overexpression of Skp2 enhances FOXO polyubiquitination[180] and that loss of PTEN and thus activation of PKB enhances the expression of Skp2[186] (Figure 1). At present it is unknown whether lysine 48-linked polyubiquitination of FOXO is the only type of ubiquitin modification, or whether there are more ubiquitin or ubiquitin-like modifications. It is interesting to note here that several proteins that like FOXO can bind to DNA can be monoubiquitinated, e.g. FANCD2. As a result FANCD2 interacts stronger with chromatin[187].

Notably, ε-amino groups of lysines and α-amino groups of N-terminal residues of proteins can be modified by both acetylation and ubiquitination. These posttranslational modifications are mutually exclusive and obviously could therefore be interdependent. Indeed, acetylation of several proteins, e.g. Smad7 and p53, protects them from ubiquitin-mediated degradation[93,188]. As ubiquitination often is not specific to certain lysine residues, it remains to be seen whether competition between protein acetylation and ubiquitination is a wide-spread phenomenon.

Outline of this thesis

FOXO transcription factors play an important role in essential biological processes such as differentiation, proliferation, apoptosis, DNA repair, metabolism and stress resistance. Phosphorylation is the modification that was first found on FOXOs and much of the subsequent studies focused on this type of modification, studying the roles of kinases like PKB, SGK and JNK in the regulation of FOXO. However, proteins can also be modified and regulated by other posttranslational modifications like acetylation and ubiquitination. Only little is known about such modifications on FOXO.
This thesis describes our studies regarding posttranslational modifications of FOXO transcription factors and the consequences thereof at both the cellular and organismal level. In **Chapter 2** we provide an explanation for the increase in C.elegans lifespan by the longevity protein Sir2, which requires the worm FOXO-homologue DAF-16. We show that mammalian Sir2, also called SIRT1, directly binds to and deacetylates FOXO4 and thereby activates FOXO transcriptional activity both towards cell cycle regulation and stress resistance. Furthermore, we show that FOXOs are acetylated in response to oxidative stress by the acetyltransferase CBP **(Chapter 2)**. Sir2 activity is inhibited by nicotinamide, a product of the Sir2-mediated deacetylation reaction. Organisms from bacteria to humans have a so-called NAD^+-salvage pathway in which nicotinamide is converted. In C.elegans this job likely is performed by a nicotinamidase named PNC1, which converts nicotinamide into nicotinic acid. Potentially, by lowering the levels of nicotinamide, PNC1 increases organismal lifespan. Indeed, we found that RNAi-mediated knockdown of PNC1 decreases worm lifespan. On the contrary, overexpression of PNC1 enhanced the worm's oxidative stress resistance, which has been implicated in increasing lifespan **(Chapter 3)**.
In **Chapter 4** we show that oxidative stress also induces monoubiquitination of FOXO4. This monoubiquitination results in nuclear retention and increased transcriptional activity of FOXO4. The ubiquitin-moiety is cleaved from FOXO4 by the DUB USP7. As expected from the observed monoubiquitination neither oxidative stress nor USP7 affects FOXO4 half-life, which is about 9 hours.
cAMP has long been appreciated as a second messenger that can induce cell cycle arrest. Several models, concerning both MAPK and PI3K/PKB signalling, have been proposed to explain this phenomenon. In **Chapter 5** we demonstrate that in MEFs FOXO transcription factors, that are downstream of PI3K signalling, are required for the cAMP-mediated growth arrest and propose that this also applies to many other cell types.
To exert their diverse array of effects, FOXOs interact with other proteins. A screen to identify novel FOXO interacting transcription factors identified interferon (IFN) consensus sequence binding protein (ICSBP), nuclear factor of activated T-cells (NFATc), nuclear respiratory factor 1 (NRF1), Transcriptional Intermediary Factor 1 (TIF1), Sp1 and Homeobox D9/10 (HOXD9/10)**(Chapter 6)**. By coimmunoprecipitations we confirmed binding of ICSBP to FOXO4, which is enhanced both by oxidative stress and NFATc, another possible interactor of FOXO. NFATc and ICSBP enhance expression of interleukin 12 (IL-12) and we demonstrate that FOXO4 acts synergistically with NFATc and ICSBP to induce IL-12. IL-12 regulates T_H1 cell proliferation, so we hypothesise a novel role for FOXO4 in the adaptive immune response.

The consequences of the findings described in this thesis for our comprehension of the role of posttranslational modifications in the regulation of FOXO function in cellular and organismal behaviour are discussed in **Chapter 7**. We also propose an intriguing model in which FOXOs and p53 are compared regarding their roles in tumour suppression and aging.

References

1. Kaufmann, E. & Knochel, W. Five years on the wings of fork head. *Mech Dev* **57**, 3-20 (1996).
2. Burgering, B. M. & Kops, G. J. Cell cycle and death control: long live Forkheads. *Trends Biochem Sci* **27**, 352-60 (2002).
3. Jacobs, F. M. et al. FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics. *J Biol Chem* **278**, 35959-67 (2003).
4. Clark, K. L., Halay, E. D., Lai, E. & Burley, S. K. Co-crystal structure of the HNF-3/fork head DNA-recognition motif resembles histone H5. *Nature* **364**, 412-20 (1993).
5. Weigelt, J., Climent, I., Dahlman-Wright, K. & Wikstrom, M. Solution structure of the DNA binding domain of the human forkhead transcription factor AFX (FOXO4). *Biochemistry* **40**, 5861-9 (2001).
6. Furuyama, T., Nakazawa, T., Nakano, I. & Mori, N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* **349**, 629-34. (2000).
7. Kenyon, C., Chang, J., Gensch, E., Rudner, A. & Tabtiang, R. A C. elegans mutant that lives twice as long as wild type. *Nature* **366**, 461-4 (1993).
8. Tang, T. T. et al. The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. *J Biol Chem* **277**, 14255-65 (2002).
9. Dijkers, P. F., Medema, R. H., Lammers, J. W., Koenderman, L. & Coffer, P. J. Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* **10**, 1201-4 (2000).
10. Gilley, J., Coffer, P. J. & Ham, J. FOXO transcription factors directly activate bim gene expression and promote apoptosis in sympathetic neurons. *J Cell Biol* **162**, 613-22 (2003).
11. Brunet, A. et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-68. (1999).
12. Rokudai, S., Fujita, N., Kitahara, O., Nakamura, Y. & Tsuruo, T. Involvement of FKHR-dependent TRADD expression in chemotherapeutic drug-induced apoptosis. *Mol Cell Biol* **22**, 8695-708 (2002).
13. Modur, V., Nagarajan, R., Evers, B. M. & Milbrandt, J. FOXO proteins regulate tumour necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer. *J Biol Chem* **277**, 47928-37 (2002).
14. Bakker, W. J. et al. FoxO3a regulates erythroid differentiation and induces BTG1, an activator of protein arginine methyl transferase 1. *J Cell Biol* **164**, 175-84 (2004).
15. Alvarez, B., Martinez, A. C., Burgering, B. M. & Carrera, A. C. Forkhead transcription factors contribute to execution of the mitotic programme in mammals. *Nature* **413**, 744-7 (2001).
16. Schmidt, M. et al. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. *Mol Cell Biol* **22**, 7842-52 (2002).
17. Ramaswamy, S., Nakamura, N., Sansal, I., Bergeron, L. & Sellers, W. R. A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. *Cancer Cell* **2**, 81-91 (2002).

18. Martinez-Gac, L., Marques, M., Garcia, Z., Campanero, M. R. & Carrera, A. C. Control of cyclin G2 mRNA expression by forkhead transcription factors: novel mechanism for cell cycle control by phosphoinositide 3-kinase and forkhead. *Mol Cell Biol* **24**, 2181-9 (2004).
19. Kops, G. J. et al. Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors. *Mol Cell Biol* **22**, 2025-36 (2002).
20. Seoane, J., Le, H. V., Shen, L., Anderson, S. A. & Massague, J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* **117**, 211-23 (2004).
21. Medema, R. H., Kops, G. J., Bos, J. L. & Burgering, B. M. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782-7. (2000).
22. Junger, M. A. et al. The Drosophila forkhead transcription factor FOXO mediates the reduction in cell number associated with reduced insulin signaling. *J Biol* **2**, 20 (2003).
23. Puig, O., Marr, M. T., Ruhf, M. L. & Tjian, R. Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes Dev* **17**, 2006-20 (2003).
24. Furukawa-Hibi, Y., Yoshida-Araki, K., Ohta, T., Ikeda, K. & Motoyama, N. FOXO forkhead transcription factors induce G(2)-M checkpoint in response to oxidative stress. *J Biol Chem* **277**, 26729-32 (2002).
25. Tran, H. et al. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science* **296**, 530-4 (2002).
26. Mawal-Dewan, M. et al. Regulation of collagenase expression during replicative senescence in human fibroblasts by Akt-forkhead signaling. *J Biol Chem* **277**, 7857-64 (2002).
27. Kamei, Y. et al. A forkhead transcription factor FKHR up-regulates lipoprotein lipase expression in skeletal muscle. *FEBS Lett* **536**, 232-6 (2003).
28. Dansen, T. B. et al. Regulation of sterol carrier protein gene expression by the forkhead transcription factor FOXO3a. *J Lipid Res* **45**, 81-8 (2004).
29. Murakami, S. & Johnson, T. E. The OLD-1 positive regulator of longevity and stress resistance is under DAF-16 regulation in Caenorhabditis elegans. *Curr Biol* **11**, 1517-23 (2001).
30. Ookuma, S., Fukuda, M. & Nishida, E. Identification of a DAF-16 transcriptional target gene, scl-1, that regulates longevity and stress resistance in Caenorhabditis elegans. *Curr Biol* **13**, 427-31 (2003).
31. Nakae, J., Kitamura, T., Silver, D. L. & Accili, D. The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J Clin Invest* **108**, 1359-67 (2001).
32. Barthel, A. et al. Differential regulation of endogenous glucose-6-phosphatase and phosphoenolpyruvate carboxykinase gene expression by the forkhead transcription factor FKHR in H4IIE-hepatoma cells. *Biochem Biophys Res Commun* **285**, 897-902 (2001).
33. Nadal, A., Marrero, P. F. & Haro, D. Down-regulation of the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase gene by insulin: the role of the forkhead transcription factor FKHRL1. *Biochem J* **366**, 289-97 (2002).
34. Durham, S. K. et al. FKHR binds the insulin response element in the insulin-like growth factor binding protein-1 promoter. *Endocrinology* **140**, 3140-6 (1999).
35. Furuyama, T., Kitayama, K., Yamashita, H. & Mori, N. Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation. *Biochem J* **375**, 365-71 (2003).
36. Daitoku, H., Yamagata, K., Matsuzaki, H., Hatta, M. & Fukamizu, A. Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. *Diabetes* **52**, 642-9 (2003).

37. Sandri, M. et al. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. *Cell* **117**, 399-412 (2004).
38. Skurk, C. et al. The FOXO3a transcription factor regulates cardiac myocyte size downstream of AKT signaling. *J Biol Chem* **280**, 20814-23 (2005).
39. Stitt, T. N. et al. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. *Mol Cell* **14**, 395-403 (2004).
40. Nemoto, S. & Finkel, T. Redox regulation of forkhead proteins through a p66shc-dependent signaling pathway. *Science* **295**, 2450-2 (2002).
41. Ayyadevara, S. et al. Lifespan extension in hypomorphic daf-2 mutants of Caenorhabditis elegans is partially mediated by glutathione transferase CeGSTP2-2. *Aging Cell* **4**, 299-307 (2005).
42. Kitamura, Y. I. et al. FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction. *Cell Metab* **2**, 153-63 (2005).
43. Kops, G. J. et al. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* **419**, 316-21 (2002).
44. van den Heuvel, A. P., Schulze, A. & Burgering, B. M. Direct control of caveolin-1 expression by FOXO transcription factors. *Biochem J* **385**, 795-802 (2005).
45. Puig, O. & Tjian, R. Transcriptional feedback control of insulin receptor by dFOXO/FOXO1. *Genes Dev* **19**, 2435-46 (2005).
46. Tang, T. T. & Lasky, L. A. The forkhead transcription factor FOXO4 induces the down-regulation of hypoxia-inducible factor 1 alpha by a von Hippel-Lindau protein-independent mechanism. *J Biol Chem* **278**, 30125-35 (2003).
47. Nakae, J. et al. Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nat Genet* **32**, 245-53 (2002).
48. Galili, N. et al. Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. *Nat Genet* **5**, 230-5 (1993).
49. Davis, R. J., D'Cruz, C. M., Lovell, M. A., Biegel, J. A. & Barr, F. G. Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. *Cancer Res* **54**, 2869-72 (1994).
50. Borkhardt, A. et al. Cloning and characterization of AFX, the gene that fuses to MLL in acute leukemias with a t(X;11)(q13;q23). *Oncogene* **14**, 195-202 (1997).
51. So, C. W. & Cleary, M. L. MLL-AFX requires the transcriptional effector domains of AFX to transform myeloid progenitors and transdominantly interfere with forkhead protein function. *Mol Cell Biol* **22**, 6542-52 (2002).
52. Berndt, N. Roles and regulation of serine/threonine-specific protein phosphatases in the cell cycle. *Prog Cell Cycle Res* **5**, 497-510 (2003).
53. Rena, G., Guo, S., Cichy, S. C., Unterman, T. G. & Cohen, P. Phosphorylation of the transcription factor forkhead family member FKHR by protein kinase B. *J Biol Chem* **274**, 17179-83 (1999).
54. Biggs, W. H., 3rd, Meisenhelder, J., Hunter, T., Cavenee, W. K. & Arden, K. C. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc Natl Acad Sci U S A* **96**, 7421-6 (1999).
55. Kops, G. J. et al. Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**, 630-4 (1999).
56. van der Heide, L. P., Jacobs, F. M., Burbach, J. P., Hoekman, M. F. & Smidt, M. P. FoxO6 transcriptional activity is regulated by Thr26 and Ser184, independent of nucleo-cytoplasmic shuttling. *Biochem J* **391**, 623-9 (2005).
57. Fried, H. & Kutay, U. Nucleocytoplasmic transport: taking an inventory. *Cell Mol Life Sci* **60**, 1659-88 (2003).

58. Brownawell, A. M., Kops, G. J., Macara, I. G. & Burgering, B. M. Inhibition of nuclear import by protein kinase B (Akt) regulates the subcellular distribution and activity of the forkhead transcription factor AFX. *Mol Cell Biol* **21**, 3534-46 (2001).
59. Mingot, J. M., Bohnsack, M. T., Jakle, U. & Gorlich, D. Exportin 7 defines a novel general nuclear export pathway. *Embo J* **23**, 3227-36 (2004).
60. Brunet, A. et al. Protein kinase SGK mediates survival signals by phosphorylating the forkhead transcription factor FKHRL1 (FOXO3a). *Mol Cell Biol* **21**, 952-65 (2001).
61. Hertweck, M., Gobel, C. & Baumeister, R. C. elegans SGK-1 is the critical component in the Akt/PKB kinase complex to control stress response and life span. *Dev Cell* **6**, 577-88 (2004).
62. Rena, G. et al. Two novel phosphorylation sites on FKHR that are critical for its nuclear exclusion. *Embo J* **21**, 2263-71 (2002).
63. Woods, Y. L. et al. The kinase DYRK1A phosphorylates the transcription factor FKHR at Ser329 in vitro, a novel in vivo phosphorylation site. *Biochem J* **355**, 597-607 (2001).
64. von Groote-Bidlingmaier, F. et al. DYRK1 is a co-activator of FKHR (FOXO1a)-dependent glucose-6-phosphatase gene expression. *Biochem Biophys Res Commun* **300**, 764-9 (2003).
65. Rena, G., Prescott, A. R., Guo, S., Cohen, P. & Unterman, T. G. Roles of the forkhead in rhabdomyosarcoma (FKHR) phosphorylation sites in regulating 14-3-3 binding, transactivation and nuclear targetting. *Biochem J* **354**, 605-12 (2001).
66. Hu, M. C. et al. IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* **117**, 225-37 (2004).
67. Nishiyama, T., Kii, I. & Kudo, A. Inactivation of Rho/ROCK signaling is crucial for the nuclear accumulation of FKHR and myoblast fusion. *J Biol Chem* **279**, 47311-9 (2004).
68. Bois, P. R. & Grosveld, G. C. FKHR (FOXO1a) is required for myotube fusion of primary mouse myoblasts. *Embo J* **22**, 1147-57 (2003).
69. Hribal, M. L., Nakae, J., Kitamura, T., Shutter, J. R. & Accili, D. Regulation of insulin-like growth factor-dependent myoblast differentiation by Foxo forkhead transcription factors. *J Cell Biol* **162**, 535-41 (2003).
70. Barthel, A., Schmoll, D., Kruger, K. D., Roth, R. A. & Joost, H. G. Regulation of the forkhead transcription factor FKHR (FOXO1a) by glucose starvation and AICAR, an activator of AMP-activated protein kinase. *Endocrinology* **143**, 3183-6 (2002).
71. De Ruiter, N. D., Burgering, B. M. & Bos, J. L. Regulation of the Forkhead transcription factor AFX by Ral-dependent phosphorylation of threonines 447 and 451. *Mol Cell Biol* **21**, 8225-35 (2001).
72. Essers, M. A. et al. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *Embo J* **23**, 4802-12 (2004).
73. Oh, S. W. et al. JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proc Natl Acad Sci U S A* **102**, 4494-9 (2005).
74. Wang, M. C., Bohmann, D. & Jasper, H. JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. *Cell* **121**, 115-25 (2005).
75. Legube, G. & Trouche, D. Regulating histone acetyltransferases and deacetylases. *EMBO Rep* **4**, 944-7 (2003).
76. Polevoda, B. & Sherman, F. Nalpha -terminal acetylation of eukaryotic proteins. *J Biol Chem* **275**, 36479-82 (2000).
77. Yang, X. J. The diverse superfamily of lysine acetyltransferases and their roles in leukemia and other diseases. *Nucleic Acids Res* **32**, 959-76 (2004).
78. Kouzarides, T. Acetylation: a regulatory modification to rival phosphorylation? *Embo J* **19**, 1176-9 (2000).

79. Kalkhoven, E. CBP and p300: HATs for different occasions. *Biochem Pharmacol* **68**, 1145-55 (2004).
80. Iyer, N. G., Ozdag, H. & Caldas, C. p300/CBP and cancer. *Oncogene* **23**, 4225-31 (2004).
81. Kaida, A. et al. Identification of a novel p300-specific-associating protein, PRS1 (phosphor ibosylpyrophosphate synthetase subunit 1). *Biochem J* **391**, 239-47 (2005).
82. Parker, D. et al. Phosphorylation of CREB at Ser-133 induces complex formation with CREB-binding protein via a direct mechanism. *Mol Cell Biol* **16**, 694-703 (1996).
83. Dhalluin, C. et al. Structure and ligand of a histone acetyltransferase bromodomain. *Nature* **399**, 491-6 (1999).
84. Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H. & Nakatani, Y. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**, 319-24 (1996).
85. Herrera, J. E., Bergel, M., Yang, X. J., Nakatani, Y. & Bustin, M. The histone acetyltransferase activity of human GCN5 and PCAF is stabilized by coenzymes. *J Biol Chem* **272**, 27253-8 (1997).
86. Hamamori, Y. et al. Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. *Cell* **96**, 405-13 (1999).
87. Kawasaki, H. et al. ATF-2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation. *Nature* **405**, 195-200 (2000).
88. Chen, H., Lin, R. J., Xie, W., Wilpitz, D. & Evans, R. M. Regulation of hormone-induced histone hyperacetylation and gene activation via acetylation of an acetylase. *Cell* **98**, 675-86 (1999).
89. Poizat, C., Sartorelli, V., Chung, G., Kloner, R. A. & Kedes, L. Proteasome-mediated degradation of the coactivator p300 impairs cardiac transcription. *Mol Cell Biol* **20**, 8643-54 (2000).
90. Girdwood, D. et al. P300 transcriptional repression is mediated by SUMO modification. *Mol Cell* **11**, 1043-54 (2003).
91. Frank, S. R. et al. MYC recruits the TIP60 histone acetyltransferase complex to chromatin. *EMBO Rep* **4**, 575-80 (2003).
92. Chen, L. & Widom, J. Mechanism of transcriptional silencing in yeast. *Cell* **120**, 37-48 (2005).
93. Gronroos, E., Hellman, U., Heldin, C. H. & Ericsson, J. Control of Smad7 stability by competition between acetylation and ubiquitination. *Mol Cell* **10**, 483-93 (2002).
94. Sakaguchi, K. et al. DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev* **12**, 2831-41 (1998).
95. Bereshchenko, O. R., Gu, W. & Dalla-Favera, R. Acetylation inactivates the transcriptional repressor BCL6. *Nat Genet* **32**, 606-13 (2002).
96. Kawaguchi, Y., Ito, A., Appella, E. & Yao, T. P. Charge modification at multiple C-terminal lysine residues regulates p53 oligomerization and its nucleus-cytoplasm trafficking. *J Biol Chem* (2005).
97. de Ruijter, A. J., van Gennip, A. H., Caron, H. N., Kemp, S. & van Kuilenburg, A. B. Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J* **370**, 737-49 (2003).
98. Blander, G. & Guarente, L. The Sir2 family of protein deacetylases. *Annu Rev Biochem* **73**, 417-35 (2004).
99. Bertos, N. R., Wang, A. H. & Yang, X. J. Class II histone deacetylases: structure, function, and regulation. *Biochem Cell Biol* **79**, 243-52 (2001).
100. Marmorstein, R. Structure of histone deacetylases: insights into substrate recognition and catalysis. *Structure* **9**, 1127-33 (2001).
101. Yoshida, M., Kijima, M., Akita, M. & Beppu, T. Potent and specific inhibition of mammalian histone deacetylase both in vivo and in vitro by trichostatin A. *J Biol Chem* **265**, 17174-9 (1990).

102. Bjerling, P. et al. Functional divergence between histone deacetylases in fission yeast by distinct cellular localization and in vivo specificity. *Mol Cell Biol* **22**, 2170-81 (2002).
103. Gregoretti, I. V., Lee, Y. M. & Goodson, H. V. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. *J Mol Biol* **338**, 17-31 (2004).
104. Brachmann, C. B. et al. The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. *Genes Dev* **9**, 2888-902 (1995).
105. Frye, R. A. Phylogenetic classification of prokaryotic and eukaryotic Sir2-like proteins. *Biochem Biophys Res Commun* **273**, 793-8 (2000).
106. Frye, R. A. Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity. *Biochem Biophys Res Commun* **260**, 273-9 (1999).
107. Tanny, J. C. & Moazed, D. Coupling of histone deacetylation to NAD breakdown by the yeast silencing protein Sir2: Evidence for acetyl transfer from substrate to an NAD breakdown product. *Proc Natl Acad Sci U S A* **98**, 415-20 (2001).
108. Tanner, K. G., Landry, J., Sternglanz, R. & Denu, J. M. Silent information regulator 2 family of NAD- dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. *Proc Natl Acad Sci U S A* **97**, 14178-82 (2000).
109. Liou, G. G., Tanny, J. C., Kruger, R. G., Walz, T. & Moazed, D. Assembly of the SIR complex and its regulation by O-acetyl-ADP-ribose, a product of NAD-dependent histone deacetylation. *Cell* **121**, 515-27 (2005).
110. Rine, J. & Herskowitz, I. Four genes responsible for a position effect on expression from HML and HMR in Saccharomyces cerevisiae. *Genetics* **116**, 9-22 (1987).
111. Gottschling, D. E., Aparicio, O. M., Billington, B. L. & Zakian, V. A. Position effect at S. cerevisiae telomeres: reversible repression of Pol II transcription. *Cell* **63**, 751-62 (1990).
112. Kaeberlein, M., McVey, M. & Guarente, L. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. *Genes Dev* **13**, 2570-80. (1999).
113. Tissenbaum, H. A. & Guarente, L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature* **410**, 227-30. (2001).
114. Rogina, B. & Helfand, S. L. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci U S A* **101**, 15998-6003 (2004).
115. Lin, S. J., Ford, E., Haigis, M., Liszt, G. & Guarente, L. Calorie restriction extends yeast life span by lowering the level of NADH. *Genes Dev* **18**, 12-6 (2004).
116. Anderson, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O. & Sinclair, D. A. Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. *Nature* **423**, 181-5 (2003).
117. Cohen, H. Y. et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* **305**, 390-2 (2004).
118. Ford, J., Jiang, M. & Milner, J. Cancer-specific functions of SIRT1 enable human epithelial cancer cell growth and survival. *Cancer Res* **65**, 10457-63 (2005).
119. Howitz, K. T. et al. Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature* **425**, 191-6 (2003).
120. Wood, J. G. et al. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* **430**, 686-9 (2004).
121. Min, J., Landry, J., Sternglanz, R. & Xu, R. M. Crystal structure of a SIR2 homolog-NAD complex. *Cell* **105**, 269-79 (2001).
122. Avalos, J. L. et al. Structure of a Sir2 enzyme bound to an acetylated p53 peptide. *Mol Cell* **10**, 523-35 (2002).
123. Blander, G. et al. SIRT1 shows no substrate specificity in vitro. *J Biol Chem* **280**, 9780-5 (2005).

124. Brunet, A. et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* **303**, 2011-5 (2004).
125. Daitoku, H. et al. Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. *Proc Natl Acad Sci U S A* **101**, 10042-7 (2004).
126. Motta, M. C. et al. Mammalian SIRT1 represses forkhead transcription factors. *Cell* **116**, 551-63 (2004).
127. van der Horst, A. et al. FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). *J Biol Chem* **279**, 28873-9 (2004).
128. Kobayashi, Y. et al. SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative stress. *Int J Mol Med* **16**, 237-43 (2005).
129. Yeung, F. et al. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *Embo J* **23**, 2369-80 (2004).
130. Luo, J. et al. Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* **107**, 137-48. (2001).
131. Vaziri, H. et al. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* **107**, 149-59. (2001).
132. Zhao, X., Sternsdorf, T., Bolger, T. A., Evans, R. M. & Yao, T. P. Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications. *Mol Cell Biol* **25**, 8456-64 (2005).
133. Fulco, M. et al. Sir2 regulates skeletal muscle differentiation as a potential sensor of the redox state. *Mol Cell* **12**, 51-62 (2003).
134. Picard, F. et al. Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. *Nature* **429**, 771-6 (2004).
135. Nemoto, S., Fergusson, M. M. & Finkel, T. SIRT1 functionally interacts with the metabolic regulator and transcriptional coactivator PGC-1{alpha}. *J Biol Chem* **280**, 16456-60 (2005).
136. Rodgers, J. T. et al. Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* **434**, 113-8 (2005).
137. Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D. & Escalante-Semerena, J. C. Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science* **298**, 2390-2 (2002).
138. Bell, S. D., Botting, C. H., Wardleworth, B. N., Jackson, S. P. & White, M. F. The interaction of Alba, a conserved archaeal chromatin protein, with Sir2 and its regulation by acetylation. *Science* **296**, 148-51 (2002).
139. Bouras, T. et al. SIRT1 deacetylation and repression of p300 involves lysine residues 1020/1024 within the cell cycle regulatory domain 1. *J Biol Chem* **280**, 10264-76 (2005).
140. Muth, V., Nadaud, S., Grummt, I. & Voit, R. Acetylation of TAF(I)68, a subunit of TIF-IB/SL1, activates RNA polymerase I transcription. *Embo J* **20**, 1353-62 (2001).
141. Takata, T. & Ishikawa, F. Human Sir2-related protein SIRT1 associates with the bHLH repressors HES1 and HEY2 and is involved in HES1- and HEY2-mediated transcriptional repression. *Biochem Biophys Res Commun* **301**, 250-7 (2003).
142. Rosenberg, M. I. & Parkhurst, S. M. Drosophila Sir2 is required for heterochromatic silencing and by euchromatic Hairy/E(Spl) bHLH repressors in segmentation and sex determination. *Cell* **109**, 447-58 (2002).
143. Senawong, T. et al. Involvement of the histone deacetylase SIRT1 in chicken ovalbumin upstream promoter transcription factor (COUP-TF)-interacting protein 2-mediated transcriptional repression. *J Biol Chem* **278**, 43041-50 (2003).
144. Moretti, P. & Shore, D. Multiple interactions in Sir protein recruitment by Rap1p at silencers and telomeres in yeast. *Mol Cell Biol* **21**, 8082-94 (2001).
145. Lee, S. E., Paques, F., Sylvan, J. & Haber, J. E. Role of yeast SIR genes and mating type in directing DNA double-strand breaks to homologous and non-homologous repair paths. *Curr Biol* **9**, 767-70 (1999).

146. Pappas, D. L., Jr., Frisch, R. & Weinreich, M. The NAD(+)-dependent Sir2p histone deacetylase is a negative regulator of chromosomal DNA replication. *Genes Dev* **18**, 769-81 (2004).
147. Lemieux, M. E. et al. The Sirt1 deacetylase modulates the insulin-like growth factor signaling pathway in mammals. *Mech Ageing Dev* **126**, 1097-105 (2005).
148. Bordone, L. et al. Sirt1 Regulates Insulin Secretion by Repressing UCP2 in Pancreatic beta Cells. *PLoS Biol* **4**, e31 (2005).
149. Hoppe, G. J. et al. Steps in assembly of silent chromatin in yeast: Sir3-independent binding of a Sir2/Sir4 complex to silencers and role for Sir2-dependent deacetylation. *Mol Cell Biol* **22**, 4167-80 (2002).
150. Perrot, V. & Rechler, M. M. The coactivator p300 directly acetylates the forkhead transcription factor Foxo1 and stimulates Foxo1-induced transcription. *Mol Endocrinol* **19**, 2283-98 (2005).
151. Yang, Y., Hou, H., Haller, E. M., Nicosia, S. V. & Bai, W. Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation. *Embo J* **24**, 1021-32 (2005).
152. Mahmud, D. L. et al. Phosphorylation of forkhead transcription factors by erythropoietin and stem cell factor prevents acetylation and their interaction with coactivator p300 in erythroid progenitor cells. *Oncogene* **21**, 1556-62. (2002).
153. Fukuoka, M. et al. Negative regulation of forkhead transcription factor AFX (Foxo4) by CBP-induced acetylation. *Int J Mol Med* **12**, 503-8. (2003).
154. Andersen, K. M., Hofmann, K. & Hartmann-Petersen, R. Ubiquitin-binding proteins: similar, but different. *Essays Biochem* **41**, 49-67 (2005).
155. Pickart, C. M. Mechanisms underlying ubiquitination. *Annu Rev Biochem* **70**, 503-33 (2001).
156. Koegl, M. et al. A novel ubiquitination factor, E4, is involved in multiubiquitin chain assembly. *Cell* **96**, 635-44 (1999).
157. Chen, P., Johnson, P., Sommer, T., Jentsch, S. & Hochstrasser, M. Multiple ubiquitin-conjugating enzymes participate in the in vivo degradation of the yeast MAT alpha 2 repressor. *Cell* **74**, 357-69 (1993).
158. Hofmann, R. M. & Pickart, C. M. Noncanonical MMS2-encoded ubiquitin-conjugating enzyme functions in assembly of novel polyubiquitin chains for DNA repair. *Cell* **96**, 645-53 (1999).
159. Pickart, C. M. & Eddins, M. J. Ubiquitin: structures, functions, mechanisms. *Biochim Biophys Acta* **1695**, 55-72 (2004).
160. Huibregtse, J. M., Scheffner, M. & Howley, P. M. E6-AP directs the HPV E6-dependent inactivation of p53 and is representative of a family of structurally and functionally related proteins. *Cold Spring Harb Symp Quant Biol* **59**, 237-45 (1994).
161. Huang, L. et al. Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science* **286**, 1321-6 (1999).
162. Borden, K. L. RING domains: master builders of molecular scaffolds? *J Mol Biol* **295**, 1103-12 (2000).
163. Zheng, N. et al. Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. *Nature* **416**, 703-9 (2002).
164. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H. & Zhang, H. p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol* **9**, 661-4 (1999).
165. Yaron, A. et al. Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. *Nature* **396**, 590-4 (1998).
166. Fang, S. & Weissman, A. M. A field guide to ubiquitylation. *Cell Mol Life Sci* **61**, 1546-61 (2004).
167. Nijman, S. M. et al. A genomic and functional inventory of deubiquitinating enzymes. *Cell* **123**, 773-86 (2005).

168. Johnston, S. C., Larsen, C. N., Cook, W. J., Wilkinson, K. D. & Hill, C. P. Crystal structure of a deubiquitinating enzyme (human UCH-L3) at 1.8 A resolution. *Embo J* **16**, 3787-96 (1997).
169. Amerik, A. Y. & Hochstrasser, M. Mechanism and function of deubiquitinating enzymes. *Biochim Biophys Acta* **1695**, 189-207 (2004).
170. Nanao, M. H. et al. Crystal structure of human otubain 2. *EMBO Rep* **5**, 783-8 (2004).
171. Goodrich, J. S., Clouse, K. N. & Schupbach, T. Hrb27C, Sqd and Otu cooperatively regulate gurken RNA localization and mediate nurse cell chromosome dispersion in Drosophila oogenesis. *Development* **131**, 1949-58 (2004).
172. Li, F., Macfarlan, T., Pittman, R. N. & Chakravarti, D. Ataxin-3 is a histone-binding protein with two independent transcriptional corepressor activities. *J Biol Chem* **277**, 45004-12 (2002).
173. Kovalenko, A. et al. The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. *Nature* **424**, 801-5 (2003).
174. Lin, H., Yin, L., Reid, J., Wilkinson, K. D. & Wing, S. S. Divergent N-terminal sequences of a deubiquitinating enzyme modulate substrate specificity. *J Biol Chem* **276**, 20357-63 (2001).
175. van der Knaap, J. A. et al. GMP synthetase stimulates histone H2B deubiquitylation by the epigenetic silencer USP7. *Mol Cell* **17**, 695-707 (2005).
176. Nijman, S. M. et al. The deubiquitinating enzyme USP1 regulates the Fanconi anemia pathway. *Mol Cell* **17**, 331-9 (2005).
177. Reiley, W., Zhang, M., Wu, X., Granger, E. & Sun, S. C. Regulation of the deubiquitinating enzyme CYLD by IkappaB kinase gamma-dependent phosphorylation. *Mol Cell Biol* **25**, 3886-95 (2005).
178. Henry, K. W. et al. Transcriptional activation via sequential histone H2B ubiquitylation and deubiquitylation, mediated by SAGA-associated Ubp8. *Genes Dev* **17**, 2648-63 (2003).
179. Li, M. et al. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* **416**, 648-53 (2002).
180. Huang, H. et al. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. *Proc Natl Acad Sci U S A* **102**, 1649-54 (2005).
181. Matsuzaki, H., Daitoku, H., Hatta, M., Tanaka, K. & Fukamizu, A. Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proc Natl Acad Sci U S A* **100**, 11285-90 (2003).
182. Plas, D. R. & Thompson, C. B. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. *J Biol Chem* **278**, 12361-6 (2003).
183. Reich, N. C., Oren, M. & Levine, A. J. Two distinct mechanisms regulate the levels of a cellular tumor antigen, p53. *Mol Cell Biol* **3**, 2143-50 (1983).
184. Pagano, M. et al. Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. *Science* **269**, 682-5 (1995).
185. Aoki, M., Jiang, H. & Vogt, P. K. Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. *Proc Natl Acad Sci U S A* **101**, 13613-7 (2004).
186. Mamillapalli, R. et al. PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2). *Curr Biol* **11**, 263-7 (2001).
187. Wang, X., Andreassen, P. R. & D'Andrea, A. D. Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. *Mol Cell Biol* **24**, 5850-62 (2004).
188. Ito, A. et al. MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. *Embo J* **21**, 6236-45 (2002).
189. Essers, M. *Forkhead Box O and the Control of Cellular Oxidative Stress*. Thesis (2004).

CHAPTER

2

FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2^{SIRT1}

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FOXO4 Is Acetylated upon Peroxide Stress and Deacetylated by the Longevity Protein hSir2^{SIRT1}*

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FOXO transcription factors have important roles in metabolism, cellular proliferation, stress tolerance, and aging. FOXOs are negatively regulated by protein kinase B/c-Akt-mediated phosphorylation. Here we show that FOXO factors are also subject to regulation by reversible acetylation. We provide evidence that the acetyltransferase CREB-binding protein (CBP) binds FOXO resulting in acetylation of FOXO. This acetylation inhibits FOXO transcriptional activity. Binding of CBP and acetylation are induced after treatment of cells with peroxide stress. Deacetylation of FOXOs involves binding of the NAD-dependent deacetylase hSir2^{SIRT1}. Accordingly, hSir2^{SIRT1}-mediated deacetylation precludes FOXO inhibition through acetylation and thereby prolongs FOXO-dependent transcription of stress-regulating genes. These data demonstrate that acetylation functions in a second pathway of negative control for FOXO factors and provides a novel mechanism whereby hSir2^{SIRT1} can promote cellular survival and increase lifespan.

The Forkhead box, class O subfamily of forkhead transcription factors (FOXO)[1] consists of the functionally related proteins FOXO1, FOXO3a, and FOXO4 (also known as FKHR, FKHRL1, and AFX, respectively; Ref. 1). The growth factor-stimulated phosphatidylinositol 3-kinase-protein kinase B (PKB)/c-Akt pathway negatively regulates FOXO factors by phosphorylation-mediated nuclear exclusion (2–4). This pathway is evolutionarily conserved between *Caenorhabditis elegans* and humans. DAF-16, the *C. elegans* homologue of mammalian FOXO, is also controlled by phosphatidylinositol 3-kinase/PKB signaling. DAF-16 regulates daver formation in larvae, and responses to various environmental stresses and longevity in adult worms (5–8). In parallel, mammalian FOXO transcription factors have been implicated in regulating metabolism, cell cycle progression, and stress tolerance (9, 10; reviewed in Ref. 11).

In *C. elegans*, overexpression of the NAD-dependent deacetylase Sir2 (silent information regulator 2) increases lifespan, which requires DAF-16 (12). The *Sir2* family of genes is a highly conserved group of genes with seven human homologues, of which the *SIRT1* gene encodes the closest homologue of yeast and *C. elegans* Sir2, hence named hSir2^{SIRT1} (13). Recently, deacetylation of p53 by hSir2^{SIRT1} has been demonstrated, and it has been suggested that this functions in increasing cellular resistance against stress. However, subsequent studies (14) showed that in HEK293T cells, which lack functional p53, activation of hSir2^{SIRT1} by resveratrol treatment still increases cellular resistance against gamma-radiation, thus suggesting alternative pathways. As DAF-16 is necessary for lifespan extension by Sir2, FOXOs may well function in such an alternative pathway.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfection, and Treatment—HEK293T, DL23 (DLD-1 human colon carcinoma cells expressing a conditionally active version of FOXO3a; Ref. 10), and A14 cells (human insulin receptor overexpressing mouse NIH3T3 cells (15) and C2C12 mouse myoblasts were maintained in Dulbecco's modified Eagle's medium supplemented with glutamine, penicillin/streptomycin, and 10% fetal bovine serum. In all experiments, cells were cultured in the presence of 10% fetal bovine serum, unless stated otherwise. HEK293T cells were transiently transfected using FuGENE6 reagent according to the manufacturer's suggestions (Roche Applied Science). A14 cells were transfected using the calcium phosphate method. Total amounts of transfected DNA were equalized using pBluescript II KS(+). 1 μM trichostatin A (ICN), 5 mM nicotinamide (Nam, Supelco), 1 μg/ml insulin, 20 ng/ml epidermal growth factor (EGF), 500 nM 4-hydroxy-tamoxifen (4-OHT), and 20–500 μM hydrogen peroxide (H_2O_2, Merck) were added as indicated. All experiments were performed at least three times and representative results are shown.

Plasmids, Oligonucleotides, and Recombinant Proteins—pMT2-HA-FOXO4, pRP261-GST-FOXO4-DB (3), pcDNA3-HA-FOXO3a.A3 (10), pcDNA3.1-hSir2^{SIRT1} (16), pBabe-puro (17), 6× DBE (18), and p27GL-1609 (19) have been described before. pcDNA3.1-myc-FOXO4 was created by ligating a Klenow-blunted SalI/NotI fragment from pMT2-HA-FOXO4 into Klenow-blunted BamHI/NotI digested pcDNA3.1-myc. GLOFLAG3-FLAG-FOXO4 was created by ligating a Klenow-blunted SalI/NotI fragment from pMT2-HA-FOXO4 into mungbean-blunted BamHI/NotI-digested GLOFLAG3. pcDNA3.1-myc-hSir2^{SIRT1} and pRP265-GST-hSir2^{SIRT1} were created by ligating a BamHI/XbaI fragment from pcDNA3.1-hSir2^{SIRT1} into BamHI/XbaI-digested pcDNA3.1-myc and pRP265, respectively.

pRc/RSV-CBP-HA and pRP265-GST-p300-HAT were kindly provided by R. Giles (20) and R. Vries (21), respectively. Gal4-DBD-CBP-HA constructs were a kind gift of E. Kalkhoven (22). pRL-TK (Tk-Renilla luciferase) was purchased from Promega.

siRNA duplexes to down-regulate hSir2^{SIRT1} expression (duplex 1,

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[1] The abbreviations used are: FOXO, Forkhead box, class O subfamily of forkhead transcription factors; Nam, nicotinamide; EGF, epidermal growth factor; 4-OHT, 4-hydroxy-tamoxifen; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; MnSOD, manganese superoxide dismutase; HA, hemagglutinin; PKB, protein kinase B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, short interfering RNA; DBE, Daf-16 family protein binding element; RIPA, radioimmune precipitation assay buffer.

sense sequence, CAACUUGUACGACGAAGACdTdT; duplex 2, sense sequence, GCUGAUGAACCGCUUGCUAdTdT; duplex 3, sense sequence, CGUCUUAUCCUCUAGUUCUdTdT) were purchased from Dharmacon RNA Technologies and cotransfected using OligofectAMINE according to the manufacturer (Invitrogen).

GST-FOXO4-DB, GST-p300-HAT, and GST-tagged hSir2^{SIRT1} were purified from bacteria using a standard GST-fusion protein-purification protocol (23).

Antibodies—Monoclonal 12CA5 and 9E10 antibodies were produced using hybridoma cell lines. Monoclonal antibodies recognizing the FLAG-M2 epitope, p27^{kip1}, and GAPDH were obtained from Sigma, Transduction Laboratories, and Chemicon, respectively. Polyclonal antibodies recognizing acetylated lysine residues (α-AcLys), actin, CBP, Gal4-DBD, MnSOD, and pS473-PKB were obtained from Cell Signaling Technologies, Santa Cruz Biotechnology (3×), Stressgen BioReagents, and New England Biolabs, respectively. Polyclonal antibodies recognizing FOXO3a were purchased from Sigma and Santa Cruz Biotechnology. Polyclonal antibodies recognizing FOXO4 (19) and PKB (15) have been described before. Polyclonal antibody recognizing hSir2^{SIRT1} was raised in a rabbit using an N-terminally ^{6}His-tagged fragment consisting of amino acids 506–747 of hSir2^{SIRT1}.

Western Blot Analysis—Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon). Western blot analysis was performed under standard conditions using the indicated antibodies.

FOXO Activity—To determine the expression of endogenous p27^{kip1} and MnSOD, HEK293T cells were transfected with empty vector or HA-FOXO4 together with pBabe-puro. Either constructs encoding Gal4-DBD-CBP-HA or myc-hSir^{SIRT1} were cotransfected or cells were treated using hydrogen peroxide or Nam for twenty-four hours. Twenty-four hours before harvest, 1 μg/ml puromycin was added to the cultures to select for transfected cells. Cells were lysed in 1× Laemmli sample buffer, and samples were analyzed by Western blot analysis. If indicated, DL23 cells treated for twenty-four hours with 4-OHT and/or Nam were used.

For luciferase assays, either A14 cells transiently transfected with a reporter construct bearing six canonical FOXO binding sites (6× DBE-luciferase) or a reporter cell line (A14-p27luc) stably expressing the human p27^{kip1}-promoter linked to luciferase (p27GL-1609) were used. Cells were treated as above. Luciferase counts were normalized using Tk-Renilla-luciferase. Samples were analyzed according to the manufacturer's instructions (Promega). To control for effects on basal transcription, the fold induction of luciferase expression was determined by dividing luciferase ratios from samples transfected with FOXO4 by the ratios from parallel samples transfected with empty vector. Statistical analysis was performed using a Student's t test. $p < 0.05$ was considered significant.

Flow cytometric analysis to determine the percentage of cells in the G_1-phase or S-phase of the cell cycle was essentially done as described (3). Cells were either transfected with HA-FOXO3a.A3 and hSir2^{SIRT1} or treated with 4-OHT (500 nM) and/or Nam (5 mM). Statistical analysis was performed using a Student's t test. $p < 0.05$ was considered significant.

For the measurement of reactive oxygen species, DL23 and A14 cells were loaded with 10 μg/ml CM-H_2DCFDA probe (Molecular Probes) in HEPES buffered saline for 5 min at 37 °C. Cells were incubated in Dulbecco's modified Eagle's medium containing 10 mM HEPES, 10 mM bicarbonate, pH 7.2, without phenol red at 37 °C and treated with 150 μM H_2O_2. Fluorescence was collected with a Leica DMR microscope. Excitation was done with a monochromator (SPEX Industries) at 490 nm (slit = 8 nm), >515 nm dichroic mirror, and a >515 nm emission filter. Ultra-low light conditions were applied only during image collection, under which no photo-activation of the cellular H_2DCFDA could be observed. Images were collected by integration of 20 frames at 10-s interval (fixed gain, intensifier/camera/grabber, during all experiments). Off-line analysis was done by quantification of the average pixel intensities of at least five individual cells/image during time in at least two separate experiments. Statistical analysis was performed using a Student's t test. $p < 0.05$ was considered significant.

Co-immunoprecipitation—For exogenous proteins, HEK293T cells were transfected with the indicated constructs. For CBP/FOXO4 co-immunoprecipitations, cells were left untreated or were treated with insulin plus EGF or hydrogen peroxide only for 30 min (see Fig. 4*A*) or 60 min (see Fig. 4*C*) or with insulin plus EGF for 30 min before adding hydrogen peroxide for 30 min (see Fig. 4*A*, as indicated). For hSir2^{SIRT1}/FOXO4 co-immunoprecipitations, cells were treated with Nam (5 mM) or hydrogen peroxide (200 μM) as indicated. Forty hours after transfection, cells were lysed in RIPA buffer. Immunoprecipitation samples were analyzed by Western blot analysis. For endogenous proteins, C2C12 or HEK293T cells were treated using 500 μM H_2O_2 for 1 h and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 5% glycerol, 5 mM EDTA, 100 mM NaCl, protease inhibitors). Immunoprecipitations were carried out essentially as described for exogenous proteins.

Acetylation—HEK293T cells were transfected with the indicated constructs. Cells were treated with Nam for 1 h or with hydrogen peroxide for the indicated times. Forty hours after transfection, cells were lysed in acetylation buffer (50 mM Tris-HCl, pH 7.5, 0.4% Triton X-100, 5 mM EDTA, 100 mM NaCl, protease inhibitors) or RIPA buffer. FOXO4 immunoprecipitation samples were analyzed by Western blot analysis.

In Vitro and in Vivo Deacetylation—For *in vitro* deacetylation, 2.5 μg GST-FOXO4-DB were incubated with 0.05 μCi [^{14}C]-acetyl-CoA (ICN) and 2.0 μg GST-p300-HAT in reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 0.5% Nonidet P-40, 150 mM NaCl (21) for 45 min at 30 °C. Subsequently, deacetylation buffer (50 mM Tris-HCl, pH 8.8, 4 mM $MgCl_2$, 0.2 mM dithiothreitol, 2 μM trichostatin A plus 1 mM NAD^+ if indicated (24) and 2.0 μg of GST-tagged wt-hSir2^{SIRT1} were added to a final volume of 100 μl. Samples were incubated for another 90 min at 37 °C, and the reaction was terminated using 5× Laemmli sample buffer. Samples were separated by SDS-PAGE and analyzed by autoradiography.

For *in vivo* deacetylation, HEK293T cells were transfected with the indicated constructs. Forty hours posttransfection cells were lysed in RIPA buffer, and myc-FOXO4 was immunoprecipitated from cleared lysates using 9E10 antibody. Immunoprecipitates and total lysates were subjected to Western blotting.

RESULTS

Nicotinamide Inhibits FOXO Transcriptional Activity—To investigate the possibility that hSir2^{SIRT1} controls FOXO function, we analyzed the effect of Nam, an inhibitor of the Sir2 family of deacetylases (25), on FOXO4 transcriptional activity. Nam inhibited FOXO4-mediated transcription of a luciferase reporter construct bearing six canonical FOXO DNA-binding sites (Fig. 1*A*; Ref. 18). Previously, we have shown that FOXO transcriptionally regulates the expression of the cell-cycle inhibitor p27^{kip1} (19). In support of this, expression of FOXO4 in HEK293T cells increased p27^{kip1} expression (Fig. 1*B*). Importantly, Nam inhibited the expression of the endogenous *p27^{kip1}* gene induced by FOXO4 (Fig. 1*B*). Inhibition of p27^{kip1} expression by Nam in these cells was not due to PKB activation, as Nam treatment under these conditions did not change phosphorylation of PKB on Ser-473, which is indicative of PKB activity. Also, in A14 cells stably expressing the luciferase gene driven by the p27^{kip1} promoter (A14-p27luc), FOXO4-induced luciferase expression was reduced by Nam (data not shown). These data demonstrate that the Sir2-inhibitor Nam inhibits FOXO4 transcriptional activity, possibly via hSir2^{SIRT1}.

Next we investigated whether Nam also affects the biological activity of FOXO transcription factors. Previously, we have employed DL23 cells, DLD-1 human colon carcinoma cells expressing an inducible conditionally active version of FOXO3a and lacking functional p53, and demonstrated in these cells transcriptional regulation of p27^{kip1} expression and induction of a G_1 cell-cycle arrest by FOXO (10). Treatment of DL23 cells with 4-OHT induced p27^{kip1} expression (Fig. 1*C*, *left panel*). This induction was reduced by treatment of these cells with Nam (Fig. 1*C*, *left panel*). Furthermore, FOXO3a activation imposed a cell-cycle arrest, which was partially relieved by treatment with Nam, in keeping with its effect on p27^{kip1} expression (Fig. 1*C*, *right panel*). In addition to imposing a cell-cycle arrest, FOXO activation increases cellular resistance against oxidative stress by transcriptional regulation of the gene encoding manganese superoxide dismutase (MnSOD) (10). Activation of FOXO in DL23 cells by 4-OHT treatment increased MnSOD expression, and, similar to FOXO-induced p27^{kip1} expression, MnSOD levels were reduced in cells treated with Nam (Fig. 1*C*, *left panel*). In keeping with MnSOD mediating FOXO-induced cellular resistance against oxidative

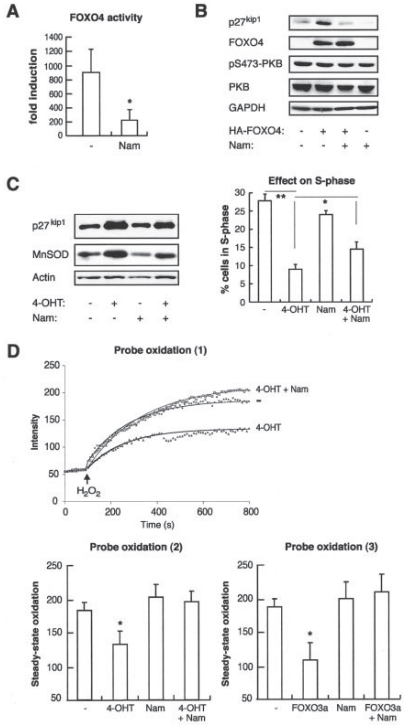

FIG. 1. **Nam inhibits the transcriptional and biological activity of FOXO4.** *A*, A14 cells were transfected with empty vector or a construct encoding HA-FOXO4 together with 6× DBE-luciferase and treated with Nam. All samples were assayed in triplicate. Luciferase counts were normalized using Tk-Renilla-luciferase (luciferase ratio), and the fold induction of luciferase expression was determined by dividing luciferase ratios from samples transfected with FOXO4 by the ratios from parallel samples transfected with empty vector to control for effects on basal transcription. *, $p < 0.05$. *B*, HEK293T cells were transfected with empty vector or a construct encoding HA-FOXO4 and treated with Nam. Lysates of transfected cells were assayed for $p27^{kip1}$, HA-FOXO4, pS473-PKB, and PKB expression. GAPDH expression was used to monitor equal protein loading. *C*, DL23 cells were treated for twenty-four hours using Nam and/or 4-OHT. Total lysates were analyzed by Western blotting for the expression of $p27^{kip1}$, MnSOD, and actin (*left panel*). The percentage of DL23 cells in S-phase was determined by flow cytometry of bromodeoxyuridine-stained cells. Histograms were analyzed using ModFit LT software (*right panel*). *, $p < 0.05$; **, $p < 0.001$. *D*, the kinetics of CM-H_2CDFA probe oxidation in DL23 cells (*upper* and *lower left panels*) and A14 cells (*lower right panel*) was measured after H_2O_2 (150 μM) addition (average of 4–5 cells/experiment). Curves were fitted with a single exponent according to $f(x) = e^{-kt}$, using χ^2 minimalization. All measurements had similar half-lives ($\tau = 172 \pm 14$ s) (*left panel*). The line representing the control + Nam was omitted from the graph for reasons of clarity (it was in between the control and 4-OHT + Nam lines). Steady-state oxidation levels were measured at 800 s (*lower panels*). *, $p < 0.05$. The experiments presented are representative of at least three independent experiments.

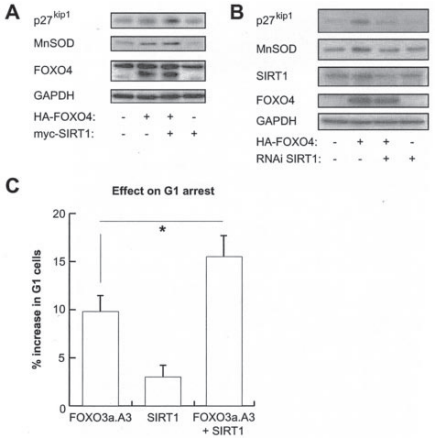

FIG. 2. **hSir2^{SIRT1} enhances FOXO4 transcriptional and biological activity.** *A*, HEK293T cells were transfected as described for Fig. 1*B* together with a myc-hSir2^{SIRT1} construct. $p27^{kip1}$, MnSOD, and HA-FOXO4 expressions were examined by Western blotting. GAPDH expression was used to monitor equal loading. *B*, HEK293T cells were transfected with three different hSir2^{SIRT1}-targeting siRNA oligonucleotides and/or HA-FOXO4. Forty hours after transfection, cells were harvested and total lysates were assayed for $p27^{kip1}$, MnSOD, hSir2^{SIRT1}, and FOXO4 expression. GAPDH was used to check for equal loading. *C*, A14 cells were transfected with empty vector or HA-FOXO3a.A3 together with hSir2^{SIRT1}. Cells were harvested forty hours posttransfection, and the increase in percentage of cells in the G_1-phase of the cell cycle as compared with untransfected cells was determined by flow cytometry of propidium iodide-stained cells. Histograms were analyzed using ModFit LT software. *, $p < 0.05$. The experiments presented are representative of at least three independent experiments.

stress, H_2O_2-induced oxidative stress was reduced in cells treated with 4-OHT, whereas Nam treatment inhibited the effect of FOXO3a (Fig. 1*D*, *upper* and *lower left panels*). In addition, A14 cells expressing active FOXO3a after retroviral transduction displayed increased resistance against H_2O_2-induced oxidative stress, and this was also reversed by Nam treatment (Fig. 1*D*, *lower right panel*). These results show that inhibition of Sir2 function by Nam treatment inhibits FOXO-induced gene expression of $p27^{kip1}$ and MnSOD and, consequently, FOXO-induced cell-cycle arrest and resistance against oxidative stress.

hSir2^{SIRT1} Stimulates FOXO Transcriptional Activity—Next we determined whether the deacetylase inhibitor Nam indeed exerts its inhibitory effect on FOXO-mediated transcription through hSir2^{SIRT1}. We first tested the effect of hSir2^{SIRT1} expression on FOXO4 activity. To this end, hSir2^{SIRT1} and FOXO4 were transfected into HEK293T cells, and the expression of endogenous $p27^{kip1}$ and MnSOD was determined. Both genes were up-regulated by hSir2^{SIRT1} in a FOXO-dependent manner (Fig. 2*A*). Also, in A14-p27luc cells, FOXO4-induced luciferase expression was stimulated by co-expression of hSir2^{SIRT1} (data not shown). To further demonstrate involvement of hSir2^{SIRT1} in regulating FOXO4 transcriptional activity, we used siRNA to hSir2^{SIRT1}, which resulted in reduced levels of endogenous hSir2^{SIRT1} (Fig. 2*B*). In keeping with the inhibitory effect of Nam on FOXO activity, knock-down of hSir2^{SIRT1} impaired FOXO4-induced $p27^{kip1}$ expression. Expression of MnSOD, another transcriptional target of FOXOs (10), was also reduced by siRNA to hSir2^{SIRT1}. As regulation of $p27^{kip1}$ is important in mediating a FOXO-induced cell-cycle arrest, we also analyzed the effect of hSir2^{SIRT1} on FOXO-induced cell-cycle arrest. In keeping with the effect of Nam (see Fig. 1*C*), hSir2^{SIRT1} increased the percentage of cells arrested in G_1 (Fig. 2*C*). Thus, these data indicate that hSir2^{SIRT1} enhances the transcriptional activity of FOXO and thereby

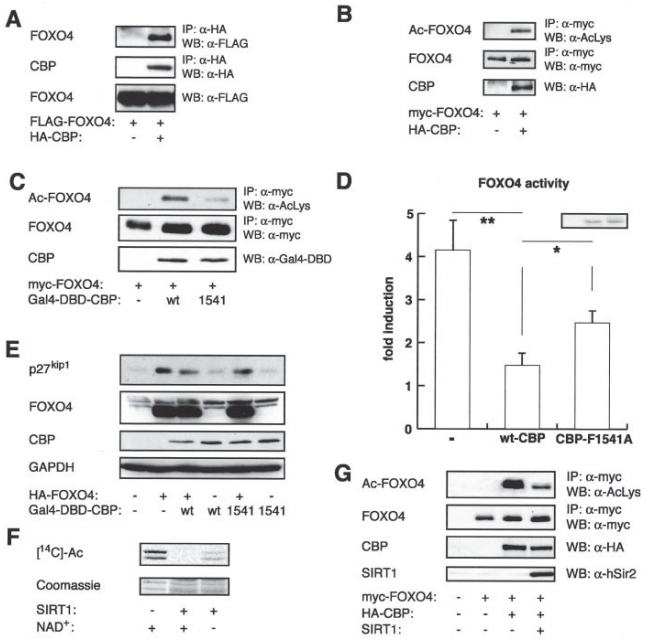

FIG. 3. **CBP inhibits FOXO4 transcriptional activity through acetylation and hSir2^{SIRT1} *deacetylates FOXO4.*** *A*, HEK293T cells were transfected with constructs encoding HA-CBP and FLAG-FOXO4. CBP immunoprecipitates and total lysates were analyzed by Western blotting using anti-HA (12CA5) and anti-FLAG-M2 antibodies. *B*, HEK293T cells were transfected with constructs encoding HA-CBP and myc-FOXO4. Immunoprecipitation samples and total lysates were analyzed by Western blotting using anti-acetylated lysine (α-*AcLys*), anti-myc (9E10), and anti-HA (12CA5) antibodies. *C*, HEK293T cells were cotransfected with a construct encoding HA-FOXO4 together with empty vector, GAL4-DBD-wt-CBP, or an acetyltransferase-impaired mutant (CBP-F1541A). Immunoprecipitation samples and total lysates were analyzed by Western blotting using anti-acetylated lysine (α-*AcLys*), anti-myc (9E10), and anti-Gal4-DBD antibodies. *D*, A14-p27luc cells were transfected with the constructs used in Fig. 2*C*. Samples were analyzed as described for Fig. 1*A*. Similar results were obtained with two other independent A14-p27luc cell lines. *Inset*, CBP expression. *, $p < 0.05$; **, $p < 0.01$. *E*, HEK293T cells were transfected as described for Fig. 1*B* together with the CBP constructs used in Fig. 2, *C* and *D*. p27^{kip1}, HA-FOXO4, and GAL4-DBD-CBP expression were examined by Western blotting. GAPDH expression was used to monitor equal loading. *F*, bacterially expressed GST-FOXO4-DB was acetylated using GST-p300-HAT and [^{14}C]acetyl CoA. Subsequently, bacterially expressed GST-hSir2^{SIRT1} was added in the presence or absence of NAD$^+$ for 90 min. Samples were assayed by Coomassie blue staining and autoradiography. *G*, HEK293T cells were transfected with constructs encoding myc-FOXO4, HA-CBP, and hSir2^{SIRT1}. Immunoprecipitation samples and total lysates were analyzed by Western blotting using anti-acetylated lysine (α-*AcLys*), anti-myc (9E10), anti-HA (12CA5), and anti-hSir2^{SIRT1} antibodies. The experiments presented are representative of at least three independent experiments.

modulates its biological activity.

CBP Acetylates FOXO and Inhibits FOXO Transcriptional Activity—As Sir2 deacetylases are generally thought to act as repressors of transcription via deacetylating histones (26) we hypothesized that hSir2^{SIRT1} directly deacetylates FOXO transcription factors. Acetylation of proteins is mediated by acetyltransferases such as the related proteins p300 and CBP; binding between p300/CBP and FOXO as well as FOXO acetylation have been reported (27–29). To confirm these findings, HA-CBP and FLAG-FOXO4 were co-expressed in HEK293T cells. Indeed FLAG-FOXO4 was found to bind to HA-CBP (Fig. 3*A*). More importantly, binding of CBP to FOXO4 induced acetylation of FOXO4 (Fig. 3*B*). Also, co-expression of CBP and FOXO3a resulted in acetylation of FOXO3a (data not shown), suggesting that CBP is able to mediate acetylation of other FOXO transcription factors. Similarly, co-expression of p300 and FOXO4 induced acetylation of FOXO4 (data not shown), indicating that CBP and p300 are functionally equivalent in FOXO4 acetylation.

Because inhibiting hSir2^{SIRT1} activity by either Nam or siRNA-mediated knockdown of hSir2^{SIRT1} inhibited transcriptional activity of FOXO, we next determined the effect of CBP-mediated acetylation of FOXO4 on its transcriptional activity. In A14-p27luc cells expressing wt-CBP, FOXO4 activity was considerably impaired as compared with control cells expressing FOXO4 only (Fig. 3*D*). This repression was due to the acetyltransferase activity of CBP as co-expression of the CBP-mutant F1541A, which possesses reduced but not absent acetyltransferase activity (Fig. 3*C*; Ref. 30), impaired FOXO4 transcriptional activity to a smaller extent. We also analyzed the effect of CBP expression on the endogenous FOXO target gene p27^{kip1}. Consistent with the reporter assays, wt-CBP inhibited the induction of p27^{kip1} expression by FOXO4, whereas the F1541A mutant displayed a smaller effect because of residual activity (Fig. 3*E*). Taken together, these data indicate that CBP inhibits FOXO4 transcriptional activity by acetylation, in agreement with the observed inhibition of transcriptional activity of FOXO4 by the deacetylase inhibitor Nam.

hSir2^{SIRT1} Deacetylates FOXO4—As CBP and p300 can acetylate FOXO transcription factors, we tested whether hSir2^{SIRT1} could deacetylate FOXO4 *in vitro*. To this end, bacterially expressed FOXO4-DNA-binding domain (GST-FOXO4-

DB) was *in vitro* acetylated by a GST-tagged p300 acetyltransferase domain in the presence of [^{14}C]acetyl-CoA (21). Subsequently, the reactions were incubated with GST-tagged hSir2^{SIRT1} in the absence or presence of the essential cofactor NAD^+ (24). GST-hSir2^{SIRT1} completely deacetylated GST-FOXO4-DB in an NAD-dependent manner (Fig. 3*F*). The incomplete inhibition of deacetylase activity by omitting NAD^+ was possibly due to traces of NAD^+ in the GST-hSir2^{SIRT1} purification. Next, we tested deacetylation by hSir2^{SIRT1} *in vivo*. In HEK293T cells, a clear deacetylation of CBP-acetylated FOXO4 was observed upon cotransfection with hSir2^{SIRT1} (Fig. 3*G*). Taken together, these results show that hSir2^{SIRT1} can directly deacetylate FOXO4 both *in vitro* and *in vivo* and thereby stimulate FOXO transcriptional and biological activity.

Hydrogen Peroxide Induces Acetylation of FOXO and Inhibits FOXO Activity—To investigate whether extracellular signaling could regulate acetylation/deacetylation of FOXO4, we tested the effects of insulin/insulin-like growth factor-1, a known regulator of FOXO function, and hydrogen peroxide. Hydrogen peroxide is known to promote acetylation of proteins like p53 and histones (31, 32), and FOXO transcription factors have been shown to be important in regulating stress responses (10). First, we investigated whether hydrogen peroxide could affect binding of CBP to FOXO4. Therefore, HA-CBP was immunoprecipitated from HEK293T cells co-expressing myc-FOXO4. Increasing amounts of hydrogen peroxide enhanced the interaction between HA-CBP and myc-FOXO4 (Fig. 4*A*), suggesting that peroxide stress may induce acetylation of FOXO4 by CBP. Furthermore, activation of phosphatidylinositol 3-kinase/PKB signaling by treating cells with insulin and epidermal growth factor decreased basal binding between HA-CBP and myc-FOXO4. As CBP is localized exclusively within the nucleus, the decreased binding observed is likely due to the fact that phosphatidylinositol 3-kinase/PKB signaling induces relocalization of myc-FOXO4 to the cytosol. Importantly, activation of phosphatidylinositol 3-kinase/PKB signaling could not prevent the increase of HA-CBP/myc-FOXO4 complex formation induced by hydrogen peroxide treatment, indicating that the effect of peroxide is dominant over growth factor stimulation in this respect. Next, we determined whether endogenous FOXO and CBP could interact. We detected endogenous interaction between CBP and both FOXO4 and FOXO3a. Similar to the transient expression studies, H_2O_2 treatment increased the interaction between FOXO and CBP, although the magnitude differed (Fig. 4*B*). Finally, we tested whether enhanced binding of CBP to FOXO4 after treatment of cells with hydrogen peroxide resulted in increased acetylation of FOXO4. Thus, HEK293T cells transfected with a myc-FOXO4 construct were treated with different concentrations of peroxide and followed up for several periods of time. FOXO4 was immunoprecipitated and acetylation was determined by Western blotting. Hydrogen peroxide treatment indeed induced acetylation of FOXO4 (Fig. 4*C*). Also, treatment of cells cotransfected with FLAG-FOXO4 and HA-CBP with hydrogen peroxide resulted in increased acetylation in the fraction of FLAG-FOXO4 bound to HA-CBP (Fig. 4*D*). As noted, we detected acetylation by endogenous acetyltransferases only at later timepoints than would be suggested by the binding of CBP to FOXO4 (Fig. 4, *A* and *B*). As we used a pan-acetyllysine-antibody, we attribute this result to the low sensitivity of the antibody toward FOXO4, precluding detection of a low stoichiometry. This is further suggested by the observation that upon overexpression of CBP, we detected acetylation at shorter timepoints after peroxide treatment. Therefore, increased binding of FOXO4 to CBP after hydrogen peroxide treatment likely leads to increased acetylation.

As hydrogen peroxide induces acetylation of FOXO4, we expected that peroxide would have the same effect as CBP or Nam on FOXO4 transcriptional activity. To study this, FOXO4 was transfected in HEK293T cells, and the expression of endogenous p27^{kip1} was determined upon treatment of these cells with various concentrations of hydrogen peroxide for twenty-four hours. Indeed, hydrogen peroxide inhibited p27^{kip1} expression (Fig. 4*E*). Inhibition of p27^{kip1} expression was not due to activation of PKB by hydrogen peroxide, as under these conditions we could not detect any changes in the Ser-473 phosphorylation of PKB. Taken together, these data indicate that hydrogen peroxide inhibits FOXO4 transcriptional activity through induction of FOXO4 acetylation.

Hydrogen Peroxide Stimulates Binding of hSir2^{SIRT1} to FOXO—Finally, because acetylation of FOXO4 occurs in conjunction with binding of the acetyltransferase CBP, we also investigated whether deacetylation by hSir2^{SIRT1} involves direct binding. Therefore, we expressed myc-hSir2^{SIRT1}, HA-FOXO4, and treated cells with Nam or hydrogen peroxide for one hour to induce acetylation of HA-FOXO4. Complex formation between hSir2^{SIRT1} and FOXO4 could only be detected after peroxide treatment (Fig. 4*F*), suggesting that hSir2^{SIRT1} binds to acetylated FOXO4. On the contrary, Nam was not able to stabilize the interaction of hSir2^{SIRT1} with FOXO4, indicating that the effect of Nam on FOXO transcriptional activity can not be accounted for by mere binding of hSir^{SIRT1} to FOXO. We also tested whether FOXO and hSir2^{SIRT1} interacted endogenously. Antibody quality precluded the detection of a clear interaction between FOXO4 and hSir2^{SIRT1}, but in HEK293T cells, an interaction between endogenous FOXO3a and hSir2^{SIRT1} was detected, and this interaction was weakly induced by peroxide treatment (Fig. 4*G*). Taken together, these data show that hSir2^{SIRT1} directly binds to FOXO, which is induced by peroxide stress.

DISCUSSION

Our results suggest a model in which FOXO transcription factors are subject to regulation by reversible acetylation. Acetylation of FOXOs results in inhibition of their transcriptional and biological activities, which is reversed by the longevity protein hSir2^{SIRT1}. Inhibition through acetylation of FOXO transcription factors is functionally equivalent to the previously described inhibition by PKB-mediated phosphorylation.

We show that the acetyltransferase CBP binds to and acetylates FOXO4, thereby inhibiting its transcriptional activity. Previous reports on CBP/p300 binding to FOXO (27, 28) suggested that CBP/p300 binding is required for activation of FOXO-dependent transcription. However, there are several important differences between these and our studies that may explain this apparent discrepancy. First, Nasrin *et al.* (28) inferred a role for CBP/p300 in the activation of FOXO because they observed that the viral protein E1A, which can inhibit CBP co-activator function, also inhibits FOXO transcriptional activity on an insulin-like growth factor BP1 promoter. However, E1A is known to have a plethora of targets within the transcriptional machinery and thus, by itself, a block by E1A is not sufficient proof of the involvement of CBP/p300 (for a review, see Ref. 33). Second, whereas we have also analyzed the effect of CBP on the regulation of endogenous gene transcription, other studies have relied on transient reporter assays in which a proper chromatin context is likely to be lacking. Third, the role of CBP/p300 can be promoter-context-dependent, and accordingly, different FOXO-responsive genes may respond differently to the presence of CBP/p300. Differential responsiveness could relate to the fact that we have to discriminate between the effect of CBP/p300 as histone acetyltransferase and as acetyltransferase acting on FOXO itself. It could be

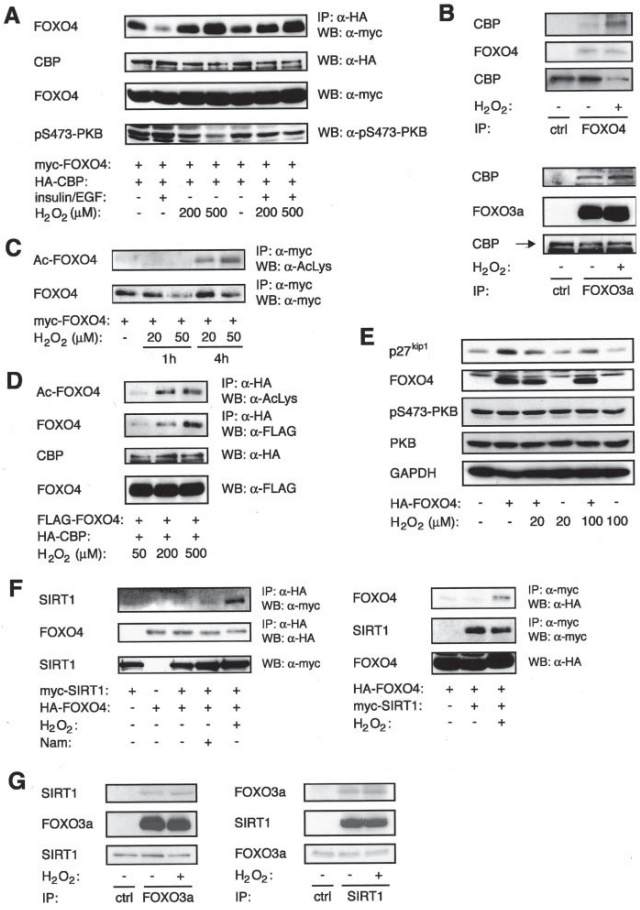

FIG. 4. **Peroxide stress induces FOXO4 acetylation, thereby inhibiting FOXO4 transcriptional activity, and induces hSir2^{SIRT1} binding to FOXO.** *A*, HEK293T cells were transfected with constructs encoding HA-CBP and myc-FOXO4. Cells were treated with the indicated concentrations of peroxide or insulin plus EGF for 30 min, and CBP was immunoprecipitated from cellular lysates. *Two lanes* at *far right*, insulin and EGF were given 30 min before adding peroxide. Immunoprecipitation samples and total lysates were analyzed by Western blotting using anti-HA (12CA5), anti-myc (9E10), and anti-pS473-PKB antibodies. *B*, C2C12 and HEK293T cells were treated for 1 h with 500 μM H_2O_2. Endogenous FOXOs (*FOXO4* from C2C12 in *upper panel*, *FOXO3a* from HEK293T in *lower panel*) were immunoprecipitated, and immune complexes were assayed for the presence of CBP. Total lysates were also checked for CBP expression. *C*, HEK293T cells were transfected with a construct encoding myc-FOXO4 and treated with the indicated concentrations of hydrogen peroxide for 1 or 4 h. FOXO4 immunoprecipitation samples were analyzed by Western blotting using anti-acetyllysine (α-*AcLys*) and anti-myc (9E10) antibodies. *D*, the experiment was performed essentially as in *A*. Immunoprecipitates were first subjected to anti-acetyllysine (α-*AcLys*) antibody before probing for FOXO4. *E*, HEK293T cells were transfected as in Fig. 1*B* and treated with peroxide. p27^{Kip1}, HA-FOXO4, pS473-PKB, and PKB expression were examined by Western blotting. GAPDH expression was used to monitor equal loading. *F*, HEK293T cells were transfected with constructs encoding myc-hSir2^{SIRT1} and HA-FOXO4. Cells were treated with 200 μM hydrogen peroxide or 5 mM Nam. Either HA-FOXO4 (*left panel*) or myc-hSir2^{SIRT1} (*right panel*) was immunoprecipitated from cellular lysates. Immunoprecipitation samples and total lysates were analyzed by Western blotting using anti-HA (12CA5) and anti-myc (9E10) antibodies. *G*, HEK293T cells were treated for 1 h with 500 μM H_2O_2. Endogenous FOXO3a (*left panel*) or hSir2^{SIRT1} (*right panel*) were immunoprecipitated, and immune complexes were assayed for the presence of hSir2^{SIRT1} and FOXO3a, respectively. Total lysates were also checked for hSir2^{SIRT1} and FOXO3a expression, respectively. The experiments presented are representative of at least three independent experiments.

possible that, with respect to different promoters, these activities of CBP/p300 may synergize or antagonize. Because CBP/p300-mediated histone acetylation is generally considered to contribute to transcriptional activation, and as we observe inhibition of FOXO transcriptional activity, we conclude that in the context of the promoters analyzed here, the direct acetylation of FOXO acts as an inhibitory signal.

The binding of CBP to FOXO4 as well as the acetylation of FOXO4 is induced upon treatment of cells with hydrogen peroxide. Furthermore, hSir2^{SIRT1} can bind to acetylated FOXO4

and consequently deacetylate FOXO4. Thus, hSir2^{SIRT1} can counteract the inhibition of FOXO induced by acetylation. Recently, regulation of p53 acetylation by hSir2^{SIRT1} has been described (16, 24), and this has been implicated in mediating cellular protection against genotoxic stresses. However, apoptosis induced by loss of hSir2^{SIRT1} is only partially rescued by dominant-negative p53 (16). Furthermore, in cells lacking functional p53, resveratrol, a presumed activator of hSir2^{SIRT1}, can still enhance protection against genotoxic stress (14). These observations suggest alternative p53-independent pathways involved in cellular protection by hSir2^{SIRT1}. Recently, we have shown that FOXO activation results in increased resistance against oxidative stress (10), and here we show that this is regulated by hSir2^{SIRT1}. We like to propose that FOXOs play an important role in the hSir2^{SIRT1}-mediated, p53-independent protection against stress. Therefore, by preventing negative regulation of FOXO due to acetylation after increased oxidative stress, hSir2^{SIRT1} can enhance cellular defenses against oxidative stress provided for by FOXO. This may be especially relevant under conditions of endogenous oxidative stress occurring during normal metabolic activities. Oxidative stress is considered to be a prime parameter of aging in all living organisms. Therefore, the results presented in this report provide a rationale for the observed increase in longevity induced by *C. elegans* Sir2, which is FOXO-dependent.

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REFERENCES

1. Kaestner, K. H., Knochel, W., and Martinez, D. E. (2000) *Genes Dev.* **14,** 142–146
2. 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
3. Kops, G. J., de Ruiter, N. D., De Vries-Smits, A. M., Powell, D. R., Bos, J. L., and Burgering, B. M. (1999) *Nature* **398,** 630–634
4. Tang, E. D., Nunez, G., Barr, F. G., and Guan, K. L. (1999) *J. Biol. Chem.* **274,** 16741–16746
5. Gottlieb, S., and Ruvkun, G. (1994) *Genetics* **137,** 107–120
6. Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993) *Nature* **366,** 461–464
7. Murakami, S., and Johnson, T. E. (1996) *Genetics* **143,** 1207–1218
8. Honda, Y., and Honda, S. (1999) *FASEB J.* **13,** 1385–1393
9. 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) *Science* **296,** 530–534
10. 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. (2002) *Nature* **419,** 316–321
11. Burgering, B. M., and Kops, G. J. (2002) *Trends Biochem. Sci* **27,** 352–360
12. Tissenbaum, H. A., and Guarente, L. (2001) *Nature* **410,** 227–230
13. Frye, R. A. (2000) *Biochem. Biophys. Res. Commun.* **273,** 793–798
14. Howitz, K. T., Bitterman, K. J., Cohen, H. Y., Lamming, D. W., Lavu, S., Wood, J. G., Zipkin, R. E., Chung, P., Kisielewski, A., Zhang, L. L., Scherer, B., and Sinclair, D. A. (2003) *Nature* **425,** 191–196
15. Burgering, B. M., and Coffer, P. J. (1995) *Nature* **376,** 599–602
16. Vaziri, H., Dessain, S. K., Ng Eaton, E., Imai, S. I., Frye, R. A., Pandita, T. K., Guarente, L., and Weinberg, R. A. (2001) *Cell* **107,** 149–159
17. Medema, R. H., Herrera, R. E., Lam, F., and Weinberg, R. A. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 6289–6293
18. Furuyama, T., Nakazawa, T., Nakano, I., and Mori, N. (2000) *Biochem. J.* **349,** 629–634
19. Medema, R. H., Kops, G. J., Bos, J. L., and Burgering, B. M. (2000) *Nature* **404,** 782–787
20. Chrivia, J. C., Kwok, R. P., Lamb, N., Hagiwara, M., Montminy, M. R., and Goodman, R. H. (1993) *Nature* **365,** 855–859
21. Vries, R. G., Prudenziati, M., Zwartjes, C., Verlaan, M., Kalkhoven, E., and Zantema, A. (2001) *EMBO J.* **20,** 6095–6103
22. Kalkhoven, E., Teunissen, H., Houweling, A., Verrijzer, C. P., and Zantema, A. (2002) *Mol. Cell. Biol.* **22,** 1961–1970
23. O'Brien, R. M., Noisin, E. L., Suwanichkul, A., Yamasaki, T., Lucas, P. C., Wang, J. C., Powell, D. R., and Granner, D. K. (1995) *Mol. Cell. Biol.* **15,** 1747–1758
24. Luo, J., Nikolaev, A. Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001) *Cell* **107,** 137–148
25. Bitterman, K. J., Anderson, R. M., Cohen, H. Y., Latorre-Esteves, M., and Sinclair, D. A. (2002) *J. Biol. Chem.* **277,** 45099–45107
26. Moazed, D. (2001) *Mol. Cell* **8,** 489–498
27. So, C. W., and Cleary, M. L. (2002) *Mol. Cell. Biol.* **22,** 6542–6552
28. Nasrin, N., Ogg, S., Cahill, C. M., Biggs, W., Nui, S., Dore, J., Calvo, D., Shi, Y., Ruvkun, G., and Alexander-Bridges, M. C. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10412–10417
29. Mahmud, D. L., Amlak, M., Deb, D. K., Platanias, L. C., Uddin, S., and Wickrema, A. (2002) *Oncogene* **21,** 1556–1562
30. Martinez-Balbas, M. A., Bannister, A. J., Martin, K., Haus-Seuffert, P., Meisterernst, M., and Kouzarides, T. (1998) *EMBO J.* **17,** 2886–2893
31. Ito, A., Lai, C. H., Zhao, X., Saito, S., Hamilton, M. H., Appella, E., and Yao, T. P. (2001) *EMBO J.* **20,** 1331–1340
32. Rahman, I., Gilmour, P. S., Jimenez, L. A., and MacNee, W. (2002) *Mol. Cell Biochem.* **234–235,** 239–248
33. Goodman, R. H., and Smolik, S. (2000) *Genes Dev.* **14,** 1553–1577

CHAPTER 3

The Caenorhabditis elegans nicotinamidase PNC-1 extends lifespan

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In preparation

The Caenorhabditis elegans nicotinamidase PNC-1 extends lifespan

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In Caenorhabditis elegans (C.elegans), increased dosage of the gene encoding the nicotinamide adenine dinucleotide (NAD)-dependent deacetylase SIR-2.1 increases lifespan in a DAF-16 dependent manner. In yeast, increasing the copy number of SIR2 also extends lifespan, which can be inhibited by nicotinamide, the end-product of SIR2-mediated NAD-breakdown. Furthermore, the yeast pyrazinamidase/nicotinamidase PNC1 can extend yeast lifespan by converting nicotinamide (Nam). Here, we report that knockdown of the C.elegans homologue of yeast PNC1 as well as growing worms on Nam- containing medium significantly decreases adult lifespan. However, in contrast to the observed increase in lifespan by increased *sir-2.1* gene dosage, we do not observe that an increased gene dosage of *pnc-1* extends lifespan, yet it increases survival under conditions of oxidative stress. These data imply that the conversion of nicotinamide and hence increased activity of Sir2 is an evolutionary conserved pathway for lifespan extension.

In recent years substantial progress has been made in studying aging. In model organisms like yeast, Caenorhabditis elegans (C.elegans) and mice a number of genes were found, mutations in which either positively or negatively affect lifespan (reviewed in Finch and Ruvkun, 2001[1]). For example, in C.elegans, reduction of the activity of the insulin-like receptor DAF-2, or the type I phosphatidylinositol-3-kinase AGE-1, extends the lifespan of adult worms. This lifespan extension is caused by activation of DAF-16[2], which is the homologue of human forkhead box O (FOXO) transcription factors, probably through enhanced stress resistance[3]. Likewise, increased dosage of the gene encoding the nicotinamide adenine dinucleotide(NAD)-dependent deacetylase SIR-2.1 increases worm lifespan in a DAF-16-dependent manner[4]. The Sir2 protein is also conserved from yeast (SIR2) to humans (SIRT1) and, in yeast, increasing the copy number of SIR2 also extends lifespan[5]. The activity of Sir2 can be inhibited by nicotinamide (Nam), the end-product of Sir2-mediated NAD breakdown[6]. In yeast, nicotinamide can be converted into nicotinic acid by a protein named PNC1. And, by degrading nicotinamide, yeast nicotinamidase PNC1 increases longevity as well[7,8].

Here we address whether C.elegans PNC-1 (gene ID: Y38C1AA.3) extends adult worm lifespan as been shown for its yeast counterpart. To this end, we performed longevity assays with N2 worms fed on bacteria expressing control RNAi or RNAi against *pnc-1* or *sir-2.1*. Consistent with the increase in lifespan by extra copies of *sir-2.1*[4], we observed lifespan shortening using RNAi against *sir-2.1* (17.1±0.57 vs. 20.6±0.89 days, power=0.906; Fig. 1A). Interestingly, worms fed on bacteria containing RNAi against *pnc-1* also lived significantly shorter than worms growing on empty vector control bacteria (16.0±0.50 vs. 20.6±0.89 days, power=0.986; Fig. 1A). Similarly, N2 worms on plates containing the Sir2-

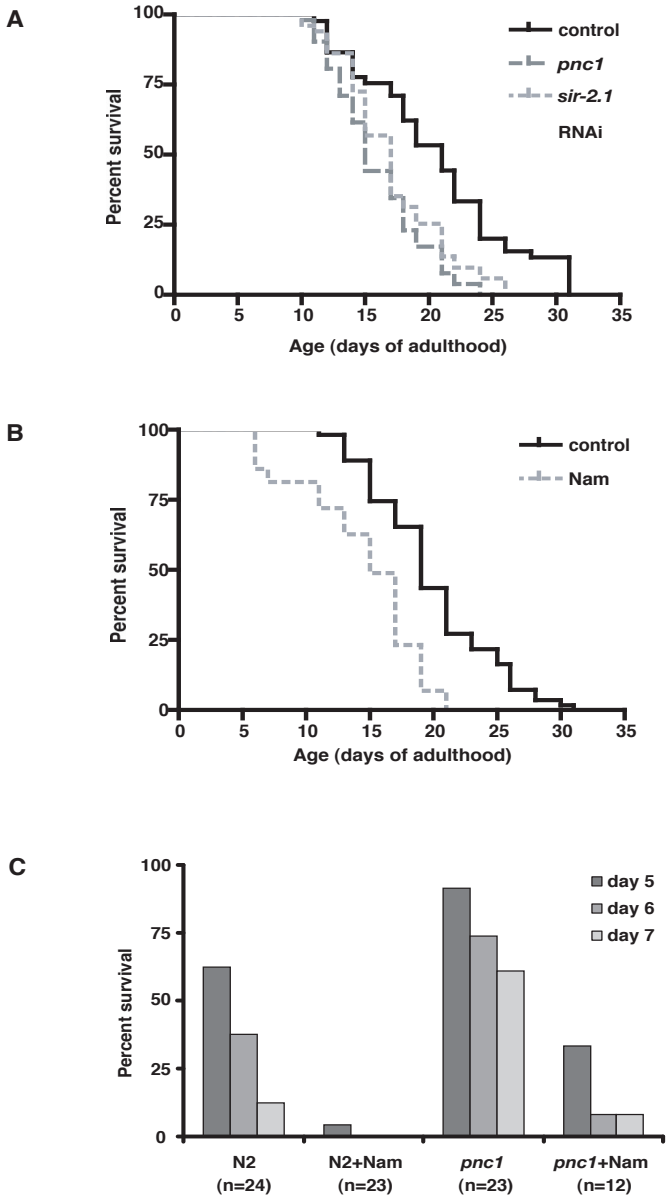

Figure 1. Regulation of C.elegans lifespan by PNC-1. **(A)** Loss of *pnc-1* or *sir-2.1* reduces lifespan. Survival curves show that RNAi-mediated knockdown of *pnc-1* or *sir-2.1* results in a shorter mean lifespan (16.0±0.50 days, n=52 and 17.1±0.57 days, n=51 resp.) as compared to control RNAi (20.6±0.89 days, n= 45) (power=0.986 and 0.906 resp., Kaplan-Meier survival analysis). Data presented are representative of three experiments. **(B)** Growing worms on the Sir2-inhibitor Nam reduces lifespan. Survival curves show that worms growing on Nam have a shorter mean lifespan (14.4±0.72 days, n=43) than control animals growing in the absence of Nam (19.8±0.64 days, n= 55) (power=0.999, Kaplan-Meier survival analysis). Data presented are representative of three experiments. **(C)** *pnc-1* transgenic worms are more resistant to paraquat-induced oxidative stress than N2 worms. The percentage of animals that are still alive at the indicate days is indicated. Data presented are based on one experiment.

inhibitor Nam lived significantly shorter than N2 worms grown in the absence of Nam (14.4±0.72 vs. 19.8±0.64 days, power=0.999; Fig. 1B). These results suggest that the C.elegans PNC-1, like its yeast homologue, increases lifespan. To study this in more detail, we made a transgenic worm strain containing extra copies of the *pnc-1* gene under control of the *let-858* promoter, driving expression in all cells. These worms have an approximately 4-fold higher expression of PNC1, as determined by real-time PCR (data not shown). Unexpectedly, increasing the dosage of PNC-1 did not extend the lifespan of adult worms compared to N2 worms (19.7±0.82 vs. 20.6±0.89 days, power=0.115). These results may indicate that under normal conditions the levels of PNC-1 are sufficient to minimize negative effects of endogenous Nam on lifespan. Indeed, when we challenged *pnc-1* transgenic worms and wild-type worms with 5 mM paraquat (PQ), an inducer of oxidative stress, and determined survival, we did observe higher survival of transgenic worms compared to N2 worms (Fig. 1C). Nam still diminished survival of *pnc-1* transgenic worms, which suggests that the levels of PNC-1 are not sufficient to completely counteract the effect of Nam. Taken together, these results indicate that *pnc-1* extends lifespan only under conditions of stress, which might be more common in a natural habitat than in a laboratory setting. Furthermore, as PNC-1 converts Nam, an inhibitor of SIR-2.1, and increases stress resistance we propose that PNC-1 acts upstream of SIR-2.1 and DAF-16 to extend lifespan.

PNC-1 is evolutionary conserved and the functional counterpart of PNC-1 in mammals is NAMPT (nicotinamide phosphoribosyltransferase, also named PBEF or Visfatin), which converts nicotinamide into nicotinamide mononucleotide[9]. Consistent with it being an equivalent of PNC-1, overexpression of NAMPT increases SIRT1 activity[10]. Whether this increase in Sir2 activity increases lifespan in mammals still has to be proven. However, our data suggest that lowering Nam-levels by increasing its conversion is beneficial for multicellular organisms as well.

Materials and Methods

Worms

General methods for culturing and manipulating worms used in these studies were as described[11]. Worms were cultured on NGM plates at 20°C. Strains used were wild-type Bristol N2, and bjIs63 [*pnc-1/dpy20 gpa-15::gfp* 7.19] (FZ0216).

Transgenic animals were obtained by injection of *pnc-1*, *dpy-20* (pMH86) and *gpa-15::gfp* plasmid DNAs into the gonad of *dpy-20(e1362)* worms. Transgenic arrays were integrated by irradiating animals with 40 Gy of gamma radiation from a 137 Cs source[12]. Transgenic worms were out-crossed twice to wild-type N2 animals before phenotypic analysis.

Constructs

For transgene synthesis, the *pnc1* gene (~ 1-kb) was amplified by PCR on a C.elegans cDNA library (obtained from M. Vidal) and cloned into pGEM-T (Promega) using the primers 5'-CTACTGGCTA GCATGTTTCCCTGCCCAAAGCTT-3' and 5'-GCTGCATCTAGACTACTTCTTCACGATCCTTT G-3' and cloned as an *Nhe*I/*Xba*I fragment into L2865 pPD103.05[13], kindly provided by A. Fire.

For RNAi, the *pnc-1* and the *sir-2.1* genes were amplified by PCR on the cDNA library mentioned above and cloned into pGEM-T (Promega) using the primers 5'-AAATAAGAATGCGGCCGCATG TTTCCCTGC-3' and 5'-CGGTCGGCTCGAGGATCACAACTGCGTCAATATTC-3' for *pnc-1* and 5'-AAATAAGAATGCGGCCGCCACAAACTACAACTCACACTAGC-3' and 5'-CGGTCGGCTC GAGTCAGATACGCATTTCTTCACAC-3' for *sir-2.1*. NotI/XhoI restriction fragments were cloned into L4440 DoubleT-7 script II vector[14], kindly provided by A. Fire.

Real-time PCR

Total RNA was isolated from N2 and *pnc-1* transgenic worms using 'Easy RNA isolation from C.elegans: A TRIZOL based method' [Worm Breeder's Gazette 14(3): 10 (June 1, 1996), Easy RNA isolation from C.elegans: A TRIZOL based method, Rebecca D. Burdine, Michael J. Stern]. cDNA was synthesized using random primers according to the manufacturer (Promega). Real-time PCR was performed using SYBR green RT-PCR reagents (Applied Biosystems). 25 μl reactions contained 1x SYBR green PCR buffer, 4 mM $MgCl_2$, 1 mM dNTPs, 1.25 Units AmpliTaq Gold, x μl template and 0.6 mM of each primer. Primers used were: 5'-TTGCGTTCGTTTACTTGCCTG-3' and 5'-CCGTGATCCCGGAATGCAGCG-3' for pnc-*1* and 5'-GGAGCATGCATTTCGCTCA-3' and 5'-ACGACAATGAATATGGATACTCGAAC-3' for *gpd-1* as a household gene. Reactions were performed in a Chromo 4 Real-Time detector (BioRad) as follows: 95°C for 10 minutes, then 40 cycles: 95°C for 15 seconds, 58°C for 15 seconds, 72°C for 15 seconds. Data were analyzed using Opticon Monitor 3 software (BioRad). Expression levels of *pnc1* were corrected for the expression of *gpd-1* using the C_t values (by the following formula: $2^{(PNC\text{-}1\text{-}GPD\text{-}1)}$).

Lifespan and survival assays

Assays were done at 20°C. For assaying lifespan, strains were synchronized by hypochlorite treatment and isolated eggs were placed on NGM agar plates to hatch. For the effect of RNAi 50 L4 larvae per condition were singled to NGM agar plates containing 1 mM IPTG, ampicillin and HT115 bacteria expressing *pnc-1*, *sir-2.1* or empty vector RNAi as indicated. Animals were transferred to fresh plates every 2-3 days and were scored dead when they did not move after repeated taps with a pick. For the effect of Nam, individual L4s were transferred to plates containing OP50 bacteria, 50 μM 5'fluoro-2'deoxyuridine (FUDR, which blocks DNA synthesis and causes animals to lay eggs that do not develop and eliminates the need to transfer animals throughout the lifespan assay) and 25 mM Nam as indicated. Animals were tapped every 2-3 days and were scored as dead when they did not move after repeated taps with a pick. Statistical analyses (Kaplan-Meier survival analysis) were performed using Graphpad Prism 4 software.

To determine survival of worms on oxidative stress, we synchronized N2 and *pnc-1* transgenic worms by hypochlorite treatment and eggs were allowed to hatch on NGM agar plates containing OP50 bacteria. From these plates L4 larvae were singled to plates containing OP50 bacteria, 5 mM PQ and 25 mM Nam as indicated. Several days later the percentage of animals that had survived was determined.

Lifespan is expressed as mean±SEM (standard error of the mean). Differences were calculated using an unpaired t-test with Bonferroni correction. Powers were calculated using a p-value of 0.05, which generally is considered significant. A power of at least 0.9 was considered statistically significant.

Acknowledgments

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References

1. Finch, C. E. & Ruvkun, G. The genetics of aging. *Annu Rev Genomics Hum Genet* **2**, 435-62 (2001).
2. Kenyon, C., Chang, J., Gensch, E., Rudner, A. & Tabtiang, R. A C. elegans mutant that lives twice as long as wild type. *Nature* **366**, 461-4 (1993).

3. Johnson, T. E. et al. Longevity genes in the nematode Caenorhabditis elegans also mediate increased resistance to stress and prevent disease. *J Inherit Metab Dis* **25**, 197-206 (2002).
4. Tissenbaum, H. A. & Guarente, L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature* **410**, 227-30 (2001).
5. Kaeberlein, M., McVey, M. & Guarente, L. The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. *Genes Dev* **13**, 2570-80 (1999).
6. Bitterman, K. J., Anderson, R. M., Cohen, H. Y., Latorre-Esteves, M. & Sinclair, D. A. Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. *J Biol Chem* **277**, 45099-107 (2002).
7. Anderson, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O. & Sinclair, D. A. Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. *Nature* **423**, 181-5 (2003).
8. Gallo, C. M., Smith, D. L., Jr. & Smith, J. S. Nicotinamide clearance by Pnc1 directly regulates Sir2-mediated silencing and longevity. *Mol Cell Biol* **24**, 1301-12 (2004).
9. Rongvaux, A. et al. Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *Eur J Immunol* **32**, 3225-34 (2002).
10. Revollo, J. R., Grimm, A. A. & Imai, S. The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. *J Biol Chem* **279**, 50754-63 (2004).
11. Lewis, J. A. & Fleming, J. T. Basic culture methods. *Methods Cell Biol* **48**, 3-29 (1995).
12. Mello, C. C., Kramer, J. M., Stinchcomb, D. & Ambros, V. Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. *Embo J* **10**, 3959-70 (1991).
13. Kelly, W. G., Xu, S., Montgomery, M. K. & Fire, A. Distinct requirements for somatic and germline expression of a generally expressed Caernorhabditis elegans gene. *Genetics* **146**, 227-38 (1997).
14. Timmons, L. & Fire, A. Specific interference by ingested dsRNA. *Nature* **395**, 854 (1998).

CHAPTER 4

FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP

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Submitted

FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP

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Forkhead box O (FOXO) transcription factors are important regulators of cellular metabolism, cell cycle progression, and cell death. FOXO activity is regulated by multiple signaling pathways, most importantly by phosphoinositide-3 kinase (PI3K)/ protein kinase B (PKB/c-Akt) signaling (reviewed in [1]). Here, we demonstrate that following increased cellular oxidative stress FOXO becomes monoubiquitinated. Monoubiquitination results in FOXO nuclear localisation and increased transcriptional activity. Deubiquitination of FOXO requires the deubiquitinating enzyme USP7/ HAUSP. FOXO and USP7 interact and this interaction is enhanced following stress. Consistent with the observed monoubiquitination, cellular oxidative stress and USP7 do not change FOXO protein half-life, but USP7 regulates FOXO activity. USP7 overexpression downregulates FOXO transcriptional activity and endogenous USP7 regulates FOXO transcriptional activity towards endogenous promoters. Taken together, these data provide evidence for a new layer of FOXO control and implies USP7 as an important control element in regulating cellular stress: previously, USP7 was shown to regulate genotoxic stress through p53 and here we show that USP7 regulates metabolic stress through FOXO.

The FOXO (Forkhead box O) subfamily of forkhead transcription factors consists of four members, FOXO1, FOXO3, FOXO4 and FOXO6[2]. These proteins are involved in a number of important biological processes, such as control of cellular metabolism, cell cycle regulation, apoptosis and regulation of the stress-response[3,4] (reviewed in [1]). Initially, it was shown that the FOXO factors are regulated by protein kinase B (PKB/c-Akt)-mediated phosphorylation and that this phosphorylation results in their nuclear exclusion[5,6]. Regulation of FOXO by PKB is conserved through evolution as DAF-16, the FOXO homologue in the nematode C. elegans, is regulated in a similar manner. Recently, other signaling pathways have been described to regulate FOXO (reviewed in [7]), but it remains to be established how conserved these non-PKB regulatory pathways are. More recently, we showed that a pathway sensitive to cellular oxidative stress, involving the small GTPase Ral and the stress kinase JNK (Jun-N-terminal kinase), positively regulates the transcriptional activity of FOXO4 by phosphorylation[8,9]. Interestingly, like PKB signaling towards FOXO, regulation by JNK[10,11] and possibly Ral[12] appears to be conserved throughout evolution.

Besides phosphorylation, FOXO transcription factors can also be posttranslationally regulated by acetylation and polyubiquitination. FOXO acetylation occurs by p300, CBP (CREB-binding protein) and P/CAF (p300/CBP associated factor) and deacetylation by HDACs and Sir2. However, the consequence of (de)acetylation on FOXO activity is still unclear[13-18]. Polyubiquitination of FOXOs linked via lysine-48 of ubiquitin can be induced by growth factor signaling and this is likely mediated by Skp2[19-22], an F-box protein present in so-called SCF-complexes (Skp1, Cullin-1, F-box protein) of ubiquitin ligases. The role of K48-mediated polyubiquitination on FOXO in normal signaling is unclear as it does not appear to considerably affect protein stability[20,21].

We set out to study ubiquitination of FOXO in more detail. To this end HA-FOXO4 and his-ubiquitin were co-expressed in HEK293T cells and cells were left untreated or treated with hydrogen-peroxide, a known substrate-dependent inducer of mono- and/or polyubiquitination[23,24]. Subsequently, cells were lysed in a denaturing buffer containing 8M urea to remove any (ubiquitinated) protein binding to HA-FOXO, and ubiquitinated proteins were precipitated using Ni-NTA beads[25]. Western blot detection using an anti-HA antibody indicated that FOXO4 could be ubiquitinated (Fig. 1a, *left panel*). To verify that the bands recognized by the anti-HA antibody indeed represent ubiquitinated HA-FOXO4, we performed the same experiment, but probed with anti-FOXO4 instead of anti-HA (Fig. 1a, *right panel*). Both approaches resulted in the same pattern of ubiquitinated bands, indicating that these indeed represent ubiquitinated HA-FOXO4. Instead of the characteristic high molecular weight smear indicative for polyubiquitination, we observed two prominent bands of which the molecular weight of the lowest band corresponds to the molecular weight of FOXO4 plus ~8 kDa. This result suggested to us that FOXO4 might be monoubiquitinated at multiple sites. To further validate this possibility we used a his-ubiquitin construct in which all 7 lysine residues of ubiquitin were mutated to arginines (K7R). Ni-NTA pull down using this modified his-ubiquitin resulted in a similar pattern of FOXO4 ubiquitination (Fig. 1b), supporting our conclusion that FOXO4 can be monoubiquitinated at multiple sites.

Next, we examined the kinetics and dose-response of hydrogen peroxide-induced monoubiquitination of FOXO4. Fig. 1c shows that as soon as fifteen minutes upon addition of 50 μM hydrogen peroxide monoubiquitination of HA-FOXO4 could be detected. Moreover, this monoubiquitination was more profoundly induced by higher concentrations of peroxide and lasted for at least 30 minutes (Fig. 1c). This is in sharp contrast to the growth factor-induced polyubiquitination of FOXO, which was visible only after 12 hours of insulin treatment (data not shown and [20]). Besides hydrogen peroxide we also tested several other general stressors (e.g. anisomycin, tunicamycin, UV). However, none of these treatments induced significant monoubiquitination of HA-FOXO4 compared to hydrogen peroxide (data not shown), suggesting that the observed monoubiquitination of HA-FOXO4 specifically follows the generation of cellular oxidative stress as generated by hydrogen peroxide. Furthermore, monoubiquitination of HA-FOXO3 was also induced by hydrogen peroxide indicating that this may be a general regulatory mechanism to the O class of Forkhead transcription factors (data not shown).

Polyubiquitination of proteins normally results in proteasome-mediated protein degradation whereas monoubiquitination may mediate other signalling functions such as shown in protein trafficking and DNA repair (reviewed in [26]). Previously, we and others have shown that treatment of cells with hydrogen peroxide results in FOXO translocation to the nucleus[9,16]. Combined with the notion that monoubiquitination can alter the localisation of certain substrates (e.g. EGF receptor, reviewed in [27]), we examined the localisation of ubiquitinated

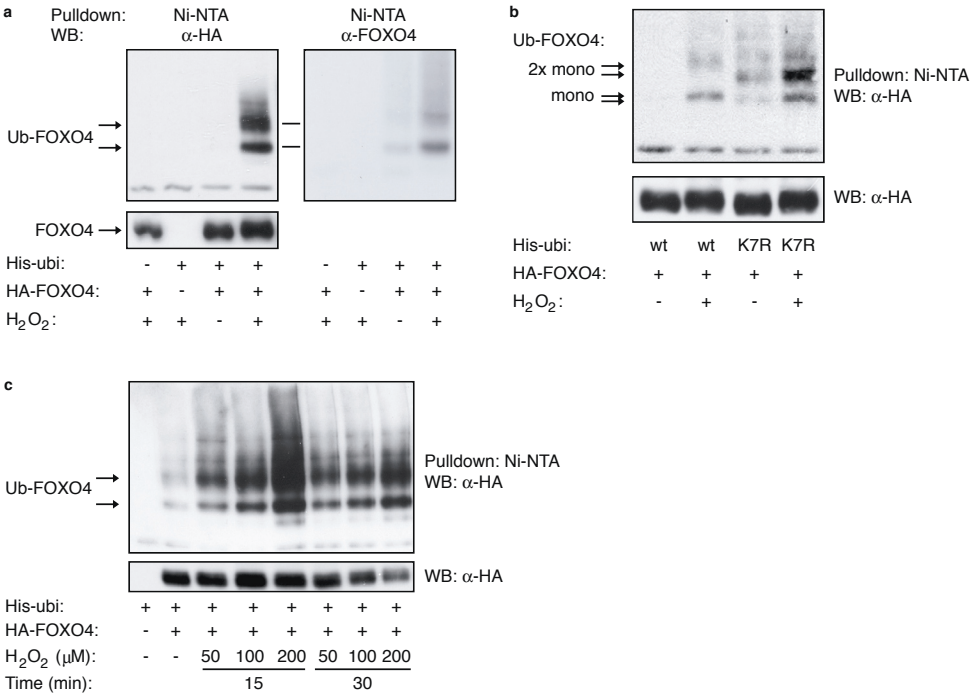

Figure 1. Hydrogen peroxide induces monoubiquitination of FOXO4. **(a)** Ubiquitinated proteins were precipitated using Ni-NTA agarose from HEK293T cells transfected with HA-FOXO4 and wild-type ubiquitin after treatment with 200 μM hydrogen peroxide for 30 minutes. Western blots were analysed for the presence of HA-FOXO4, using α-HA *(left)* and α-FOXO4 *(right)*. **(b, c)** As in **(a)**, *left panel*, using both wild-type and mutant ubiquitin in which all 7 lysine residues have been mutated to alanines **(b)**, or treated with hydrogen peroxide at the indicated concentrations and for the indicated times **(c)**.

FOXO4. To this end we first used ubiquitin-mediated fluorescence complementation as described by Fang and Kerppola (2004)[28]. A14 cells were transfected with the N-terminal part of YFP (aa 1-172) tagged to ubiquitin (YN-ubiquitin) and the C-terminal portion of YFP (aa 159-238) attached to FOXO4 (YC-FOXO4). YFP complementation was observed either in the nucleus or in a perinuclear structure (Fig. 2a). Neither we nor others, to our knowledge, ever observed clear perinuclear localisation of wildtype FOXO or various FOXO mutants. The perinuclear staining may be due to the production of a considerable amount of incorrectly folded YC-FOXO that is targeted for degradation by ubiquitination and deposited in an aggresome-like structure[29]. Therefore, to further examine the role of monoubiquitination we used an artificial fusion construct of ubiquitin fused to the N-terminus of FOXO4 (ubi-FOXO4). Previously, a similar strategy revealed the role of EGF receptor monoubiquitination in EGF receptor trafficking[30]. Consistent with the YFP complementation observed within the nucleus we observed significantly increased nuclear localisation of ubi-FOXO4 compared to wild-type FOXO4 (Fig. 2b). Taken together, these data implicate that monoubiquitination induces increased FOXO4 nuclear localisation.

Increased nuclear localisation of FOXO may result in increased FOXO transcriptional activity. Therefore, we tested the transcriptional activity of the ubi-FOXO4 fusion construct

on a FOXO-responsive reporter construct bearing six canonical FOXO-binding elements (6xDBE-luc[31]). Indeed, when corrected for expression levels, the ubi-FOXO4 construct showed approximately 10-times higher activity than wild-type FOXO4 (Fig. 2c). Notably, fusing moieties, like GFP or a tandem affinity purification (TAP) tag, to the N-terminus of FOXO4 does not change its activity (data not shown), indicating that the observed

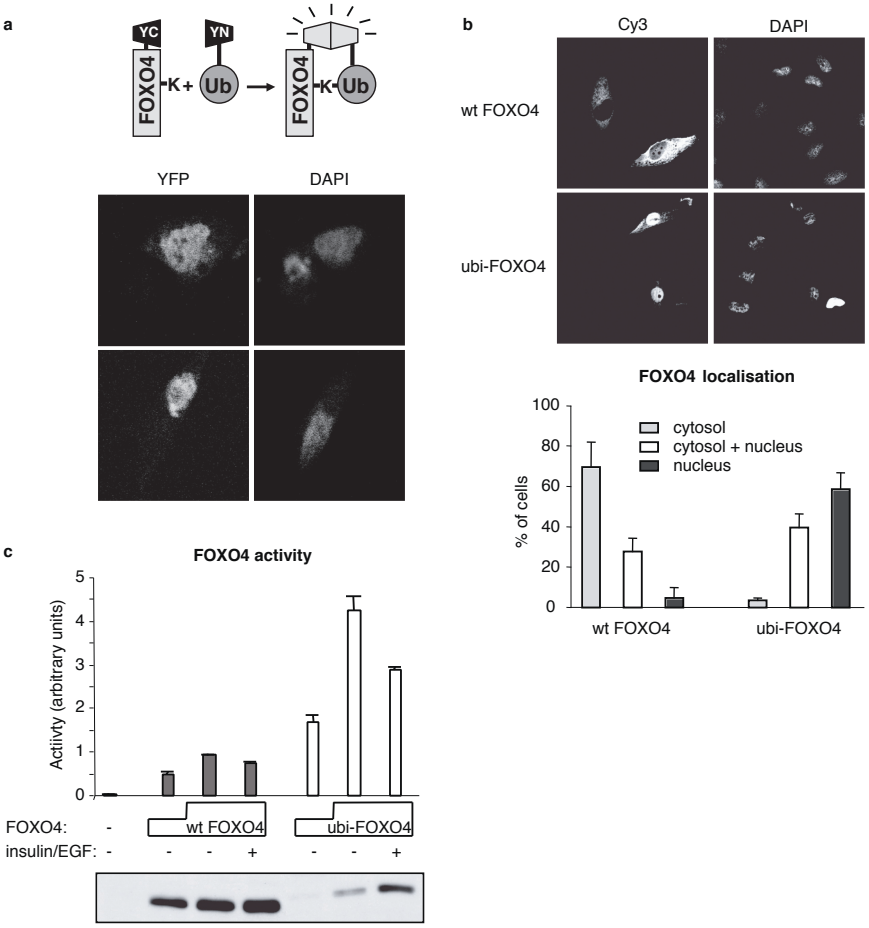

Figure 2. Effects of monoubiquitination on FOXO4. **(a)** Ubiquitin-mediated fluorescence complementation of YN-ubiquitin and YC-FOXO4 cotransfected in A14 cells (schematically, *top*). Cells were fixed and analysed using a confocal microsope *(bottom)*. Green, complemented YFP; blue, DAPI counterstaining. **(b)** A14 cells expressing wild-type or ubi-FOXO4 were stained using α-FOXO4 antibody. *(top)* Staining was analysed using a confocal microscope. Red, α-rabbit-Cy3; blue, DAPI counterstaining. *(bottom)* Fifty cells per experiment were scored for FOXO4 localisation. Data presented are the mean ± s.d. of two independent experiments. For full-colour images of panels (a) and (b), please refer to the back of the thesis. **(c)** HEK293T cells were transfected with a FOXO-responsive 6xDBE-luciferase construct together with the indicated FOXO constructs. Cells were left untreated or were treated with insulin and EGF for 16 hours. Wild-type FOXO4 and ubi-FOXO4 expression levels in whole cell extracts determined using an anti-FOXO4 antibody are shown below the corresponding bars. Representative data from three experiments are shown as mean ± s.d. of triplicates.

transcriptional increase is specific for the fused ubiquitin-moiety. Furthermore, the ubi-FOXO4 fusion can still be regulated by PKB signaling (Fig. 2c), compatible with previous results that suggested competition between insulin and oxidative stress signaling with respect to the regulation of FOXO4 activity[9,16].

Ubiquitination of proteins is counteracted by deubiquitinating enzymes (DUBs), which remove ubiquitin from proteins. An independent yeast-two-hybrid screen with a C-terminal fragment (amino acids 884-1065) of the DUB USP7/HAUSP (herpesvirus-associated ubiquitin-specific protease) was performed using a highly complex placental cDNA library previously described[32]. This screen allowed us to identify FOXO4, and more precisely the C-terminal part of FOXO4 (amino acids 360-501) as interacting with USP7. To confirm binding of USP7 to FOXO4 we first tagged FOXO4 with a TAP-tag, and isolated cell lines derived from the parental C2C12 line, stably expressing TAP-FOXO4 at near-endogenous levels (A.B.Brenkman *et al*., manuscript in preparation). Purification of TAP-FOXO4 from these cells showed binding of endogenous USP7 to TAP-FOXO4 (Fig. 3a). Second, we analyzed the interaction between FOXO4 and USP7 in more detail using exogenously expressed proteins. Indeed, HA-FOXO4 and myc-USP7 interacted and this interaction could be enhanced by hydrogen peroxide (Fig. 3b, *left panel*), consistent with induction of monoubiquitination by oxidative stress (Fig. 1). Moreover, FOXO3 could also bind to USP7 indicating that within the FOXO class of transcription factors this interaction is not specific to FOXO4 (Fig. 3b, *middle panel*). Recently, USP7 has been shown to bind to and to deubiquitinate p53[33,34]. Furthermore, a direct interaction between p53 and FOXO3 has been suggested[16]. To exclude the possibility that USP7 binds to FOXOs via p53, we exploited H1299 human lung carcinoma cells, which lack p53 (Fig. 3b, *right panel*). Also in these cells a hydrogen peroxide sensitive interaction between myc-USP7 and HA-FOXO4 was observed, indicating that this interaction occurs independently of p53.

The interaction between USP7 and proteins such as p53, Mdm2 and Mdmx results in deubiquitination of these USP7 substrates[33-35]. We therefore analyzed whether USP7 can deubiquitinate FOXO4. Co-expression of wild-type USP7, but not a catalytically inactive mutant (USP7-CS), almost completely blocked monoubiquitination of HA-FOXO4 induced by hydrogen peroxide (Fig. 3c and 3e). Again, this effect of USP7 is not dependent on p53 as the same result was obtained in H1299 cells (Fig. 3d). The family of DUB proteins consists of approximately sixty members[36]. The DUB USP14 is suggested to lack activity towards polyubiquitin chains and hence may be a specific DUB for monoubiquitinated proteins[37]. Therefore, to test the specificity of USP7 as a DUB for monoubiquitinated FOXO4 we analyzed the ability of HA-USP14 to deubiquitinate FLAG-FOXO4 (Fig. 3e). As shown, HA-USP14 displayed no activity towards FLAG-FOXO4 and in agreement with

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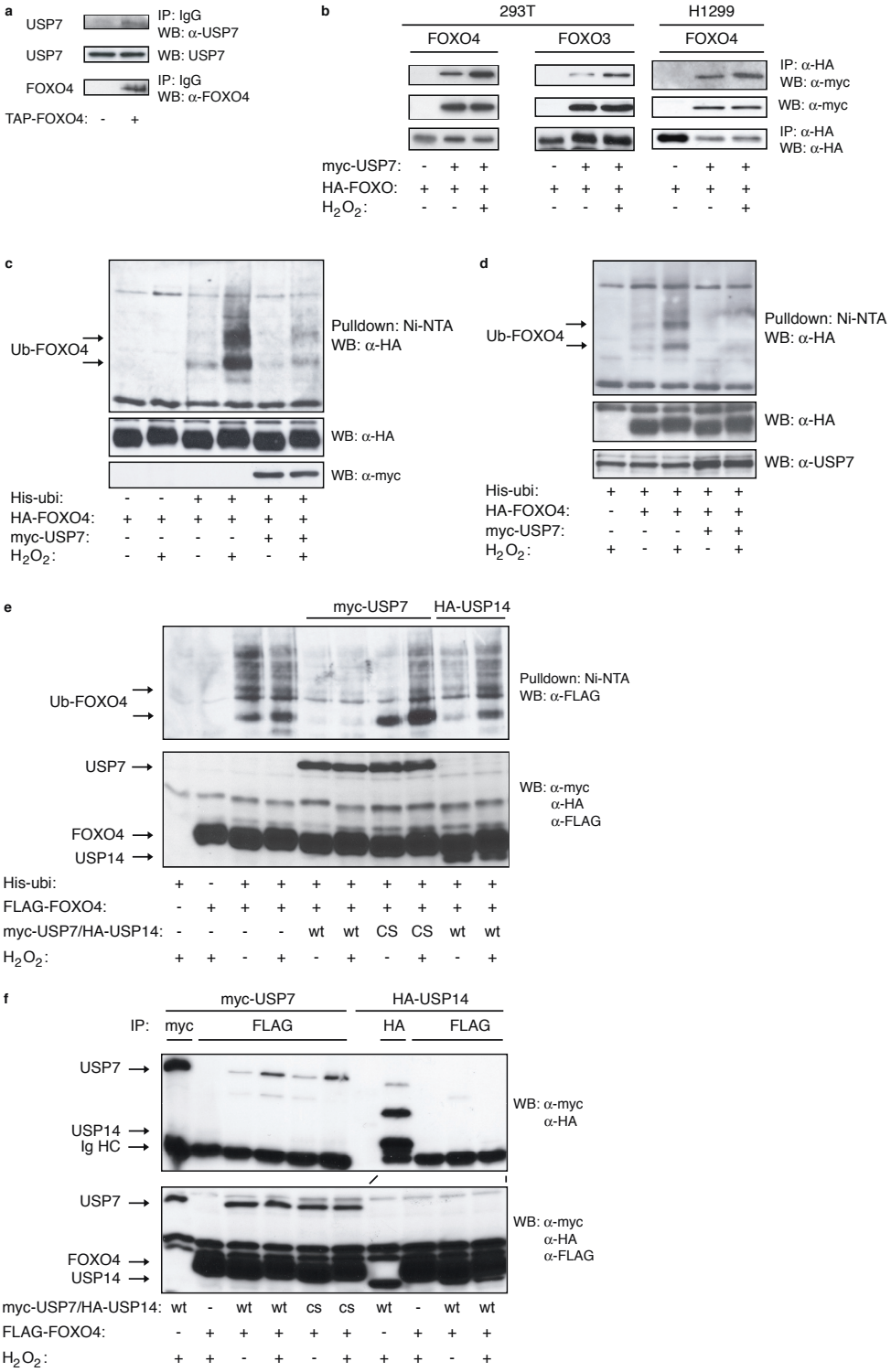
Figure 3. USP7 interacts with and deubiquitinates FOXO4. **(a)** Nuclei from C2C12 cells stably expressing TAP-FOXO4 were purified and TAP-FOXO4 was immunoprecipitated. Immunoprecipitation samples were analysed by western blotting using α-USP7 and α-FOXO4 antibodies. **(b)** HA-FOXO4 or HA-FOXO3 was immunoprecipitated from HEK293T *(left and middle)* or H1299 (p53 null, *right*) cells treated with 200 μM hydrogen peroxide for 30 minutes. Western blots were probed for USP7 and the indicated FOXO protein. **(c,d)** Ubiquitinated proteins were precipitated from HEK293T **(c)** or H1299 **(d)** cells treated with hydrogen peroxide (as in Fig. 1b) in the absence or presence of wild-type USP7. Samples were analysed for HA-FOXO4 levels. **(e)** As in **(c)**, to show dependence on enzymatic activity and specificity of USP7, respectively myc-USP7-CS and HA-USP14 were used. **(f)** Co-immunoprecipitation of FOXO4 and USP7 or USP14 in HEK293T cells treated with hydrogen peroxide (as in **(b)**).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this we did not observe binding of HA-USP14 to FLAG-FOXO4 (Fig. 3f), suggesting that the interaction of FOXO4 with USP7 and the deubiquitination of FOXO4 by USP7 are specific.
Based on our observation that monoubiquitination induces nuclear localisation and increased FOXO transcriptional activity we analyzed the effect of USP7 on FOXO transcriptional activity. Overexpression of wild-type USP7, but not that of the catalytically inactive mutant inhibited FOXO transcriptional activity as measured in a reporter assay using the 6xDBE-luc (Fig. 4a). To assess the involvement of endogenous USP7 in the control of endogenous FOXO we used siRNA against endogenous USP7 and FOXOs. Two independent siRNAs against endogenous USP7 induced expression of $p27^{kip1}$, a well-established FOXO4 target gene[38] (Fig. 4b and 4c). This is consistent with the observed inhibition of FOXO transcriptional activity by overexpressed USP7. However, to confirm that this increase in $p27^{kip1}$ expression was dependent on the presence of FOXO we cosilenced FOXOs by siRNA. siRNA against endogenous FOXO proteins abolished the increase in $p27^{kip1}$ expression induced by USP7 knockdown (Fig. 4c), suggesting that indeed the increase of $p27^{kip1}$ expression induced by siRNA against USP7 is mediated by FOXO.
Finally, we tested whether the observed ubiquitination is involved in the regulation of FOXO protein stability (Fig. 4d). Under normal conditions we determined a half-life of FOXO4 of approximately 9 hours, in agreement with results obtained by Matsuzaki *et al*. (2003)[20]. Treatment of cells with hydrogen peroxide or co-expression of USP7 did not significantly change HA-FOXO4 half-life. In this experiment a puromycine resistance marker was co-expressed and transfected cells were selected by adding puromycin to the culture medium. This enabled us to not only determine the half-life of the transfected HA-FOXO4, but that of endogenous FOXO3 as well. Again expression of USP7 and/or treatment of cells with hydrogen peroxide did not considerably change FOXO3 half-life. In agreement with this we noted that knockdown of USP7 did not increase FOXO3 levels either (Fig. 4c). Taken together, these data show that over the time course studied, hydrogen peroxide-induced ubiquitination and USP7-mediated deubiquitination have no major effect on FOXO protein stability. This reinforces our observation that FOXO4 activity under these conditions is regulated through monoubiquitination.

Here, we report monoubiquitination of FOXO4 following increased cellular oxidative stress due to treatment of cells with hydrogen peroxide. FOXO4 binds to the deubiquitinating enzyme USP7/HAUSP and this binding is increased by cellular oxidative stress. Binding of USP7 results in removal of the ubiquitin moiety on FOXO4 induced by hydrogen peroxide treatment. We show that monoubiquitinated FOXO4 is localised within the nucleus and displays increased transcriptional activity. Consequently, we show that overexpression of USP7 inhibits, and loss of USP7 expression stimulates FOXO4 activity. Since FOXO4 is known to be regulated by PI3K/PKB signaling, our results suggest that targeting USP7 by small-molecule inhibitors could be an alternative way to activate FOXO and thus to negatively regulate the oncogenic PI3K/PKB signaling pathway, which may be of therapeutic potential in cancer.
Ubiquitination of FOXOs has been observed in several studies. However, these studies all relate to polyubiquitination[19-21]. Initially, it was shown that PKB substrates in general, including FOXOs, are targeted for polyubiquitination by growth factors through PI3K/PKB signaling[20,21]. However, in these studies the kinetics of polyubiquitination, (detectable after ~12 hrs), as well as of protein degradation are relatively slow (half-life of ~9 hrs). In chicken

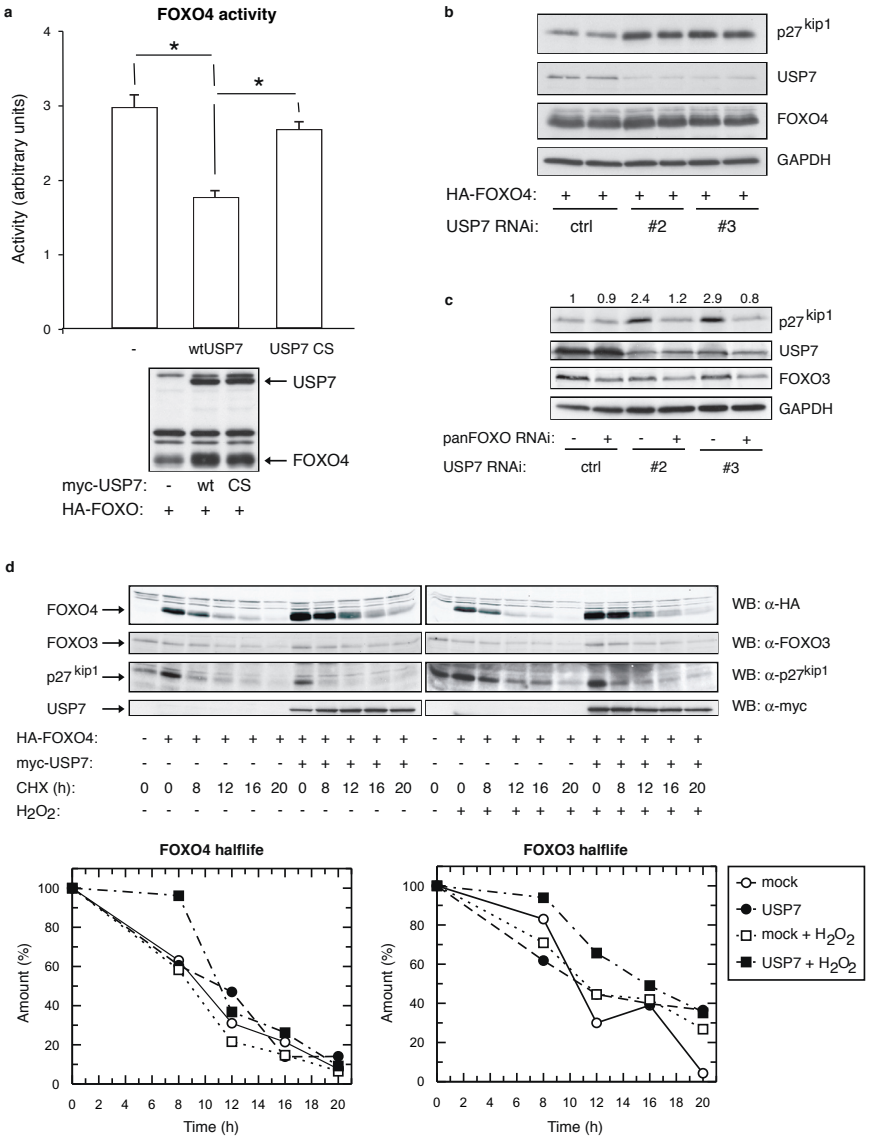

Figure 4. USP7 regulates FOXO activity. **(a)** Luciferase assay using HEK293T cells expressing a FOXO-responsive luciferase-reporter, Tk-Renilla, HA-FOXO4 and wild-type USP7 or USP7-CS. Representative data are shown as mean ± s.d. of triplicates. Asterisk indicates $p < 0.001$ (Student's t-test). **(b)** Western blot analysis for $p27^{kip1}$ expression of puromycin-selected HEK293T cells transfected with HA-FOXO4 and a non-targeting siRNA oligo or one of two independent siRNAs against USP7. **(c)** Western blot analysis for $p27^{kip1}$ expression of HEK293T cells transfected with siRNAs against USP7 and siRNA targeting all three FOXO homologues. A non-targeting siRNA oligo was used as a control. The relative level of expression as determined with the Odyssey Infra-red imaging system is indicated above the blots for $p27^{kip1}$. **(d)** Half-life analysis of HEK293T cells expressing HA-FOXO4 and Myc-USP7. Puromycin-selected cells were treated with 50 μM hydrogen peroxide for 24 hours and with cycloheximide for the indicated times. Protein levels were equalized and samples were analysed using the indicated antibodies *(top)*. Using the Odyssey Infra-red imaging system relative expression levels were calculated and displayed in a graph *(bottom)*.

embryo fibroblasts (CEFs) transformed through overexpression of activated forms of PI3K and/or PKB, PDGF treatment resulted in a strong reduction in FOXO half-life[22]. This is probably due to increased expression of the F-box protein Skp2 in these transformed CEFs since it has been shown that overexpression of Skp2 enhances FOXO polyubiquitination[19] and that loss of PTEN and thus activation of PKB enhances the expression of Skp2[39]. These data would suggest that enhanced degradation of PKB targets such as FOXO is a gain of function for cells transformed through the PI3K pathway and would be consistent with the observation that forced FOXO activation inhibits cellular transformation of these cells[38]. In normal cells the kinetics of FOXO polyubiquitination are clearly different from the kinetics of ubiquitination we observe here, supporting the notion that cellular oxidative stress induces monoubiquitination of FOXO.

Recently, it has become clear that remarkable similarities exist between p53 and FOXO. Not only in their shared ability to induce cell cycle arrest and apoptosis but apparently also in their mode of regulation. Previously we and others showed that similar to p53, FOXOs are regulated by acetylation by p300/CBP and deacetylation by Sir2[15-18,40,41]. Here we extend this similarity by showing regulation of FOXO by monoubiquitination and USP7. We note that the commonality between p53 and FOXO in their cellular response as well as in their shared regulatory network may have important implications for our understanding of aging in higher eukaryotes as this process is likely to involve both the FOXO and the p53 pathway.

Methods

Cell culture, transfection and treatment.

HEK293T, A14 (human insulin receptor overexpressing mouse NIH3T3 cells[42]) and H1299 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glutamine, penicillin/streptomycin and 10% FBS. In all experiments cells were cultured in the presence of 10% FBS. HEK293T cells were transiently transfected using FuGENE6 reagent according to the manufacturer (Roche). A14 cells were transfected using the calcium phosphate method. H1299 cells were transfected using PEI (1 μg/μl, polyethylenimine, Polysciences) in a 2:1 ratio to the amount of DNA. Total amounts of transfected DNA were equalized using pBluescript KSII+. siRNA oligos were transfected using Xtremegene according to the manufacturer (Roche).

Cycloheximide (Sigma), puromycin, insulin, epidermal growth factor (EGF) and hydrogen peroxide (H_2O_2, Merck) were added at 10 μg/ml, 2 μg/ml, 1 μg/ml, 20 ng/ml and 50-200 μM respectively as indicated.

Plasmids, oligos and recombinant proteins.

pMT2-HA-FOXO4[6], GLOFLAG3-FLAG-FOXO4[15], pBabe-puro[43] and 6xDBE[31] have been described before.

pMT2-HA-ubiquitin-FOXO4 was made by inserting SalI (5'-ACGCGTCGACAATGCAAATTTT CGTCAAAAC-3', 5'- ACGCGTCGACACTAGCGAATTCTGACCAC-3') digested PCR product (YN-ubiquitin, kindly provided by T. Kerppola, was used as a template) into SalI digested pMT2-HA-FOXO4. YC-FOXO4 was created by ligating an XhoI/Klenow-blunted SalI fragment from pMT2-HA-FOXO4 into XhoI/Klenow-blunted HindIII pcDNA-YFP[159-238], kindly provided by J.A. Rodriguez. pBabe-TAP-FOXO4 was created using Gateway Technology (Invitrogen) and full length FOXO4 cDNA. Resulting plasmids were verified by automated sequencing. GloMyc-USP7 and GloMyc-USP7-C223S (USP7-CS) have been described before[35]. HA-USP14 and His-ubiquitin were kind gifts of A. Borodovsky and D. Bohmann respectively. pRL-TK (Tk-Renilla luciferase) was purchased from Promega.

Non-targeting siRNA duplex (siCONTROL),_siRNA against panFOXO (sense sequence: 5'-AAGGAUAAGGGCGACAGACC-dTdT-3'), and duplexes to downregulate USP7 expression (duplex 2, sense sequence: 5'-ACCCUUGGACAAUAUUCCUdTdT-3'; duplex 3, sense sequence: 5'-AGUCGUUCAGUCGUCGUAUdTdT-3') were purchased from Dharmacon RNA technologies.

Antibodies.
Monoclonal 12CA5 and 9E10 antibodies were produced using hybridoma cell lines and purification on proteinA-agarose columns. Monoclonal antibodies recognizing the FLAG-M2 epitope, $p27^{kip1}$, the penta-His epitope and GAPDH were obtained from Sigma, Transduction Laboratories, Qiagen and Chemicon respectively. Polyclonal antibodies recognizing FOXO3 and USP7 (BL851) were purchased from Santa Cruz Biotechnology and Bethyl Laboratories respectively. The polyclonal antibody recognizing FOXO4[38] has been described before.

Western blot analysis.
Protein samples were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF, Immobilon). Western blot analysis was performed under standard conditions using the indicated antibodies. For quantification of protein levels, membranes were probed with fluorophore-conjugated antibodies and analysed using the Odyssey Infra-red imaging system and software according to the manufacturer (LI-COR).

FOXO activity.
To determine the expression of endogenous $p27^{kip1}$, HEK293T cells were transfected with HA-FOXO4 and pBabe-puro using FuGENE and/or with siRNA oligos. Twenty-four hours before harvest 2 μg/ml puromycin was added to the cultures to select for transfected cells. Cells were lysed in RIPA lysis buffer, equalized using Bradford reagent and 5x Laemmli sample buffer was added to a final concentration of 1x. Samples were analysed by Western blot analysis.
For luciferase assays, HEK293T cells transiently transfected with a luciferse reporter construct bearing six canonical FOXO binding elements (6xDBE-luc) were transfected with the indicated constructs. Luciferase counts were normalized using Tk-Renilla-luciferase. Samples were analysed according to the manufacturer (Promega). Representative data are shown as mean ± s.d. of triplicates. If indicated a Student's t-test was performed using GraphPad statistical analysis.

Co-immunoprecipitation.
HEK293T or H1299 cells were transfected with the indicated constructs. Cells were left untreated or were treated with hydrogen peroxide for 30 minutes. Forty hours posttransfection cells were lysed. FOXO proteins were immunoprecipitated from whole cell lysates (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1.5 mM $MgCl_2$, 5 mM EDTA, 100 mM NaCl, protease inhibitors). TAP-FOXO4 was immunoprecipitated from isolated nuclei (isolated using a standard nuclear-cytosolic fractionation method) from C2C12 cells stably expressing TAP-FOXO4 at near-endogenous levels, using IgG sepharose beads. Immunoprecipitation samples were analysed by Western blot analysis.

Ubiquitination.
HEK293T or H1299 cells were transfected with the indicated constructs. Cells were treated with hydrogen peroxide with the indicated concentrations and for the indicated times. Forty hours posttransfection cells were lysed in urea lysis buffer (8M urea, 100 mM Na_2HPO_4/NaH_2PO_4 [pH 8.0], 10 mM Tris-HCl [pH 8.0], 0.2% TritonX-100, 5 mM NEM, protease inhibitors). Ubiquitinated proteins were precipitated from urea lysates using Ni-NTA agarose beads essentially as described[25] and analysed by Western blotting for the presence of FOXO4.

FOXO half-life analysis.
HEK293T were transfected with pBabe-puro plasmid and the indicated constructs. Twenty-four hours posttransfection puromycin and 50 μM hydrogen peroxide were added for 24 hours. Cells were

treated with cycloheximide for 8, 12, 16 or 20 hours and lysed in RIPA lysis buffer. Protein levels were equalized using Bradford reagent. Samples were analysed by Western blotting. Relative FOXO4 and FOXO3 protein expression levels were calculated.

Ubiquitin-mediated fluorescence complementation.
Ubiquitin-mediated fluorescence complementation was essentially done as described[28]. A14 cells were transfected with YC-FOXO4 and YN-ubiquitin. Forty hours posttransfection cells were fixed using 4% paraformaldehyde and nuclei were stained using DAPI. Fluorescence was visualized using a Zeiss LSM 510 confocal microcope.

Immunofluorescence.
A14 cells were transfected with HA-FOXO4, HA-ubiquitin-FOXO4. Forty hours posttransfection cells were fixed in 4% paraformaldehyde and the different FOXO4 proteins were stained using α-FOXO4 antibody and anti-rabbit-Cy3. Nuclei were visualized using DAPI. Fluorescence was captured using a Zeiss LSM 510 confocal microcope. Fifty cells per experiment were scored for FOXO4 localisation (nuclear, cytosolic or both). Data presented are the mean ± s.d. of two independent experiments.

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References

1. Burgering, B. M. & Kops, G. J. Cell cycle and death control: long live Forkheads. *Trends Biochem Sci* **27**, 352-60 (2002).
2. Katoh, M. Human FOX gene family (Review). *Int J Oncol* **25**, 1495-500 (2004).
3. Tran, H. et al. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science* **296**, 530-4 (2002).
4. Kops, G. J. et al. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* **419**, 316-21 (2002).
5. Brunet, A. et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-68 (1999).
6. Kops, G. J. et al. Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**, 630-4 (1999).
7. Tran, H., Brunet, A., Griffith, E. C. & Greenberg, M. E. The many forks in FOXO's road. *Sci STKE* **2003**, RE5 (2003).
8. De Ruiter, N. D., Burgering, B. M. & Bos, J. L. Regulation of the Forkhead transcription factor AFX by Ral-dependent phosphorylation of threonines 447 and 451. *Mol Cell Biol* **21**, 8225-35 (2001).
9. Essers, M. A. et al. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *Embo J* **23**, 4802-12 (2004).
10. Wang, M. C., Bohmann, D. & Jasper, H. JNK extends life span and limits growth by antagonizing cellular and organism-wide responses to insulin signaling. *Cell* **121**, 115-25 (2005).
11. Oh, S. W. et al. JNK regulates lifespan in Caenorhabditis elegans by modulating nuclear translocation of forkhead transcription factor/DAF-16. *Proc Natl Acad Sci U S A* **102**, 4494-9 (2005).

12. Nanji, M., Hopper, N. A. & Gems, D. LET-60 RAS modulates effects of insulin/IGF-1 signaling on development and aging in Caenorhabditis elegans. *Aging Cell* **4**, 235-45 (2005).
13. Mahmud, D. L. et al. Phosphorylation of forkhead transcription factors by erythropoietin and stem cell factor prevents acetylation and their interaction with coactivator p300 in erythroid progenitor cells. *Oncogene* **21**, 1556-62 (2002).
14. Fukuoka, M. et al. Negative regulation of forkhead transcription factor AFX (Foxo4) by CBP-induced acetylation. *Int J Mol Med* **12**, 503-8 (2003).
15. van der Horst, A. et al. FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). *J Biol Chem* **279**, 28873-9 (2004).
16. Brunet, A. et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* **303**, 2011-5 (2004).
17. Motta, M. C. et al. Mammalian SIRT1 represses forkhead transcription factors. *Cell* **116**, 551-63 (2004).
18. Daitoku, H. et al. Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. *Proc Natl Acad Sci U S A* **101**, 10042-7 (2004).
19. Huang, H. et al. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. *Proc Natl Acad Sci U S A* **102**, 1649-54 (2005).
20. Matsuzaki, H., Daitoku, H., Hatta, M., Tanaka, K. & Fukamizu, A. Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proc Natl Acad Sci U S A* **100**, 11285-90 (2003).
21. Plas, D. R. & Thompson, C. B. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. *J Biol Chem* **278**, 12361-6 (2003).
22. Aoki, M., Jiang, H. & Vogt, P. K. Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. *Proc Natl Acad Sci U S A* **101**, 13613-7 (2004).
23. Onishi, Y. et al. Identification of mono-ubiquitinated LDH-A in skeletal muscle cells exposed to oxidative stress. *Biochem Biophys Res Commun* **336**, 799-806 (2005).
24. Cao, C. et al. Ubiquitination and degradation of the Arg tyrosine kinase is regulated by oxidative stress. *Oncogene* **24**, 2433-40 (2005).
25. Peng, J. et al. A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol* **21**, 921-6 (2003).
26. Sigismund, S., Polo, S. & Di Fiore, P. P. Signaling through monoubiquitination. *Curr Top Microbiol Immunol* **286**, 149-85 (2004).
27. Dikic, I. Mechanisms controlling EGF receptor endocytosis and degradation. *Biochem Soc Trans* **31**, 1178-81 (2003).
28. Fang, D. & Kerppola, T. K. Ubiquitin-mediated fluorescence complementation reveals that Jun ubiquitinated by Itch/AIP4 is localized to lysosomes. *Proc Natl Acad Sci U S A* **101**, 14782-7 (2004).
29. Lelouard, H. et al. Dendritic cell aggresome-like induced structures are dedicated areas for ubiquitination and storage of newly synthesized defective proteins. *J Cell Biol* **164**, 667-75 (2004).
30. Haglund, K. et al. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat Cell Biol* **5**, 461-6 (2003).
31. Furuyama, T., Nakazawa, T., Nakano, I. & Mori, N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* **349**, 629-34 (2000).
32. Colland, F. et al. Functional proteomics mapping of a human signaling pathway. *Genome Res* **14**, 1324-32 (2004).
33. Li, M. et al. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* **416**, 648-53 (2002).
34. Li, M., Brooks, C. L., Kon, N. & Gu, W. A dynamic role of HAUSP in the p53-Mdm2 pathway. *Mol Cell* **13**, 879-86. (2004).
35. Meulmeester, E. et al. Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. *Mol Cell* **18**, 565-76 (2005).
36. Amerik, A. Y. & Hochstrasser, M. Mechanism and function of deubiquitinating enzymes. *Biochim Biophys Acta* **1695**, 189-207 (2004).

37. Yin, L., Krantz, B., Russell, N. S., Deshpande, S. & Wilkinson, K. D. Nonhydrolyzable diubiquitin analogues are inhibitors of ubiquitin conjugation and deconjugation. *Biochemistry* **39**, 10001-10 (2000).
38. Medema, R. H., Kops, G. J., Bos, J. L. & Burgering, B. M. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782-7 (2000).
39. Mamillapalli, R. et al. PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2). *Curr Biol* **11**, 263-7 (2001).
40. Luo, J. et al. Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* **107**, 137-48 (2001).
41. Vaziri, H. et al. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* **107**, 149-59 (2001).
42. Burgering, B. M. & Coffer, P. J. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599-602 (1995).
43. Medema, R. H., Herrera, R. E., Lam, F. & Weinberg, R. A. Growth suppression by p16ink4 requires functional retinoblastoma protein. *Proc Natl Acad Sci U S A* **92**, 6289-93 (1995).

CHAPTER 5

Activation of FoxO transcription factors contributes to the antiproliferative effect of cAMP

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Activation of FoxO transcription factors contributes to the antiproliferative effect of cAMP

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cAMP is a potent inhibitor of cell proliferation in a variety of cell lines. Downregulation of cyclin D1 and upregulation of the cell cycle inhibitor $p27^{Kip1}$ are two mechanisms by which cAMP may induce a G1-arrest. Here we show that cAMP inhibits proliferation of cells that constitutively express cyclin D1 or are deficient for Rb, demonstrating that changes in these cell cycle regulators do not account for the cAMP-induced growth effects in mouse embryo fibroblasts (MEFs). Interestingly, the antiproliferative effect of cAMP mimics the effect previously observed for FoxO transcription factors. These transcription factors are under negative control of protein kinase B (PKB). We show that in MEFs cAMP strongly induces transcriptional activation of FoxO4 through the inhibition of PKB. Accordingly, not only $p27^{Kip1}$ but also the FoxO target MnSOD is upregulated by cAMP. Importantly, introduction of dominant-negative FoxO partially rescues cAMP-induced inhibition of proliferation. From these results we conclude that inhibition of PKB and subsequent activation of FoxO transcription factors mediates an antiproliferative effect of cAMP.

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Introduction

cAMP is an important regulator of proliferation, through modulation of cAMP-dependent protein kinase A (PKA). Depending on the cell type, cAMP can either stimulate or inhibit proliferation. Inhibition of proliferation by cAMP takes place in the G1-phase of the cell cycle, and involves the cyclin-dependent kinase (CDK) complexes cyclin D1-CDK4/6 and cyclin E/A-CDK2 as well as the CDK inhibitors (CKIs) $p21^{Cip1}$ and $p27^{Kip1}$. Indeed, inhibition of cell proliferation by cAMP has most often been reported to correlate with and partially depend on a decrease in cyclin D1 or an increase in $p27^{Kip1}$ (Sewing *et al*., 1993; Kato *et al*., 1994; Vadiveloo *et al*., 1997; Williamson *et al*., 1997; Kim *et al*., 2001b; van Oirschot *et al*., 2001). The main pathways involved in the regulation of $p27^{Kip1}$ and cyclin D1 are the MAPK and PI3K pathways (Lavoie *et al*., 1996; Aktas *et al*., 1997; Muise-Helmericks *et al*., 1998; Medema *et al*., 2000). Inhibition of the MAPK pathway by cAMP is well documented and takes place at the level of Raf kinase (Burgering *et al*., 1993; Cook and McCormick, 1993), which is phosphorylated by PKA (Wu *et al*., 1993; Hafner *et al*., 1994; Mischak *et al*., 1996; Dumaz *et al*., 2002). Inhibition of the MAPK pathway alone, however, cannot account for the G1-arrest induced by cAMP (McKenzie and Pouyssegur, 1996; Dumaz *et al*., 2002; Balmanno *et al*., 2003). Also, the PI3K pathway may be inhibited by cAMP, but this has only been shown in a restricted number of cell types and the mechanism is elusive. It has been suggested that cAMP inhibits the pathway at the level of PI3K itself, resulting in disturbed PIP3 formation and PDK1 membrane localization (Kim *et al*., 2001a; Lee and Kay, 2003).

Cyclin D1 in complex with CDK4 or CDK6 promotes proliferation by inhibiting the retinoblastoma (Rb) protein, which on its turn blocks E2F transcription factors and thus the induction of genes involved in DNA replication (Sherr and Roberts, 1999). MAPK regulates cyclin D1 levels transcriptionally, via the ATF/CREB family of transcription factors and the CREB response element (CRE) in the cyclin D1 promoter (Lee *et al*., 1999). The PI3K/PKB pathway regulates cyclin D1 levels transcriptionally via regulation of FoxO transcription factors (Ramaswamy *et al*., 2002; Schmidt *et al*., 2002), at the translational level involving p70S6 kinase (Muise-Helmericks *et al*., 1998; Takuwa *et al*., 1999), and post-transcriptionally by targeting it for degradation using GSK3β (Diehl *et al*., 1998).

The cell cycle inhibitor $p27^{Kip1}$ acts during late G1-phase by binding and inhibiting CDK2-cyclin E/A complexes (Sherr and Roberts, 1999). Cells can only progress through the cell cycle when $p27^{Kip1}$ is dissociated from the CDK2-cyclin E/A complexes, and this is

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in general achieved by degradation of $p27^{Kip1}$ (Pagano *et al*., 1995; Malek *et al*., 2001). Degradation of $p27^{Kip1}$ is usually initiated through phosphorylation by CDK2 (Muller *et al*., 1997; Vlach *et al*., 1997), but may also involve calpains (Delmas *et al*., 2003), caspases (Loubat *et al*., 1999) or jab1 (Tomoda *et al*., 1999). Furthermore, $p27^{Kip1}$ protein can be regulated at the transcriptional level by FoxO transcription factors (Medema *et al*., 2000) or by translational regulation (Agrawal *et al*., 1996; Hengst and Reed, 1996). Finally, $p27^{Kip1}$ activity can also be diminished by sequestering it in cyclin D-CDK complexes (Sherr and Roberts, 1999) or by nuclear exclusion, which is preceded by phosphorylation of $p27^{Kip1}$ by protein kinase B (PKB) or KIS (Boehm *et al*., 2002; Fujita *et al*., 2002; Liang *et al*., 2002; Shin *et al*., 2002; Viglietto *et al*., 2002).

The picture that emerges is one in which cAMP affects different cell cycle components by multiple mechanisms. This, together with cell-type differences has obstructed identification of a uniform mechanism of cAMP-induced cell cycle arrest. For example, it has been hypothesized that the combined downregulation of cyclin D1 and upregulation of $p27^{Kip1}$ would be sufficient (L'Allemain *et al*., 1997), but actual proof for this is lacking. Interestingly, upregulation of $p27^{Kip1}$ and downregulation of cyclin D1 is part of the program by which FoxO transcription factors induce a G1 arrest in nonhematopoietic cells (Medema *et al*., 2000; Ramaswamy *et al*., 2002; Schmidt *et al*., 2002). These FoxO transcription factors are negatively regulated by the PI3K/PKB pathway, and direct phosphorylation of FoxOs by PKB leads to nuclear exclusion (Brunet *et al*., 1999; Brownawell *et al*., 2001). Therefore, we have tested here the involvement of FoxO in a cAMP-induced growth arrest. We show that in mouse embryo fibroblasts (MEFs) apart from the MAPK pathway, the PKB/FoxO pathway plays a prominent role in the antiproliferative effect of cAMP.

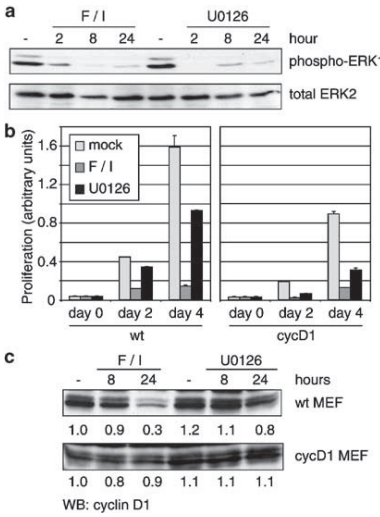

Figure 1 Inhibition of the MAPK pathway and cyclin D1 by cAMP is not sufficient for induction of a G1-arrest. (**a**) ERK-phosphorylation is inhibited to the same extent by cAMP and U0126. MEFs were stimulated for the indicated time points with forskolin/IBMX (F/I), U0126 or were left untreated. The amount of phosphorylated ERK1 and -2 and total ERK2 were visualized on a Western blot. (**b**) Wild type (wt) and cycD1 MEFs are sensitive to cAMP-induced growth inhibition. Proliferation of wt and cycD1 MEFs was measured upon mock, forskolin/IBMX or U0126 treatment. The amount of cells was measured using crystal violet staining as read-out at 0, 2 and 4 days after stimulation. Error bars represent standard deviation (s.d.). (**c**) Cyclin D1 protein levels are downregulated by cAMP in wt, but not in cycD1 MEFs. Wt and cycD1 MEFs were stimulated with forskolin/IBMX or U0126 for the indicated time points. Total amounts of cyclin D1 were visualized by immunoblotting and quantified by densitometric scanning and corrected for a control signal. Fold inductions in comparison to lane 1 were calculated and plotted below the panels

Results

Inhibition of the MAPK pathway and cyclin D1 by cAMP is not sufficient for the induction of a G1-arrest in cycling MEFs

We choose to study the growth inhibitory effects of cAMP in MEFs based on the fact that these cells are known to be responsive to cAMP and the existence of MEFs, derived from mice in which various cell cycle regulators have been deleted by targeted gene disruption. First, we compared the effects of the cAMP-elevating compound forskolin (in combination with the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX)) and that of the MEK-inhibitor U0126 on wild-type MEFs grown in the presence of serum. Whereas both compounds inhibited the ERK pathway equally well (Figure 1a), forskolin/IBMX was clearly more effective in inhibiting proliferation (Figure 1b left panel). We then investigated the level of cyclin D1 under both conditions and found that cyclin D1 was clearly downregulated by forskolin/IBMX, but only weakly and transiently downregulated by U0126 (Figure 1c upper panel). To see if this difference in cyclin D1 expression could explain the difference in growth rate, we made use of cycD1 MEFs, in which cyclin D1 is expressed under the control of a constitutive promoter at levels comparable to those seen in proliferating cells (Schmidt *et al*., 2002). As expected, forskolin/IBMX did not change cyclin D1 expression in these cells (Figure 1c lower panel). Despite this, the inhibition of growth by forskolin/IBMX was only slightly less as compared to that of wild-type cells. Furthermore, forskolin/IBMX was still more potent in inhibiting proliferation than U0126 (Figure 1b right panel and Table 1). These findings are consistent with other data showing a partial involvement of the MAPK pathway and/or cyclin D1 in a cAMP-induced cell cycle arrest (McKenzie and Pouyssegur, 1996; Dumaz *et al*., 2002; Balmanno *et al*., 2003).

Table 1 Comparison of growth inhibition by cAMP and U0126 in different cell lines

	Treatment			
	Forskolin/IBMX		*U0126*	
Cell type	*Inhibition (%)*	P-*value*	*Inhibition (%)*	P-*value*
wt MEF ($n=3$)	90±1.7	—	43±4.2	—
cycD1 MEF ($n=2$)	86±0.3	0.0452*	65±1.2	0.0062*
p27$^{-/-}$ MEF ($n=4$)	64±6.6	0.0012*	51±7.1	0.1712
Rb$^{-/-}$ 3T3 ($n=2$)	82±1.8	0.0178*	34±3.8	0.0983

Cells were treated with forskolin/IBMX, U0126 or left untreated, and were grown until untreated cells reached confluency. At this time point, the amount of cells was measured and the percentage of growth inhibition in comparison to untreated cells was calculated of two to four independent experiments. The percentage of growth inhibition plus standard deviations and the statistical significance (*P*-value) in comparison to wt MEFs are shown. *P*-values with higher confidence than 95% are indicated with an asterix (*)

Subsequently, we tested the role of Rb, which acts downstream of cyclin D1, in the antiproliferative effect of cAMP. However, proliferation of Rb-deficient 3T3 cells was still inhibited by forskolin/IBMX, to a comparable level as seen in cycD1 MEFs (Figure 2a and Table 1). Interference of apoptosis in the measurement of proliferation could be excluded, as there was no significant increase in apoptotic cells (Figure 2b, sub-G1 fraction). Furthermore, forskolin/IBMX clearly induced a G1-arrest in the Rb$^{-/-}$ cells, comparable to wild-type cells (Figure 2b and Supplementary Figure), whereas U0126 led to a minor increase. Changes seen in cyclin D1 levels after forskolin/IBMX treatment were identical to those seen in wild-type MEFs (Figure 2c). We conclude that promoting the inhibitory action of Rb on E2F is not the only mechanism for a cAMP-induced G1 arrest in MEFs. This is in agreement with our observation that constitutively expressing cyclin D1 hardly affects the antiproliferative effect of cAMP.

p27^{Kip1} plays an important role in inhibition of proliferation by cAMP

To see if in addition to MAPK, p27^{Kip1} is involved in cAMP-induced G1-arrest of MEFs, we first measured the protein levels of this cell cycle inhibitor. A clear increase in p27^{Kip1} levels up to at least 48 h after stimulation with forskolin/IBMX was seen, whereas U0126 gave a weaker and more transient increase (Figure 3a). Furthermore, stimulation with forskolin/IBMX induced translocation of p27^{Kip1} to the nucleus (Figure 3b), where it is known to exert its inhibitory action. In contrast, hardly any p27^{Kip1} translocated after stimulation with U0126 (data not shown). To investigate the importance of p27^{Kip1} for inhibition of proliferation by cAMP, we made use of p27^{Kip1}-deficient MEFs. Although these cells grow slower than wild-type cells, proliferation of the p27$^{-/-}$ MEFs was significantly less inhibited by treatment with forskolin/IBMX as compared to wild-type cells. Furthermore, the residual inhibition of proliferation by cAMP was almost comparable to the effect of U0126 on proliferation (Figure 3c and Table 1). In addition, cell cycle analysis showed that the increase in p27^{Kip1}-deficient cells in G1

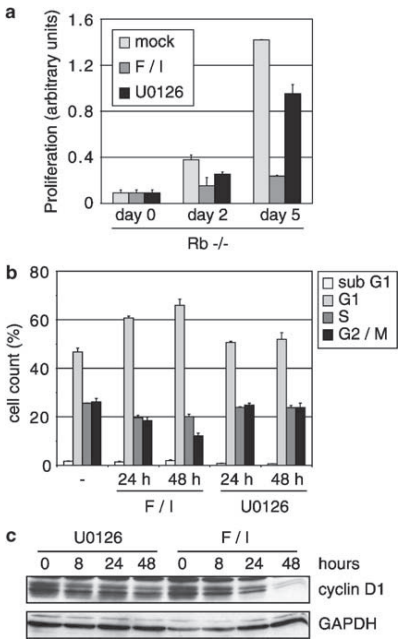

Figure 2 Effect of Rb-deficiency on cAMP-induced G1 arrest. (**a**) Rb-deficient cells are inhibited in proliferation by cAMP. Proliferation of Rb$^{-/-}$ cells was assayed as in Figure 1a, with error bars representing s.d. (**b**) cAMP induces a G1-arrest in Rb-deficient cells. Rb$^{-/-}$ cells were assayed for their cell cycle profile. Cells were treated for 24 or 48 h with forskolin/IBMX (F/I) or U0126 or were left untreated. The cell cycle profile was determined by FACS analysis, using propidium iodide as staining. The percentage of cells per phase of the cell cycle was calculated and plotted. Error bars represent s.d. (**c**) Cyclin D1 is downregulated by cAMP in Rb-deficient cells. Rb$^{-/-}$ cells were stimulated with U0126 and forskolin/IBMX for the indicated time points. Cyclin D1 protein levels and GAPDH as a control were visualized by immunoblotting

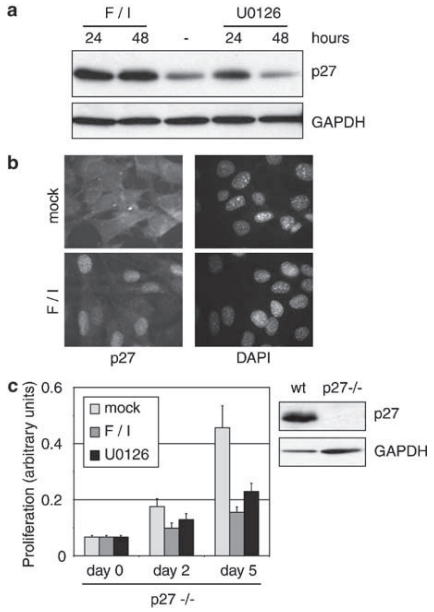

Figure 3 $p27^{Kip1}$ plays an important role in inhibition of proliferation by cAMP. (**a**) $p27^{Kip1}$ protein levels are elevated by cAMP. Wild-type MEFs were stimulated for 24 or 48 h with forskolin/IBMX (F/I) or U0126, or were left untreated. Total $p27^{Kip1}$ levels were visualized by immunoblotting with GAPDH levels as a control. (**b**) cAMP induces nuclear localization of $p27^{Kip1}$. Cells were stimulated as indicated and fixed to slides. $p27^{Kip1}$ protein was stained by immunofluorescence and nuclei were stained with DAPI. (**c**) Inhibition of proliferation of p27-deficient cells is partially reversed by cAMP. $p27^{-/-}$ MEFs were subjected to a proliferation assay as in Figure 1a. The average proliferation, calculated from three independent experiments, is shown, with error bars representing s.d. The presence of $p27^{Kip1}$ protein was checked on a Western blot with GAPDH as a control

was only 5%, which is clearly lower than the 15% increase seen in wild-type cells (Supplementary Figure). Together, this demonstrates that upregulation of $p27^{Kip1}$ in MEFs contributes to cAMP-induced growth arrest but is not solely responsible for this effect.

cAMP regulates $p27^{Kip1}$ levels via transcriptional activity of FoxO transcription factors

To test if cyclin D1 in the absence of $p27^{Kip1}$ upregulation would overcome a cAMP-induced G1-arrest, we tried to generate $p27^{Kip1}$ knockout MEFs, constitutively expressing cyclin D1. However, whereas we could express cyclin D1 in wild-type MEFs, $p27^{Kip1}$ knockout MEFs expressing cyclin D1 appeared unstable. We then reasoned that FoxO transcription factors have been shown to inhibit proliferation by regulating both cyclin D1 and $p27^{Kip1}$ levels (Medema *et al*., 2000; Ramaswamy *et al*., 2002; Schmidt *et al*., 2002). To investigate whether these transcription factors play a role in a cAMP-induced arrest, the effect of forskolin/IBMX on FoxO activity was tested. A reporter construct containing six DAF-16 family protein-binding elements (DBEs), which is known to be activated by FoxO family members (Furuyama *et al*., 2000), was used for this purpose. Luciferase activity was increased by co-transfection of FoxO4 and clearly elevated upon stimulation with cAMP (Figure 4a). cAMP was not able to increase luciferase activity in the absence of co-transfected FoxO4 or in the presence of an active FoxO4 mutant, which is mutated at its PKB phosphorylation sites (FoxO4-A3) (Figure 4a and data not shown), indicating that cAMP indeed regulates 6xDBE activity via FoxO transcription factors. The next step was to investigate whether regulation of the $p27^{Kip1}$ promoter by cAMP was FoxO-dependent. To this end, a reporter construct containing the $p27^{Kip1}$ promoter (−1609 to +178) (Kwon *et al*., 1996) was used. Treatment of cells with forskolin/IBMX elevated FoxO4-induced luciferase activity, whereas in the absence of co-transfected FoxO4, cAMP was not able to stimulate the $p27^{Kip1}$ promoter (Figure 4b). Moreover, reporter gene activity induced by active FoxO4 was not enhanced by forskolin/IBMX treatment (Figure 5d). Furthermore, we investigated whether protein levels of $p27^{Kip1}$ are increased by cAMP via FoxOs. We transiently transfected MEFs with active PKB (gagPKB), the DNA binding domain of FoxO4 (FoxO4-DB), which acts as dominant-negative FoxO, or active FoxO4, and examined the effect of cAMP on the total levels of $p27^{Kip1}$. Whereas active FoxO4 in the absence of cAMP increased $p27^{Kip1}$ levels, the basal level of $p27^{Kip1}$ was lower in the presence of active PKB or dominant-negative FoxO4. Furthermore, the increase of $p27^{Kip1}$ levels caused by cAMP was clearly reduced by overexpression of active PKB and to a lesser extent by dominant-negative FoxO4. No complete inhibition of the cAMP-induced $p27^{Kip1}$ levels was seen, which may be explained by the fact that not all cells were transfected or by additional inputs on the $p27^{Kip1}$ promoter. Active FoxO4 could, as reported before, induce $p27^{Kip1}$ protein levels, but interestingly, forskolin/IBMX did not increase this (Figure 4c). Together, these data show that cAMP can regulate FoxO transcriptional activity and that PKB and FoxO mediate cAMP-induced expression of $p27^{Kip1}$.

If cAMP is able to regulate FoxO transcription factors, FoxO targets other than $p27^{Kip1}$ are predicted to be regulated as well. We were particularly interested in the FoxO target manganese superoxide dismutase (MnSOD), as we previously showed that MnSOD by scavenging reactive oxygen species (ROS) plays a role in protecting cells from going into apoptosis (Kops *et al*., 2002). Thus, MnSOD might protect cAMP-arrested cells, which are not apoptotic (data not shown). Indeed, MnSOD protein levels were increased in MEFs after stimulation with forskolin/IBMX for 24 or 48 h, whereas U0126 did not have an effect (Figure 4d).

cAMP-induced G1-arrest involves inhibition of PKB, leading to enhanced activity of FoxO transcriptional factors

We next addressed the question as to how cAMP regulates FoxO transcription factors. Given that the PI3K/PKB pathway directly affects FoxO activity (Kops *et al*., 1999), we measured PKB phosphorylation as a read-out for PI3K activity. Due to the fact that we grew the cells in the continuous presence of serum, PKB activity is elevated as compared to that in serum-starved cells, with insulin treatment giving only a mild further increase. Phosphorylation of both serine 473 and threonine 308 of PKB was rapidly decreased upon stimulation with forskolin/IBMX and this inhibition was sustained (Figure 5a and b), thus correlating with $p27^{Kip1}$ upregulation. In contrast, U0126 led to a reduced activity of PKB only at later time points. Furthermore, inhibition of the PI3K pathway using the inhibitor LY294002 resulted in an increase in G1 in Rb-deficient cells, almost comparable to the effect of forskolin/IBMX (data not shown). Thus, cAMP-mediated growth arrest might be largely dependent on activation of FoxOs due to inhibition of PI3K/PKB by cAMP. To confirm that the observed decrease in PKB phosphorylation affected phosphorylation of PKB sites in FoxO members, total cell lysates were probed with a phospho-specific antibody against threonine 32 of FoxO3a. Indeed, phosphorylation of this PKB site was strongly diminished by forskolin/IBMX treatment up to at least 48 h (Figure 5c). To further corroborate this, we tested the effect of constitutively active PKB (gagPKB) on cAMP-induced, FoxO-mediated activation of the $p27^{Kip1}$ promoter. Both activation of the $p27^{Kip1}$-promoter by FoxO4 and the increased activity due to forskolin/IBMX treatment were completely inhibited by co-transfection of active PKB. As expected, forskolin/IBMX treatment did not increase the transcriptional activity of FoxO4-A3, which cannot be phosphorylated by PKB (Figure 5d). Together, these experiments show that cAMP mediates activation of FoxO transcription factors via inhibition of PKB, which results in enhanced transcription from the $p27^{Kip1}$-promoter.

To prove that FoxO transcription factors are indeed important in a cAMP-induced cell cycle arrest, the DNA binding domain of FoxO4 (FoxO4-DB) was over-expressed in MEFs. This protein is expected to act as a dominant negative for all FoxO family members. We assessed the inhibition of proliferation by determining the increase in the G1-phase of the cell cycle by cAMP. A clear, albeit incomplete, reversion of cAMP-induced inhibition of proliferation was seen both at 24 and 48 h

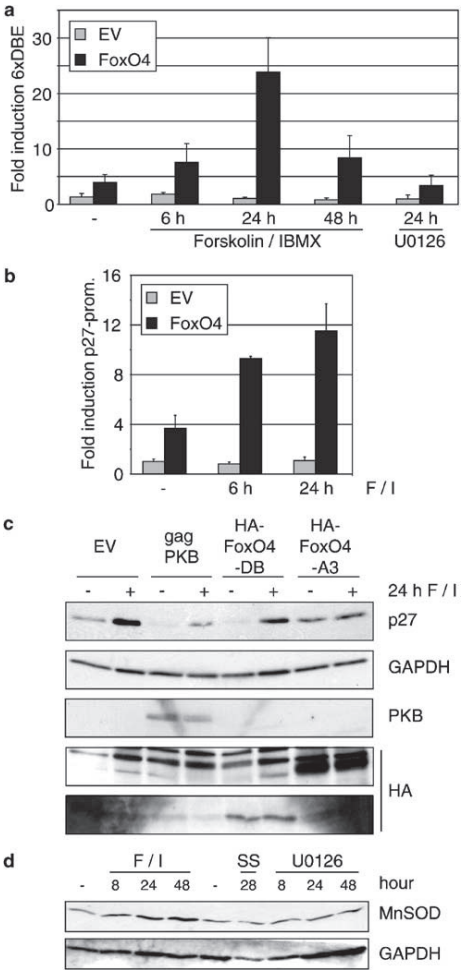

Figure 4 cAMP regulates FoxO transcriptional activity. (**a**) MEFs were transfected with the FoxO binding sites containing luciferase construct and either co-transfected with empty vector (EV) or a FoxO4 construct. Cells were stimulated for the indicated time points with forskolin/IBMX (F/I) or U0126. Fold induction of normalized luciferase counts in relation to unstimulated, empty vector transfected cells was calculated and plotted. Error bars represent s.d. (**b**) cAMP increases $p27^{Kip1}$-promoter activity in a FoxO-dependent manner. The experiment was performed as in (**a**), except that the $p27^{Kip1}$-promoter luciferase construct was used. (**c**) cAMP regulates $p27^{Kip1}$ protein levels partially via PKB and FoxOs. MEFs were transfected either with empty vector, gagPKB, HA-tagged FoxO4-A3 or HA-tagged FoxO4-DB, and cells were stimulated for 24 h with forskolin/IBMX or were left untreated. Proteins were separated and blotted and membranes were probed for total $p27^{Kip1}$ levels. Membranes were probed for PKB or HA to visualize the transfected proteins. As a control for equal loading, the blot was probed for GAPDH. (**d**) cAMP upregulates MnSOD protein levels. MEFs were stimulated with forskolin/IBMX or with U0126 for 8, 24 or 48 h or the cells were serum starved (SS) for 28 h. MnSOD levels were visualized by immunoblotting and GAPDH was used as a control

by overexpression of FoxO4-DB (Figure 5e). Together these data demonstrate a clear involvement of FoxO transcription factors in cAMP-induced cell cycle arrest.

Discussion

The mechanism of inhibition of proliferation by cAMP is a complex process, analysis of which is further complicated by cell-type-dependent differences. Consistent with previous reports, we show here that inhibition of the MAPK pathway and cyclin D1 levels cannot fully account for the cAMP-induced cell cycle arrest in MEFs, and that $p27^{Kip1}$ upregulation is clearly important as well. This is evident from the finding that cAMP-induced inhibition of proliferation is partly reversed in $p27^{Kip1}$-deficient cells. Apparently, the antiproliferative effect of cAMP in MEFs cannot be explained by upregulation of $p27^{Kip1}$ alone. In other cell types, the most likely partner in the process of inhibition of proliferation is cyclin D1. For example, in hamster fibroblasts (L'Allemain *et al*., 1997) and T cells (van Oirschot *et al*., 2001), overexpression of cyclin D at least partially overcomes the antiproliferative effect of cAMP. However, constitutive expression of cyclin D1 in MEFs has hardly any effect on cAMP-induced growth arrest. Moreover, Rb-deficient cells are also clearly inhibited in proliferation by cAMP, indicating that changes in cyclin D1 and Rb activity are not sufficient to explain the cAMP effect. It could be argued that in the Rb-deficient cells, proliferation is still inhibited due to redundancy between Rb family members. We could exclude this, as MEFs deficient for all three Rb family members, Rb, p130 and p170 (Dannenberg *et al*., 2000), are still arrested upon cAMP treatment, which is only partly due to the induction of apoptosis (HBK, unpublished observation).

Here we report that FoxO transcription factors, which are under the negative control of the PI3K/PKB

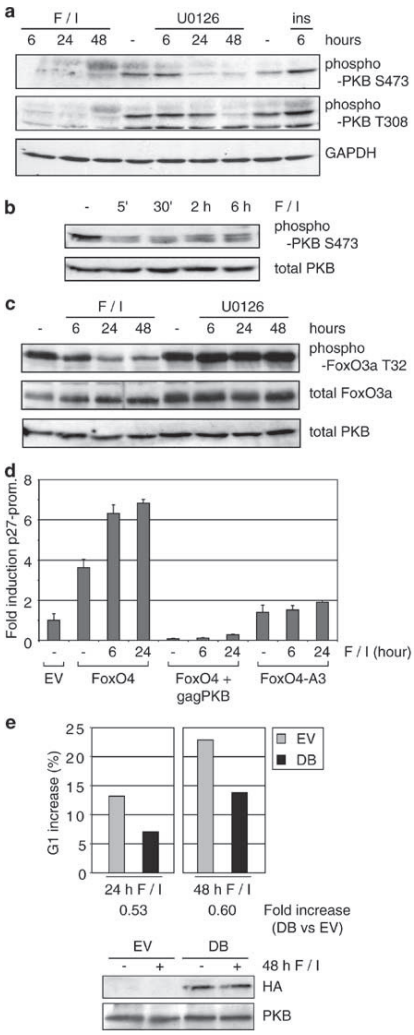

Figure 5 PKB and FoxOs are involved in cAMP-induced cell cycle arrest. (**a**) cAMP inhibits PKB-phosphorylation for long term in MEFs. MEFs were stimulated for 6, 24 or 48 h with forskolin/IBMX (F/I) or U0126, or 6 h with insulin (ins), or were left untreated. Equal protein amounts were blotted and blots were probed for both phosphorylated PKB at serine 473 and at threonine 308 and as a control blots were probed for GAPDH. (**b**) PKB phosphorylation is inhibited at early time points. MEFs were treated for the indicated time points with forskolin/IBMX. PKB, phosphorylated at serine 473, and total PKB levels as a control were visualized by immunoblotting. (**c**) Phosphorylation of FoxO by PKB is inhibited by cAMP. Cells were stimulated for 6, 24 or 48 h with forskolin/IBMX, U0126 or were left untreated. FoxO3a, phosphorylated at threonine 32, total FoxO3a and total PKB protein levels were visualized by immunoblotting. (**d**) PKB is involved in cAMP-dependent regulation of the $p27^{Kip1}$ promoter. The experiment was performed as in Figure 4b, using the $p27^{Kip1}$ luciferase construct. In addition, cells were co-transfected with active PKB (gagPKB) or transfected with an active FoxO4 mutant (FoxO4-A3). Error bars represent s.d. (**e**) FoxOs are involved in cAMP-induced cell cycle arrest. MEFs were transfected with either an empty vector (EV) or HA-tagged FoxO4-DB (DB). In two independent experiments, the cells were treated with forskolin/IBMX for either 24 or 48 h. The cell cycle profile was determined as in Figure 2b. The percentage increase in G1-phase cells of forskolin/IBMX stimulated cells compared to unstimulated cells was calculated and plotted for each experiment separately. Furthermore, the fold increase in G1 of DB-transfected cells *versus* EV-transfected cells is shown in numbers. Part of the cells used for FACS analysis was lysed before fixation to check for the presence of transfected constructs. Lysates were separated and blotted, and blots were stained for HA to visualize HA-FoxO4-DB and total PKB levels were visualized as a control

pathway, are involved in the antiproliferative effect of cAMP in MEFs. First, FoxO transcription factors can downregulate cyclin D1 and upregulate $p27^{Kip1}$ (this report and Medema *et al*., 2000; Nakamura *et al*., 2000; Ramaswamy *et al*., 2002). Furthermore, at least one other direct target of FoxO transcription factors, namely MnSOD, is upregulated following cAMP treatment. Regulation of FoxO transcription factors by cAMP is clearly observed in co-transfection experiments in which transcription from a 6xDBE luciferase construct was increased by forskolin/IBMX. In contrast, an active version of FoxO4, FoxO4-A3, did not respond to cAMP. More direct evidence for a role of FoxO transcription factors in the antiproliferative effect of cAMP comes from ectopic expression of dominant-negative FoxO4. FoxO4-DB clearly interferes in growth inhibition by cAMP, although this effect is not complete. This may be due to the fact that not all cells were transfected or that FoxO4-DB cannot fully block the action of endogenous FoxO transcription factors. An equally likely possibility however is that not all growth inhibitory effects of cAMP are mediated via activation of FoxOs. In line with this is the observation that ectopic expression of cyclin D1 does partially protect cells from a FoxO4-induced cell cycle arrest (Schmidt *et al*., 2002), whereas ectopic expression of cyclin D1 does hardly affect a cAMP-induced cell cycle arrest (Table 1). The most likely way by which cAMP activates FoxOs is via inhibition of PI3K. Although we did not directly measure decreases in PIP3 levels, the PI3K-regulated PKB was clearly less phosphorylated at S473 and T308. Moreover, phosphorylation of FoxO3a at threonine 32, which is one of the PKB-sites, was strongly decreased by cAMP treatment. Furthermore, induction of the $p27^{Kip1}$-promoter and $p27^{Kip1}$ protein levels by cAMP could both clearly be suppressed by active PKB.

Besides its antiproliferative effect, cAMP is known to protect certain cell types from apoptosis (Parvathenani *et al*., 1998; Li *et al*., 2000; Boucher *et al*., 2001). We now show that this might also be the case in mouse fibroblasts via a mechanism using MnSOD. MnSOD is a known target of FoxOs (Kops *et al*., 2002) and is clearly upregulated by cAMP, which is thus a likely consequence of stimulation of FoxOs on FoxO binding sites in the MnSOD promoter. MnSOD upregulation might provide the cells with a mechanism in which cells can be protected when PKB is inhibited.

Apart from cyclin D1 or Rb, $p21^{Cip1}$ might be involved next to $p27^{Kip1}$ in a cAMP-induced cell cycle arrest. Although we were not able to show an increase in $p21^{Cip1}$ levels upon cAMP treatment in wild-type MEFs (data not shown), this has been shown by others in other cell types (Rao *et al*., 1999; Lee *et al*., 2000). Furthermore, it has been shown that FoxO transcription factors can cooperate with TGFβ to induce $p21^{Cip1}$ levels transcriptionally (Seoane *et al*., 2004). Recently, we obtained preliminary evidence that FoxO transcription factors can regulate $p21^{Cip1}$ levels in the absence of TGFβ treatment as well (BMTB, unpublished observation). It would therefore be interesting to see if cAMP can still inhibit proliferation of p21/p27 double knock-out cells.

Materials and methods

Cell culture, plasmids, antibodies and reagents

Culturing of Rb-deficient 3T3 cells, MEFs constitutively expressing cyclin D1, $p27^{Kip1}$-deficient MEFs and wild-type MEFs has been described before (Medema *et al*., 2000; Schmidt *et al*., 2002). All cells used were cultured in DMEM, supplemented with 10% fetal bovine serum and 0.05% glutamine.

pMT2-HA-FoxO4, -DB, -A3, pSG5-gagPKB, pGL3-6xDBE-luc and pGL2-$p27^{Kip1}$-1609-luc have been described (Burgering and Coffer, 1995; Kwon *et al*., 1996; Kops *et al*., 1999; Furuyama *et al*., 2000; Medema *et al*., 2000).

Antibodies directed against the following proteins were used: phospho-Thr202/Tyr204-p44/42 MAPK, phospho-Ser473 and -Thr308-PKB (Cell Signaling), phospho-Thr32-FKHRL1 (Upstate) and FKHRL1 (sc-11351; Santa Cruz) for detection of FoxO3a, PKB (Burgering and Coffer, 1995), cyclin D1 (Immunotech), GAPDH (Chemicon), HA (12CA5; (Burgering and Coffer, 1995)), ERK2 (de Vries-Smits *et al*., 1992), p27 (Transduction Laboratories) and MnSOD (Stressgen).

The following stimuli and inhibitors were used at the following concentrations: forskolin (10 μM; ICN), IBMX (0.5 mM; Sigma), insulin (1 μg/ml; Sigma), U0126 (10 μM; Biomol Research Laboratories) and LY294002 (10 μM; Sigma).

Transfections and immunoblotting

Cells were transfected, using the calcium phosphate precipitation method, αPEI (Durocher *et al*., 2002) or using the transfection agent fugene (Roche). In the case of pBabe-puro co-transfections, the cells were selected for at least 24 h with 1 μg/ml puromycin. Following stimulation, cells were lysed by scraping in Ripa lysis buffer (20 mM Tris-HCl pH 7.5, 1% triton X-100, 0.5% Na-DOC, 0.1% SDS, 10 mM EDTA, 150 mM NaCl), supplemented with aprotinin, leupeptin, trypsin inhibitor, Na_3VO_4 and NaF. Ripa lysates were cleared by centrifugation and protein amounts were equalized, using standard Bradford protein quantification methods. Proteins were separated on SDS–polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (NEN™), following incubations with primary and secondary antibodies. Proteins were visualized using standard enhanced chemiluminescence and autoradiography.

Proliferation assay

Cells were plated in multiwell plates in triplicates, following stimulation the next day. Medium and stimuli were refreshed every 2 days and the cells were stained at 3 different days, 0–6 days after stimulation. To stain, the cells were fixed for 10 min in 10% acetic acid at RT, prior to 10 min staining at RT with 0.4% crystal violet, dissolved in 10% acetic acid. The plates were washed twice with water and dried overnight. Proliferation was quantified by measuring the optical density at wavelength 560 nm of the in 10% acetic acid re-dissolved crystal violet stain in the plates.

Flow cytometry

For determination of cell cycle distributions, untransfected cells or cells transfected with the indicated constructs together

with pBabe-puro were used. Cells were stimulated for 24 or 48 h as indicated and transfected cells were selected with 1 μg/ml puromycin for at least 24 h. Cells were detached using EDTA, containing trypsin, and washed twice. Nonadherent cells, like apoptotic cells, were collected by centrifugation of the tissue culture medium and pooled with the detached cells. Subsequently, cells were fixated in 70% ethanol for at least 16 h at 4°C. Hereafter, cells were washed and resuspended in PBS, containing 0.1% BSA, 0.25 mg/ml RNase and propidium iodide and were incubated for 30 min at 37°C prior to DNA profile measurements. Flow cytometry analysis was performed using a FACScalibur instrument (Becton Dickinson).

Luciferase assay

Cells were transfected in triplicates with renilla-luc and either 6xDBE-luc or $p27^{Kip1}$-promoter-luc, together with either pMT2-HA empty vector or pMT2-HA-FoxO4 and cells were stimulated as indicated. At 2 days after transfection, cells were lysed in passive lysis buffer (Promega) for 30 min at RT, where after lysates were cleared. Luciferase counts were measured using the dual-luciferase reporter assay system (Promega).

Immune fluorescence

Cells were plated on cover slides and stimulated for the indicated time points, followed by fixation in 4% paraformaldehyde solution in PBS. Cells were blocked with blocking buffer (0.1% saponin and 0.5% BSA in PBS), followed by incubation with an anti-$p27^{Kip1}$ antibody. Subsequently, the cells were incubated with a donkey-anti-mouse-Cy3 conjugated antibody and DAPI. Cells were visualized using a Zeiss fluorescence microscope.

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References

Agrawal D, Hauser P, McPherson F, Dong F, Garcia A and Pledger WJ. (1996). *Mol. Cell. Biol.*, **16,** 4327–4336.

Aktas H, Cai H and Cooper GM. (1997). *Mol. Cell. Biol.*, **17,** 3850–3857.

Balmanno K, Millar T, McMahon M and Cook SJ. (2003). *Mol. Cell. Biol.*, **23,** 9303–9317.

Boehm M, Yoshimoto T, Crook MF, Nallamshetty S, True A, Nabel GJ and Nabel EG. (2002). *EMBO J.*, **21,** 3390–3401.

Boucher MJ, Duchesne C, Laine J, Morisset J and Rivard N. (2001). *Biochem. Biophys. Res. Commun.*, **285,** 207–216.

Brownawell AM, Kops GJ, Macara IG and Burgering BM. (2001). *Mol. Cell. Biol.*, **21,** 3534–3546.

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J and Greenberg ME. (1999). *Cell*, **96,** 857–868.

Burgering BM and Coffer PJ. (1995). *Nature*, **376,** 599–602.

Burgering BM, Pronk GJ, van Weeren PC, Chardin P and Bos JL. (1993). *EMBO J.*, **12,** 4211–4220.

Cook SJ and McCormick F. (1993). *Science*, **262,** 1069–1072.

Dannenberg JH, van Rossum A, Schuijff L and te Riele H. (2000). *Genes Dev.*, **14,** 3051–3064.

de Vries-Smits AM, Burgering BM, Leevers SJ, Marshall CJ and Bos JL. (1992). *Nature*, **357,** 602–604.

Delmas C, Aragou N, Poussard S, Cottin P, Darbon JM and Manenti S. (2003). *J. Biol. Chem.*, **278,** 12443–12451. Epub 2003 Jan 14.

Diehl JA, Cheng M, Roussel MF and Sherr CJ. (1998). *Genes Dev.*, **12,** 3499–3511.

Dumaz N, Light Y and Marais R. (2002). *Mol. Cell. Biol.*, **22,** 3717–3728.

Durocher Y, Perret S and Kamen A. (2002). *Nucleic Acids Res.*, **30,** E9.

Fujita N, Sato S, Katayama K and Tsuruo T. (2002). *J. Biol. Chem.*, **277,** 28706–28713.

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

Hafner S, Adler HS, Mischak H, Janosch P, Heidecker G, Wolfman A, Pippig S, Lohse M, Ueffing M and Kolch W. (1994). *Mol. Cell. Biol.*, **14,** 6696–6703.

Hengst L and Reed SI. (1996). *Science*, **271,** 1861–1864.

Kato JY, Matsuoka M, Polyak K, Massague J and Sherr CJ. (1994). *Cell*, **79,** 487–496.

Kim S, Jee K, Kim D, Koh H and Chung J. (2001a). *J. Biol. Chem.*, **276,** 12864–12870. Epub 2001 Jan 26.

Kim TY, Kim WI, Smith RE and Kay ED. (2001b). *Invest. Ophthalmol. Vis. Sci.*, **42,** 3142–3149.

Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH and Burgering BM. (2002). *Nature*, **419,** 316–321.

Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL and Burgering BM. (1999). *Nature*, **398,** 630–634.

Kwon TK, Nagel JE, Buchholz MA and Nordin AA. (1996). *Gene*, **180,** 113–120.

L'Allemain G, Lavoie JN, Rivard N, Baldin V and Pouyssegur J. (1997). *Oncogene*, **14,** 1981–1990.

Lavoie JN, L'Allemain G, Brunet A, Muller R and Pouyssegur J. (1996). *J. Biol. Chem.*, **271,** 20608–20616.

Lee HT and Kay EP. (2003). *Invest. Ophthalmol. Vis. Sci.*, **44,** 3816–3825.

Lee RJ, Albanese C, Stenger RJ, Watanabe G, Inghirami G, Haines III GK, Webster M, Muller WJ, Brugge JS, Davis RJ and Pestell RG. (1999). *J. Biol. Chem.*, **274,** 7341–7350.

Lee TH, Chuang LY and Hung WC. (2000). *Oncogene*, **19,** 3766–3773.

Li J, Yang S and Billiar TR. (2000). *J. Biol. Chem.*, **275,** 13026–13034.

Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R, Franssen E and Slingerland JM. (2002). *Nat. Med.*, **8,** 1153–1160.

Loubat A, Rochet N, Turchi L, Rezzonico R, Far DF, Auberger P, Rossi B and Ponzio G. (1999). *Oncogene*, **18,** 3324–3333.

Malek NP, Sundberg H, McGrew S, Nakayama K, Kyriakides TR, Roberts JM and Kyriakidis TR. (2001). *Nature*, **413,** 323–327.

McKenzie FR and Pouyssegur J. (1996). *J. Biol. Chem.*, **271,** 13476–13483.

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

Mischak H, Seitz T, Janosch P, Eulitz M, Steen H, Schellerer M, Philipp A and Kolch W. (1996). *Mol. Cell. Biol.*, **16,** 5409–5418.

Muise-Helmericks RC, Grimes HL, Bellacosa A, Malstrom SE, Tsichlis PN and Rosen N. (1998). *J. Biol. Chem.*, **273,** 29864–29872.
Muller D, Bouchard C, Rudolph B, Steiner P, Stuckmann I, Saffrich R, Ansorge W, Huttner W and Eilers M. (1997). *Oncogene*, **15,** 2561–2576.
Nakamura N, Ramaswamy S, Vazquez F, Signoretti S, Loda M and Sellers WR. (2000). *Mol. Cell. Biol.*, **20,** 8969–8982.
Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF and Rolfe M. (1995). *Science*, **269,** 682–685.
Parvathenani LK, Buescher ES, Chacon-Cruz E and Beebe SJ. (1998). *J. Biol. Chem.*, **273,** 6736–6743.
Ramaswamy S, Nakamura N, Sansal I, Bergeron L and Sellers WR. (2002). *Cancer Cell*, **2,** 81–91.
Rao S, Gray-Bablin J, Herliczek TW and Keyomarsi K. (1999). *Exp. Cell Res.*, **252,** 211–223.
Schmidt M, Fernandez de Mattos S, van der Horst A, Klompmaker R, Kops GJ, Lam EW, Burgering BM and Medema RH. (2002). *Mol. Cell. Biol.*, **22,** 7842–7852.
Seoane J, Le HV, Shen L, Anderson SA and Massague J. (2004). *Cell*, **117,** 211–223.
Sewing A, Burger C, Brusselbach S, Schalk C, Lucibello FC and Muller R. (1993). *J. Cell Sci.*, **104,** 545–555.
Sherr CJ and Roberts JM. (1999). *Genes Dev.*, **13,** 1501–1512.
Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J and Arteaga CL. (2002). *Nat. Med.*, **8,** 1145–1152.
Takuwa N, Fukui Y and Takuwa Y. (1999). *Mol. Cell. Biol.*, **19,** 1346–1358.
Tomoda K, Kubota Y and Kato J. (1999). *Nature*, **398,** 160–165.
Vadiveloo PK, Filonzi EL, Stanton HR and Hamilton JA. (1997). *Atherosclerosis*, **133,** 61–69.
van Oirschot BA, Stahl M, Lens SM and Medema RH. (2001). *J. Biol. Chem.*, **276,** 33854–33860.
Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tsichlis P, Bellacosa A, Fusco A and Santoro M. (2002). *Nat. Med.*, **8,** 1136–1144.
Vlach J, Hennecke S and Amati B. (1997). *EMBO J.*, **16,** 5334–5344.
Williamson EA, Burgess GS, Eder P, Litz-Jackson S and Boswell HS. (1997). *Leukemia*, **11,** 73–85.
Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ and Sturgill TW. (1993). *Science*, **262,** 1065–1069.

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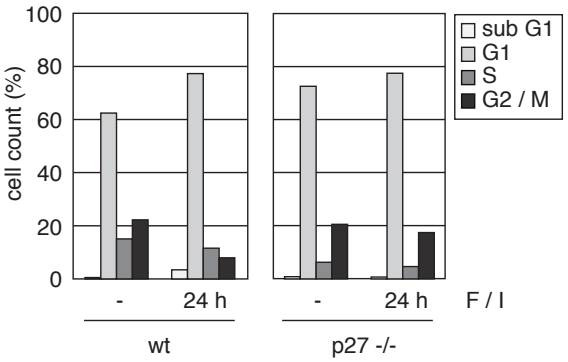
Supplementary Figure



CHAPTER 6

FOXO4 functionally interacts with ICSBP

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In preparation

FOXO4 functionally interacts with ICSBP

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The FOXO (Forkhead box O) subfamily of forkhead transcription factors are involved in a number of important biological processes, like metabolism, cell cycle regulation, apoptosis and the stress-response. Although substantial information is available on the regulation of FOXO activity, little is known about possible interactions between FOXO and other transcription factors. To this end, we performed a transcription factor-transcription factor interaction array using FOXO4 as a bait. Besides known interactors, like PPAR and Smad3/4, we identified ICSBP and NFATc as possible interactors. ICSBP is involved in the differentiation of monocytes towards mature macrophages and notably ICSBP together with NFATc induces Interleukin 12 (IL-12) production. Macrophages and dendritic cells produce IL-12 to induce and maintain Th1 cell responses. Our co-immunoprecipitations confirm that FOXO4 interacts with ICSBP in HEK293T cells and we find that NFATc induces this binding. H_2O_2 also stimulates the binding between ICSBP and FOXO4. Most notably, FOXO4 synergises with ICSBP and NFATc to induce IL-12 p40 promoter activity. Taken together, we propose a novel role for FOXO4 in concert with ICSBP and NFATc in the adaptive immune response.

The FOXO (Forkhead box O) subfamily of forkhead transcription factors consists of four members, FOXO1, FOXO3, FOXO4 and FOXO6[1]. They are involved in a number of important biological processes, like metabolism, cell cycle regulation, apoptosis and the stress-response (reviewed in [2]). In the late 1990's several groups discovered that FOXO transcription factors act in a signalling pathway downstream of tyrosine kinase receptors, namely the phosphatidylinositol-3-kinase (PI3K)-protein kinase B (PKB/Akt) pathway. These kinases negatively regulate FOXO transcription factors by their nuclear exclusion[3,4]. More recently, the pathway involving the small GTPase Ral has been shown to also regulate the transcriptional activity of FOXO4 by phosphorylation. Ral directs the peroxide-induced, Jun N-terminal kinase(JNK)-mediated phosphorylation of FOXO4 on residues 447 and 451, which acts as a stimulatory signal[5].
Lately, FOXO transcription factors were found to be regulated by two other types of post-translational modifications: acetylation and ubiquitination, indicating that FOXO transcriptional activity is tightly regulated (reviewed in[6]). A variety of binding partners for

FOXO have been described. These comprise both proteins modifying FOXO, like 14-3-3 and SIRT1, and transcription factors, e.g. androgen receptor, PPAR and Smads. To find new FOXO interactors we performed a transcription factor-transcription factor interaction array (Panomics) encompassing 244 possible transcription factor interactors. We identified two binding partners (namely PPAR and Smad3/4), which have been previously described[7,8], demonstrating the reliability of this approach. Furthermore, we identified several novel possible interactors, namely interferon (IFN) consensus sequence binding protein (ICSBP), nuclear factor of activated T-cells (NFATc), nuclear respiratory factor 1 (NRF1), Transcriptional Intermediary Factor 1 (TIF1), Sp1 and Homeobox D9/10 (HOXD9/10).
ICSBP, also called interferon regulatory factor (IRF)-8, belongs to the transcription factor family of interferon regulatory factors and is involved in the differentiation of myeloid cells towards macrophages (reviewed in Tamura and Ozato, 2002[9]). ICSBP contains two domains, namely a DNA binding domain (DBD) and an IRF association domain (IAD). The IAD interacts with proteins that contain a stretch rich in proline, glutamine, serine and threonine residues (PEST motif)[10]. The C-terminus of the IAD is related to the Smad transcription factors. Interestingly, Smads can bind to FOXO transcription factors. ICSBP regulates the transcription of many genes, including itself, containing so-called IFN-γ activation sites (GAS-elements) or IFN-stimulated responsive elements (ISRE) in their promoters[11-13]. For example, downregulation of ICSBP expression in Bcr-Abl positive myeloid cells causes a chronic myeloid leukaemia-like disorder, by causing a defect in apoptosis via downregulation of Bcl2[14].
The NFAT family of transcription factors consists of NFAT1 to NFAT5. NFATs are expressed in a variety of tissues, but are most abundant in T cells. All but NFAT5 are regulated by calcium signalling. NFATs contain a DBD that is structurally related to that of the Rel-family transcription factors and is therefore also known as Rel homology region (RHR). Besides the DBD, NFAT1 to 4 contain an NFAT homology region (NHR) that includes the docking sites for both calcineurin (NFAT phosphatase), activated by calmodulin, and NFAT kinases, like GSK3 (reviewed in Macian, 2005[15]). PKB can stimulate NFAT activity by inactivating GSK3, which normally is responsible for NFAT nuclear export (reviewed in Crabtree and Olson, 2002[16]). NFAT2a is also named NFATc. NFATc has three isoforms (A, B and C) of which C is the full-length protein that is used throughout this study.
NFATc binds to and activates together with ICSBP the IL-12 p40 promoter[17], which is active in macrophages and dendritic cells to induce and maintain Th1 cell responses. The subunits IL-12 p40 and IL-12 p35 together constitute functional IL-12[18]. A role for FOXOs in dendritic cells and/or macrophages has not been demonstrated.
Here, we show that FOXO4 can interact with ICSBP and NFATc. Importantly, NFATc enhances ICSBP binding to FOXO4 as does hydrogen peroxide. While ICSBP inhibits FOXO4 transcriptional activity, the most prominent effect is the induction of a strong synergism with ICSBP and NFATc transcriptional activity on the IL12 p40 promoter by FOXO4. These data indicate that FOXOs may have a previously unappreciated role in antigen-presenting cells and hence the adaptive immune response.

Results

To find novel interaction partners of FOXO transcription factors we performed a transcription factor-transcription factor interaction array (Fig. 1a). According to the manufacturer, we made nuclear lysates of HEK293T cells expressing FOXO4-TAP or empty vector as a

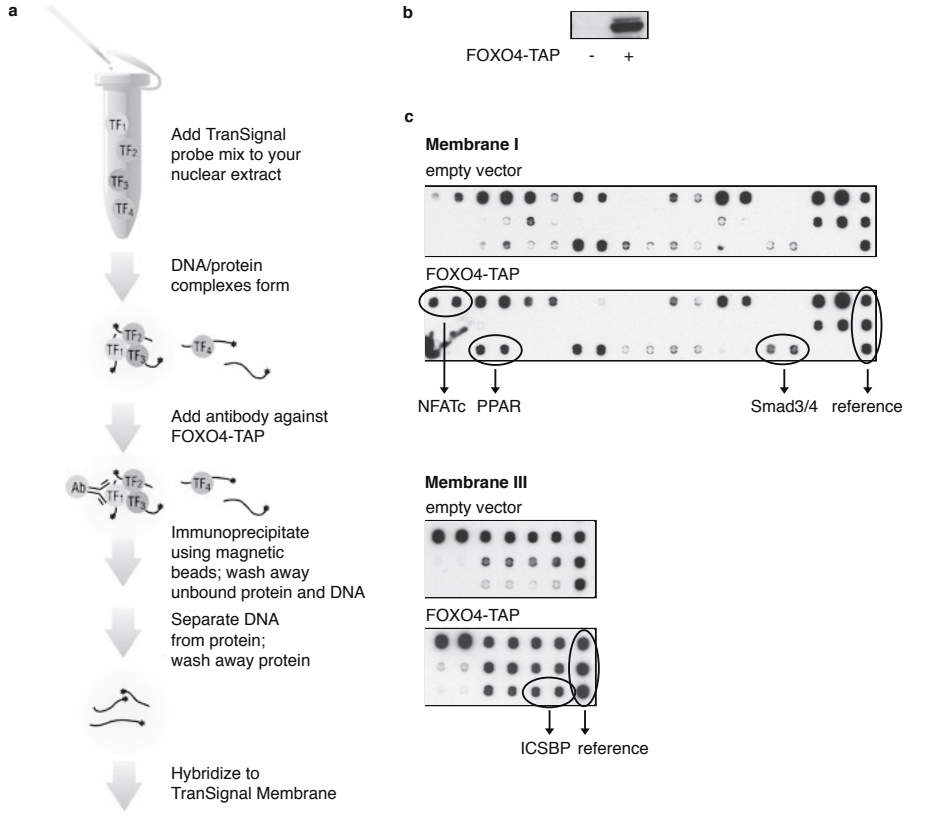


Figure 1 FOXO4-transcription factor interaction array. **(a)** Schematic representation of the protocol, adapted from Panomics. **(b)** FOXO4-TAP expression in nuclear lysates of empty vector and FOXO4-TAP transfected HEK293T cells. **(c)** Fragment of membranes I and III showing that PPAR, Smad3/4, ICSBP and NFATc bind to FOXO4-TAP.

control. These lysates were incubated with three probe mixes with different *cis*-elements recognized by 244 transcription factors in total. FOXO4-TAP was immunoprecipitated (Fig. 1b), bound probes were eluted and hybridized to three membranes (I, II and III) containing the same *cis*-elements. Fragments of the results of membranes I and III are shown in Fig. 1c, showing that ICSBP and NFATc could be novel FOXO4 interaction partners. Notably, two known interaction partners, PPAR and Smad3/4, were found, demonstrating the reliability of our approach. Furthermore, we found NRF1, TIF1, Sp1 and HOXD9/10 as possible novel FOXO interactors (data not shown).

To validate the possible interaction of FOXO4 with ICSBP we cotransfected myc-ICSBP and HA-FOXO4 in HEK293T cells and immunoprecipitated ICSBP. Indeed FOXO4 and ICSBP interacted, albeit only when cells were treated with hydrogen peroxide (Fig. 2a). Probably depending on experimental conditions, this interaction was observed in a minority of analogous co-immunoprecipitations. As NFATc can interact with ICSBP and possibly with FOXO4 (according to our array), we performed similar co-immunoprecipitations in the presence of NFATc. Notably, the interaction between FOXO4 and ICSBP was enhanced by NFATc (Fig. 2b), suggesting that these proteins can form a ternary complex.

To investigate whether the interaction between FOXO4 and ICSBP affects FOXO4 activity we employed a FOXO-responsive luciferase reporter bearing six canonical FOXO binding elements (6xDBE-luc). As shown in Fig. 3a the FOXO-mediated induction of luciferase expression is reversed by ICSBP in a concentration dependent manner. Similarly, ICSBP inhibited the activation of $p27^{kip1}$ and FasL reporter constructs by FOXO4 two to four times (Fig. 1b and c). To determine whether FOXO transcriptional activity towards an endogenous target gene was affected by ICSBP, we transfected HEK293T cells with HA-FOXO4, myc-ICSBP and pBabe-puro plasmids. Transfected cells were selected and $p27^{kip1}$ expression was analysed in lysates that were equalized for protein levels (Fig. 3d). Consistent with the luciferase data, ICSBP inhibited FOXO4 activity towards the $p27^{kip1}$ promoter.
To determine whether FOXO4 could also influence the expression of genes regulated by ICSBP and NFATc, we performed a luciferase assay using an IL-12 p40 reporter construct. Exogenous expression of FOXO4 led to a ~10-fold increase in luciferase activity and co-expression with both ICSBP and NFATc activated luciferase expression up to ~30-fold (Fig. 4a). These results suggest that FOXO4 may play an important role in stimulating IL-12 production by activated macrophages or dendritic cells and hence in regulating the adaptive immune response.

Discussion

Here, we show that a transcription factor-transcription factor interaction array is a valuable tool to discover novel binding partners of FOXO4. Besides two known interactors (PPAR and Smad3/4), we found two novel binding partners that we could validate in co-immunoprecipitation experiments: ICSBP and NFATc. Functionally, ICSBP seems to inhibit FOXO4 activity towards FOXO-regulated promoters ~2-4-fold. Conversely, we observed a ~10-fold stimulation of IL-12 p40 promoter activity, which is regulated by ICSBP and NFATc, by FOXO4. To our opinion the inhibition of FOXO4 activity by ICSBP may be due to squelching as this is minor compared to the induction of IL-12 production by FOXO4.

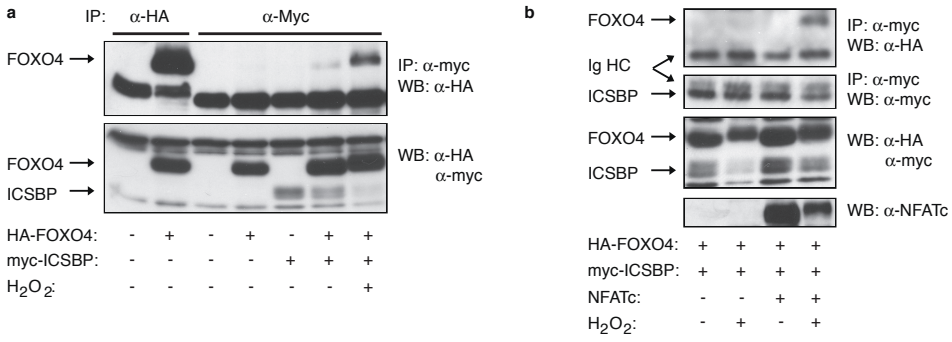


Figure 2 FOXO4 interacts with ICSBP. **(a)** HEK293T cells were transfected with HA-FOXO4 and myc-ICSBP and treated with hydrogen peroxide for 1 hr. Cells were lysed in RIPA lysis buffer, myc-ICSBP or HA-FOXO4 was immunoprecipitated and samples were analysed by western blotting with the indicated antibodies. **(b)** As in (a), but NFATc was cotransfected as indicated and antibodies were conjugated to the agarose beads to allow reprobing immunoprecipitation blots for ICSBP.

The fact that we found ICSBP in our array without the need to apply hydrogen peroxide as opposed to co-immunoprecipitation experiments in which oxidative stress or NFATc overexpression was required, indicates that our array was more sensitive.
Interestingly, we note that ICSBP contains a Smad-like IAD[10]. As Smad proteins bind to FOXO[8], the IAD of ICSBP could be responsible for the interaction with FOXO4.
Dendritic cells and macrophages secrete IL-12 upon stimulation of ICSBP and NFATc activity by IFN-γ[17]. IL-12 leads to activation of Th1 cells, which then produce IFN-γ. IFN-γ in turn can stimulate dendritic cells and macrophages, thereby forming a positive feedback loop and eliciting a strong adaptive immune response (reviewed in [19]). Based on this and our findings we propose a model (Fig. 4b), in which FOXO4 plays a stimulatory role in antigen-presenting cells and hence in the adaptive immune response by inducing IL-12 production. Interestingly, PKB has been shown to negatively regulate IL-12 p40 production in antigen-presenting cells[20,21]. Previously, Lin *et al*. (2004)[22] showed that FOXO3 could inhibit T cell activation by inhibiting the activity of NFκB. Although seemingly contradictory, IL-12 activates NFκB signalling via PKB[23,24], which could be in part through inhibition of FOXO activity, and this results in the synthesis of IFN-γ[25]. So, while FOXOs do not seem to be inhibited by IFN-γ stimulation of antigen-presenting cells and hence can stimulate IL-12 production, FOXOs are inhibited in T cells by IL-12 signalling which allows these T cells to produce IFN-γ via NFκB.
To further our findings on the role of FOXO4 in the regulation of antigen-presenting cell function, our current research focuses mainly on immunological cells.

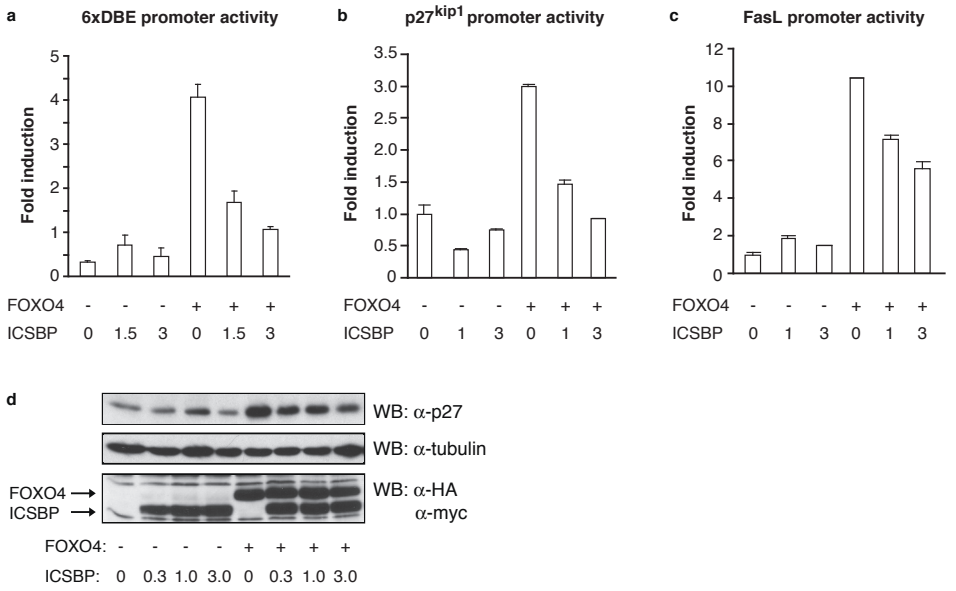


Figure 3 ICSBP inhibits FOXO4 activity. **(a, b, c)** HEK293T cells were transfected with 6xDBE (a), p27^{kip1} (b) or FasL (c) reporter plasmids together with Tk Renilla, HA-FOXO4 and myc-ICSBP as indicated. Luciferase activity was measured forty hrs after transfection. Data represent mean ± s.d. of triplicates. **(d)** HEK293T cells were transfected with HA-FOXO4, myc-ICSBP and pBabe-PURO. After puromycin selection, cells were lysed and protein levels were equalised. Samples were analysed for the expression of p27 ^{kip1}, FOXO4 and ICSBP. Tubulin was used as a loading control.

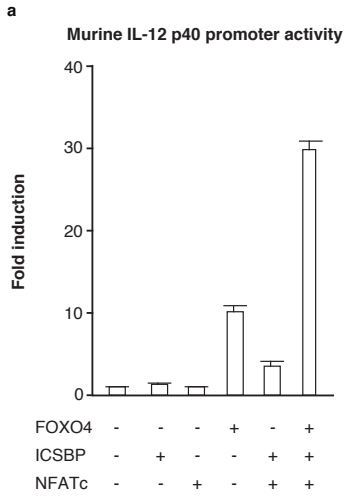
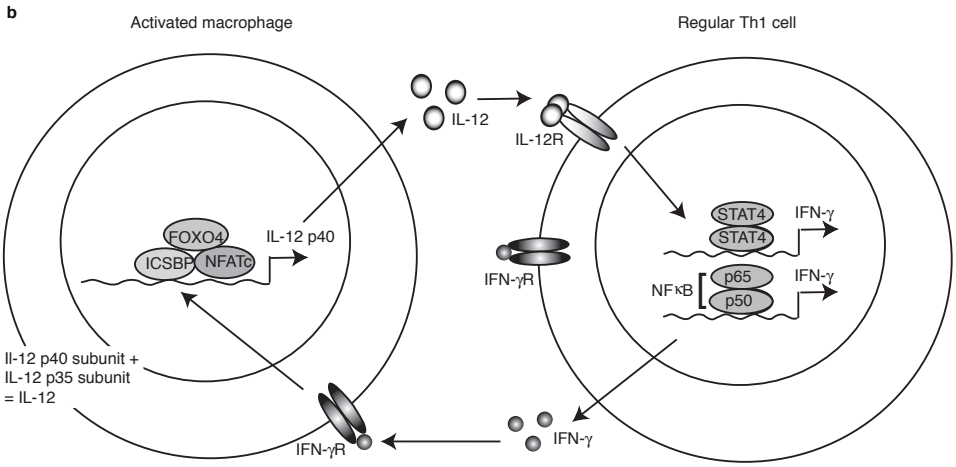


Figure 4 FOXO4 stimulates the IL-12 p40 luciferase reporter. **(a)** HEK293T cells were transfected with IL-12 p40 reporter plasmid together with Tk Renilla, HA-FOXO4, myc-ICSBP and NFATc as indicated. Luciferase activity was measured forty hrs after transfection. Data represent mean ± s.d. of triplicates. **(b)** Model describing how FOXO4 might be regulating the adaptive immune response.



Methods

Cell culture and transfection

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Biowittaker) supplemented with 10% Foetal bovine serum, penicillin/streptomycin and L-Glutamine (Biowittaker). Cells were transiently transfected using FuGENE6 according to the manufacturer (Roche). Total amounts of transfected DNA were equalized using pBluescript KSII+.

Constructs

6xDBE-luc[26], pMT2-HA-FOXO4[4], p27-luc[27] and pBabe-PURO[28] have been described before. pRSV-NFATc was kindly provided by E. Serfling[29], pcDNA3.1-myc-ICSBP was a kind gift of K. Ozato[30]. IL-12 p40-luc and FasL-luc were kind gifts of K. Murphy[31] and A. Brunet[3] respectively. pcDNA4/TO-FOXO4-TAP was created by cloning a full-length BamHI/NotI PCR-product encoding FOXO4

(forward oligo: 5'-CGCGGATCCATGAGAATTCAGCCACAGAAGGCCGCCGCGATCATAGAC CTAGATCCCGAC-3' and reverse oligo: 5'-TTTTCCTTTTGCGGCCGCGGGATCTGGCTCAAA GTTGAA-3') into pcDNA4/TO-TAP, kindly provided by H. Clevers. pRL-Tk (Tk renilla luciferase) was purchased from Promega.

Antibodies

Monoclonal mouse 12CA5 and 9E10 were produced using hybridoma cell lines. Monoclonal antibodies recognizing NFATc (MA3-024), $p27^{kip1}$ and tubulin (DM1A) were obtained from Affinity bioreagents, Transduction Laboratories and Calbiochem respectively. PAP-antibody against the TAP-tag was purchased from Sigma (P1291).

Antibody crosslinking

Protein A beads were washed three times with PBS, and incubated with antibody for 2 hrs at RT. Beads were washed twice with PBS, three times with thioethanolamine (0.2 M, pH 8.2) and then incubated with DMP/thioethanolamine (5.4 mg/ml) solution for 20 min. Subsequently, beads were washed twice and incubated for 15 min with 50 mM Tris-HCl pH 7.5 to stop crosslinking. Before use or storage, beads were washed four times with the appropriate lysis buffer.

Transcription factor-transcription factor interaction array

Ten 9-cm dishes containing HEK293T cells were transfected with FOXO4-TAP or empty vector. Forty hours after transfection nuclear extracts were prepared essentially as described[32]. The array was performed according to the manufacturer (Panomics). For FOXO4-TAP immunoprecipitation IgG sepharose beads were used. Sequences of the *cis*-elements for NFATc and ICSBP were 5'-ACGCCC AAAGAGGAAAATTTGTTTCATACA-3' and 5'-TGAGGAAACGAAACCATGAGGAAACGAA ACCA-3' respectively.

Co-immunoprecipitation

Non-confluent cells transfected as indicated were treated with 200 μM hydrogen peroxide for 1 hr and lysed in 500 μl ICSBP buffer (50 mM Tris-HCl pH 8.0, 280 mM NaCl, 0,5% Nonidet P-40, 10% Glycerol, 0.2 mM EDTA pH 8.0, 2 mM EGTA pH 9.0, 1 mM DTT, NaF, leupeptin, and aprotinin, described by Xiong) or RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% DOC, 150 mM NaCl, 2.5 mM EDTA pH 8.0, NaF, leupeptin and aprotinin) and lysates were cleared for 15 minutes at 14,000 rpm and 4ºC. Lysates were incubated for 2 hrs at 4ºC with 1 μl 12CA5 or 9E10 and 100 μl protein-A agarose beads. Immunoprecipitates were washed four times with lysis buffer, cleared from all liquid, and boiled in 25 μl 1x Laemmli sample buffer.

Immunoblot analysis

Protein samples were subjected to SDS-PAGE and transferred to PVDF membrane. Western blots were blocked for 1 hr at 4ºC in phosphate-buffered saline (PBS) containing 0.1% Tween-20, 2% Protifar and 0.5% Bovine serum albumin, processed according to standard procedures and analysed with enhanced chemiluminescene (ECL).

$p27^{kip1}$ expression

HEK293T cells were transfected with HA-FOXO4, myc-ICSBP and pBabe-PURO, 24-40 hours after transfection puromycin was added and cells were selected for 24 hours. Cells were lysed in 300 μl RIPA buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 0.5% DOC, 150 mM NaCl, 2.5 mM EDTA pH 8.0, NaF, leupeptin and aprotinin), and lysates were cleared for 15 minutes at 14,000 rpm and 4ºC.

To equalise protein levels, protein concentrations were measured using Bradford reagent. Total lysates were made by adding 5x Laemmli sample buffer.

Luciferase reporter assays

For 6xDBE, $p27^{kip1}$ and FasL promoter activity, HEK293T cells were transiently transfected with 0.5 μg reporter construct and 20 ng Tk renilla. Cells were cotransfected with 1 μg HA-FOXO4 and myc-ICSBP as indicated.

For determining the activity of the murine IL12 p40 promoter, HEK293T cells were transiently transfected with 0.5 μg IL-12 p40 luciferase construct and 20 ng Tk renilla. Cells were cotransfected with 1 μg each of HA-FOXO4, myc-ICSBP and NFATc. Cells were washed once with PBS, lysed in passive lysis buffer (PLB) and luciferase activity was analysed using a luminometer according to the manufacturer (Promega).

Acknowledgments

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References

1. Katoh, M. Human FOX gene family (Review). *Int J Oncol* **25**, 1495-500 (2004).
2. Burgering, B. M. & Kops, G. J. Cell cycle and death control: long live Forkheads. *Trends Biochem Sci* **27**, 352-60 (2002).
3. Brunet, A. et al. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-68 (1999).
4. Kops, G. J. et al. Direct control of the Forkhead transcription factor AFX by protein kinase B. *Nature* **398**, 630-4 (1999).
5. Essers, M. A. et al. FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. *Embo J* **23**, 4802-12 (2004).
6. Vogt, P. K., Jiang, H. & Aoki, M. Triple layer control: phosphorylation, acetylation and ubiquitination of FOXO proteins. *Cell Cycle* **4**, 908-13 (2005).
7. Dowell, P., Otto, T. C., Adi, S. & Lane, M. D. Convergence of peroxisome proliferator-activated receptor gamma and Foxo1 signaling pathways. *J Biol Chem* **278**, 45485-91 (2003).
8. Seoane, J., Le, H. V., Shen, L., Anderson, S. A. & Massague, J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* **117**, 211-23 (2004).
9. Tamura, T. & Ozato, K. ICSBP/IRF-8: its regulatory roles in the development of myeloid cells. *J Interferon Cytokine Res* **22**, 145-52 (2002).
10. Levi, B. Z., Hashmueli, S., Gleit-Kielmanowicz, M., Azriel, A. & Meraro, D. ICSBP/IRF-8 transactivation: a tale of protein-protein interaction. *J Interferon Cytokine Res* **22**, 153-60 (2002).
11. Kanno, Y. et al. The genomic structure of the murine ICSBP gene reveals the presence of the gamma interferon-responsive element, to which an ISGF3 alpha subunit (or similar) molecule binds. *Mol Cell Biol* **13**, 3951-63 (1993).

12. Contursi, C. et al. IFN consensus sequence binding protein potentiates STAT1-dependent activation of IFNgamma-responsive promoters in macrophages. *Proc Natl Acad Sci U S A* **97**, 91-6 (2000).
13. Driggers, P. H. et al. An interferon gamma-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex class I genes. *Proc Natl Acad Sci U S A* **87**, 3743-7 (1990).
14. Burchert, A. et al. Interferon consensus sequence binding protein (ICSBP; IRF-8) antagonizes BCR/ABL and down-regulates bcl-2. *Blood* **103**, 3480-9 (2004).
15. Macian, F. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* **5**, 472-84 (2005).
16. Crabtree, G. R. & Olson, E. N. NFAT signaling: choreographing the social lives of cells. *Cell* **109 Suppl**, S67-79 (2002).
17. Zhu, C. et al. Activation of the murine interleukin-12 p40 promoter by functional interactions between NFAT and ICSBP. *J Biol Chem* **278**, 39372-82 (2003).
18. Wolf, S. F. et al. Cloning of cDNA for natural killer cell stimulatory factor, a heterodimeric cytokine with multiple biologic effects on T and natural killer cells. *J Immunol* **146**, 3074-81 (1991).
19. Langrish, C. L. et al. IL-12 and IL-23: master regulators of innate and adaptive immunity. *Immunol Rev* **202**, 96-105 (2004).
20. Martin, M. et al. Role of the phosphatidylinositol 3 kinase-Akt pathway in the regulation of IL-10 and IL-12 by Porphyromonas gingivalis lipopolysaccharide. *J Immunol* **171**, 717-25 (2003).
21. Waggoner, S. N., Cruise, M. W., Kassel, R. & Hahn, Y. S. gC1q receptor ligation selectively down-regulates human IL-12 production through activation of the phosphoinositide 3-kinase pathway. *J Immunol* **175**, 4706-14 (2005).
22. Lin, L., Hron, J. D. & Peng, S. L. Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity* **21**, 203-13 (2004).
23. Yoo, J. K., Cho, J. H., Lee, S. W. & Sung, Y. C. IL-12 provides proliferation and survival signals to murine CD4+ T cells through phosphatidylinositol 3-kinase/Akt signaling pathway. *J Immunol* **169**, 3637-43 (2002).
24. Jones, R. G. et al. NF-kappaB couples protein kinase B/Akt signaling to distinct survival pathways and the regulation of lymphocyte homeostasis in vivo. *J Immunol* **175**, 3790-9 (2005).
25. Sica, A. et al. Interaction of NF-kappaB and NFAT with the interferon-gamma promoter. *J Biol Chem* **272**, 30412-20 (1997).
26. Furuyama, T., Nakazawa, T., Nakano, I. & Mori, N. Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues. *Biochem J* **349**, 629-34 (2000).
27. Medema, R. H., Kops, G. J., Bos, J. L. & Burgering, B. M. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782-7 (2000).
28. Medema, R. H., Herrera, R. E., Lam, F. & Weinberg, R. A. Growth suppression by p16ink4 requires functional retinoblastoma protein. *Proc Natl Acad Sci U S A* **92**, 6289-93 (1995).
29. Chuvpilo, S. et al. Alternative polyadenylation events contribute to the induction of NF-ATc in effector T cells. *Immunity* **10**, 261-9 (1999).

30. Thornton, A. M. et al. A dominant negative mutant of an IFN regulatory factor family protein inhibits both type I and type II IFN-stimulated gene expression and antiproliferative activity of IFNs. *J Immunol* **157**, 5145-54 (1996).
31. Murphy, T. L., Cleveland, M. G., Kulesza, P., Magram, J. & Murphy, K. M. Regulation of interleukin 12 p40 expression through an NF-kappa B half-site. *Mol Cell Biol* **15**, 5258-67 (1995).
32. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11**, 1475-89 (1983).

CHAPTER 7

General discussion

This thesis focuses on the regulation of FOXO transcription factors by posttranslational modifications and the consequences thereof on FOXO transcriptional activity and function.

The activity of FOXO transcription factors is regulated by receptor tyrosine kinase signalling through PI3K and PKB. PKB-mediated phosphorylation of FOXOs results in their nuclear exclusion, which renders them inactive as transcription factor (reviewed in Burgering and Kops, 2002[1]). It has long been recognized that treatment of transformed cell lines with the second messenger cAMP results in a (partial) reversal of their transformed phenotype and in most cases a cessation of cell proliferation. However, the mechanism by which cAMP induces this reversal is still unclear. Earlier studies suggested that the inhibition of MAPK activation by cAMP accounts for inhibition of proliferation (reviewed in Burgering and Bos, 1995[2]). However, this MAPK inhibition is not sufficient[3,4]. Here we show that by inhibiting the PI3K/PKB signalling pathway, cAMP activates FOXO transcriptional activity ([5] and Chapter 5), leading to inhibition of proliferation by arresting cells in the G1 phase of the cell cycle. Besides cell cycle arrest, FOXO transcription factors regulate apoptosis and oxidative stress resistance. Importantly, signalling towards FOXO transcription factors is evolutionary conserved and inactivation of insulin/IGF-signalling increases lifespan in C.elegans, Drosophila melanogaster and mice by activating FOXO activity, although formally this still has to be proven for the latter. Increased expression of the longevity protein Sir2 in C.elegans also extends lifespan via FOXO[6]. In analogy, we observed activation of FOXO activity in mammalian cells by Sir2-mediated deacetylation of FOXO and inhibition of FOXO activity by nicotinamide, an inhibitor of Sir2 ([7] and Chapter 2). Consistently, both growing worms on nicotinamide and knockdown of a nicotinamidase (PNC1) resulted in a decrease in worm lifespan (Chapter 3). Acetylation of FOXO is induced by oxidative stress ([7,8] and Chapter 2). Probably before inducing acetylation, oxidative stress induces monoubiquitination of FOXOs resulting in FOXO activation by enhanced nuclear localisation. The deubiquitinating enzyme USP7 removes this monoubiquitin moiety and thereby inhibits FOXO activity (Chapter 4). Finally, we describe some novel interaction partners for FOXO4, i.e. ICSBP and NFATc. We show that these transcription factors together are likely to be involved in the induction of IL-12 expression, suggesting a previously unrecognised role for FOXO transcription factors in the adaptive immune response (Chapter 6).

Posttranslational modifications of FOXO and their impact on FOXO activity

Regulation of FOXO phosphorylation and activity by cAMP

Through inhibiting PI3K/PKB signalling, the second messenger cyclic AMP (cAMP) inhibits proliferation of MEFs[5]. It has been suggested that this inhibition by cAMP occurs at the level of PI3K and is mediated by inhibition of the small GTPase Rap1[9]. Inhibition of proliferation by cAMP via the PI3K/PKB pathway had been demonstrated before[10,11], and was reported to correlate with and partially depend on a decrease in cyclin D1, or an increase in $p27^{kip1}$ levels[12-13]. Accordingly, it has been hypothesized that the combined downregulation of cyclin D1 and upregulation of $p27^{kip1}$ would be sufficient for cAMP-mediated growth inhibition[14], but actual proof for this is lacking. Interestingly, enhanced activity of FOXOs also leads to downregulation of cyclin D1 and upregulation of $p27^{kip1}$ levels[15,16], and indeed, cAMP inhibits proliferation of MEFs via FOXO-mediated transcriptional regulation of these

cell cycle regulatory proteins[5]. Besides the PI3K/PKB pathway, the MAPK pathway is also involved in the regulation of $p27^{kip1}$ and cyclin D1 levels[17,18]. MAPK signalling stimulates cyclin D1 expression via the ATF/CREB family of transcription factors[19] and inhibits $p27^{kip1}$ expression via phosphorylation of the Sp1 transcription factor[20]. Furthermore, MAPK posttranslationally inhibits $p27^{kip1}$ activity via nuclear exclusion[21]. Inhibition of the MAPK pathway by cAMP is well documented and takes place at the level of Raf kinase[22,23], which is phosphorylated by the cAMP-dependent PKA[24]. Inhibition of the MAPK pathway alone, however, does not account for the G1-arrest induced by cAMP[3,4]. Hypothetically, it could be that the degree of inhibition of the MAPK and PI3K/PKB pathways by cAMP is different or that the FOXO-induced changes in $p27^{kip1}$ and cyclin D expression is higher than the Sp1 and ATF/CREB-induced changes respectively.
On the other hand, cAMP can also stimulate proliferation, which coincides with the activation of the MAPK pathway. Tentatively, we speculate that FOXO transcriptional activity is not increased in these cells. So, the question as to whether to arrest growth or to proliferate seems to be a cell-type dependent phenomenon (reviewed in Stork and Schmitt[25]) and might depend on the presence of B-Raf.

Regulation of FOXO acetylation by CBP and Sir2

The signalling pathway from receptor tyrosine kinase via PI3K and PKB to FOXO is evolutionary conserved and is involved in lifespan regulation in organisms ranging from C.elegans to mice, and probably also humans[26]. In C.elegans, increased expression of the longevity protein Sir2, an NAD-dependent HDAC III family member, also extends lifespan via the FOXO family member DAF-16[6]. The mechanism by which Sir2 acts on DAF-16, however, is unknown. In mammalian cells, in which SIRT1 is the functional homologue of C.elegans Sir2, we observed activation of FOXO activity by Sir2-mediated deacetylation of FOXO and inhibition of FOXO activity by nicotinamide, an inhibitor of Sir2[8]. These findings correspond to results from several other labs[27-30], but are in contrast to data published by Motta *et al*. (2004)[31], which show that Sir2-mediated deacetylation of FOXO inhibits FOXO transcriptional activity. Also, Yang *et al*. (2005)[32] observed inhibition of FOXO activity towards target genes by Sir2, albeit that they only studied apoptosis-related genes. Notably in this respect, it has been hypothesized that depending on the target gene Sir2 either inhibits or stimulates FOXO activity[7]: Sir2 inhibits the expression of apoptosis-related genes and stimulates expression of other genes, like those involved in cell cycle regulation and stress resistance. These findings led to the "tipping the balance towards survival-theory" [33]. Part of the discrepancy between this theory and the data of Motta *et al*. could be explained by the fact that, when studying the effect of Sir2 on expression of endogenous $p27^{kip1}$, the authors overexpressed the transcriptional coactivator p300. As p300 activity is repressed by Sir2[34], downregulation of $p27^{kip1}$ could be indirect, independently of FOXO.
HDACs that can be inhibited by TSA (HDAC family I and II) probably also regulate FOXO activity by deacetylation, as TSA treatment induces FOXO acetylation ([7,29,31,35] and unpublished results) and affects FOXO localisation[29]. Future work will probably challenge the "tipping the balance-theory" proposed for Sir2 and FOXO and shed light on the effects of HDACs on FOXO activity.
Acetylation of FOXO is induced by oxidative stress[7,8,31], while UV and heat shock do not seem to stimulate FOXO acetylation ([7] and unpublished results). Acetyltransferases known to be responsible for FOXO acetylation are p300 and CBP. The debate on Sir2 effects alike,

the question as to whether these acetyltransferases are activators or repressors of FOXOs is still unclear as literature lacks reports in which these acetyltransferases are compared in one experiment. It seems, however, that acetylation of FOXO through overexpression of p300 can either stimulate or inhibit FOXO activity[27,31,36], whereas CBP consistently reduces FOXO activity[8,30,35,37]. Generally, acetylation of transcription factors can have both stimulating, as is the case for p53[38], and inhibiting, e.g. for Bcl6[39], effects on their activity. Histone acetylation, however, is generally observed to correlate with enhanced transcription. Hence, the effects of CBP on FOXO target genes, i.e. inhibition of expression, seems mediated via FOXO acetylation, whereas transcriptional activation of FOXO target genes by p300 could be via modification of histones. Accordingly, the outcome of FOXO acetylation by CBP is reduced DNA-binding and Sir2-mediated FOXO deacetylation leads to nuclear retention of FOXOs, probably due to increased DNA binding[29,35]. It is tempting to speculate that p300 activates FOXO-mediated transcription by histone acetylation and that subsequently, as a negative feedback mechanism, p300 and/or CBP acetylate FOXO. It would be interesting to know whether the acetyl-group binding bromodomain of p300 or CBP is involved in this negative feedback. This implies that overexpression of either CBP or p300 tips the balance to respectively inhibition or activation of FOXO target genes. Experimentally, inhibition of p300 activity towards FOXO, by knockdown of p300 or mutating the binding interface between p300 and FOXO, would then diminish CBP-mediated acetylation of FOXO. Furthermore, chromatin immunoprecipitation experiments using anti-FOXO antibody would yield acetylated histones only in the presence of p300.

To analyse the function of FOXO acetylation in more detail, for the different FOXOs lysine residues that can be acetylated were determined, i.e. FOXO1: Lys245, Lys248, Lys262[30]; FOXO3: Lys242, Lys259, Lys271, Lys290, Lys569[7] and FOXO4: Lys182, Lys185, Lys403[37]. Several of these sites are conserved between FOXOs and therefore of particular interest (Figure 1). Mutation of the acetylated lysine residues in FOXO1 and FOXO4 to arginines, like lysine a positively charged residue but non-acetylatable, enhanced FOXO transcriptional activity. Additionally, CBP could no longer inhibit the activity of such a mutant. At the same time, we also determined by mass-spectrometric analysis the lysine residues in FOXO4 that are acetylated (A.B. Brenkman and N. van den Broek, unpublished data). We found Lys137, Lys159, Lys170, Lys199 and Lys211 to be acetylated, but mutation of these five residues, mutation of the three conserved acetylation sites (lysines 182, 185 and 199 in FOXO4) or mutation of the residues reported by Fukuoka *et al*.[37] did not change FOXO4 transcriptional activity considerably (data not shown). In addition, when analyzing the ability of CBP to acetylate these mutants of FOXO4, we observed that they could still be acetylated, indicating that there are more lysine residues that can be modified by acetylation.

Consistent with the nicotinamide-mediated inhibition of FOXO activity in tissue culture, both growing C.elegans on nicotinamide and knockdown of the nicotinamidase PNC1 results in a decrease in worm lifespan (Chapter 3). Similar results have been published for Saccharomyces cerevisiae: PNC1, by degrading the Sir2-inhibitor nicotinamide, also increases yeast replicative lifespan[40,41]. So, our data suggest that, like yeast, multicellular organisms also profit from lowering Nam-levels by increased activity of PNC1. PNC1 expression is induced by caloric restriction[40] and as such PNC1 bridges caloric restriction to Sir2 activity and lifespan. In C.elegans, caloric restriction also increases longevity[42], which likely is Sir2-dependent[43]. Therefore, it is tempting to speculate that caloric restriction acts through enhancing the expression of PNC1. The functional counterpart of PNC1

in mammals is NAMPT (nicotinamide phosphoribosyltransferase, also called PBEF or Visfatin), which converts nicotinamide into nicotinamide mononucleotide[44]. Consistent with it being an equivalent of PNC1, overexpression of NAMPT increases Sir2 activity in mammalian cells[45]. Whether this increase in Sir2 activity increases lifespan in mammals still has to be proven (see also below).
Regulation of FOXO transcriptional activity by an acetylation-deacetylation cycle resembles regulation of p53 activity (reviewed in Bode and Dong, 2004[46]). p53 is a tumour-suppressor protein involved in similar processes as FOXO: cell cycle arrest upon cellular stress and cell death. In case of p53, acetylation is induced by genotoxic stress and stimulates DNA-binding and transcriptional activity. Notably, Sir2 regulates the acetylation status of both FOXO and p53. By deacetylating FOXO, Sir2 stimulates FOXO transcriptional activity, whereas deacetylation of p53 by Sir2 leads to its inhibition (Figure 1).

Regulation of FOXO ubiquitination by USP7

Oxidative stress can also induce monoubiquitination of FOXOs (Chapter 4). This seems to occur at earlier timepoints than observed for acetylation (5 as compared to 60 minutes, [7,8] and unpublished data). As oxidative stress results in FOXO activation by nuclear translocation, we propose that the monoubiquitination-induced nuclear localisation of FOXOs triggers FOXO acetylation. The mechanism by which monoubiquitination induces nuclear localisation is not clear. One possibility is that monoubiquitination stimulates FOXO nuclear import. For instance, the ubiquitin conjugating enzymes UbcM2, UbcH6 and UBE2E2 are translocated to the nucleus in a ubiquitin-dependent manner by the importin β family member importin 11[47]. Alternatively, nuclear localisation could be a result of increased retention in the nucleus due to cofactor and/or DNA-binding. Increased cofactor-binding has been demonstrated for PCNA, which recruits DNA polymerase η through protein-protein interaction to the site of DNA damage upon monoubiquitination of PCNA by Rad18[48]. Similarly, monoubiquitination of the DNA repair protein FancD2 allows complex formation with FancC, FancG and FancL, which target FancD2 to DNA[49].

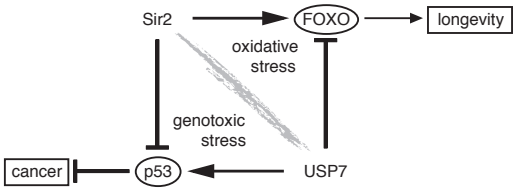


Figure 1 Trade-off between lifespan and cancer. The longevity protein Sir2 prolongs lifespan (in C.elegans, but likely also mammals) by stimulating FOXOs, which play an important role in cellular oxidative stress resistance. However, at the same time Sir2 activity leads to inhibition of p53, a protein central to genome maintenance. Hence, Sir2 activity would give an increased risk of cancer and a concomitant reduction of lifespan. On the other hand, USP7, which is claimed to act as a tumour-suppressor through p53, inhibits FOXO activity and thereby compromises longevity. This model implies that interfering with either longevity or cancer risk affects the other parameter, resulting in a limited lifespan. The only way to increase lifespan and decrease cancer risk at the same time is caloric restriction, which besides activating Sir2, which on its own may not be solely beneficial in humans, influences a whole plethora of processes.

In case of FOXOs, increased DNA-binding upon monoubiquitination would then cause their nuclear retention and this would also explain the increase in FOXO activity by monoubiquitination. To distinguish between nuclear translocation and DNA-binding, one could isolate chromatin and subsequently determine the relative amounts of ubiquitinated FOXO and not-ubiquitinated FOXO in bound and unbound fractions.
FOXOs can also be modified by polyubiquitination, which occurs after growth factor stimulation and is stimulated by the F-box protein Skp2[50-53]. However, this polyubiquitination takes several hours of growth factor stimulation to become visible. Therefore, polyubiquitination of FOXO by Skp2 may be a constitutive, but slow process. Another, not mutually exclusive, possibility is that polyubiquitination, possibly by another enzyme, functions as a negative feedback loop to inhibit monoubiquitinated, thus active, FOXO. However, we did not observe any change in FOXO half-life during an episode of oxidative stress (Chapter 4). Therefore, I propose that monoubiquitination and polyubiquitination of FOXO are two separate entities. This is supported by the fact that Skp2 does not seem to induce monoubiquitination of FOXO, because (1) we observed distinct monoubiquitination, but no polyubiquitination, upon oxidative stress (Chapter 4) and (2) Huang *et al*. (2005) did not observe any Skp2-induced monoubiquitination[50]. Knowledge of the lysines in FOXO that are ubiquitinated is essential to unravelling the natures of mono- and polyubiquitination. However, experiments in our lab to determine by mass spectrometry sites of FOXO4 ubiquitination did not yield any sites yet. Furthermore, knowing which enzyme induces

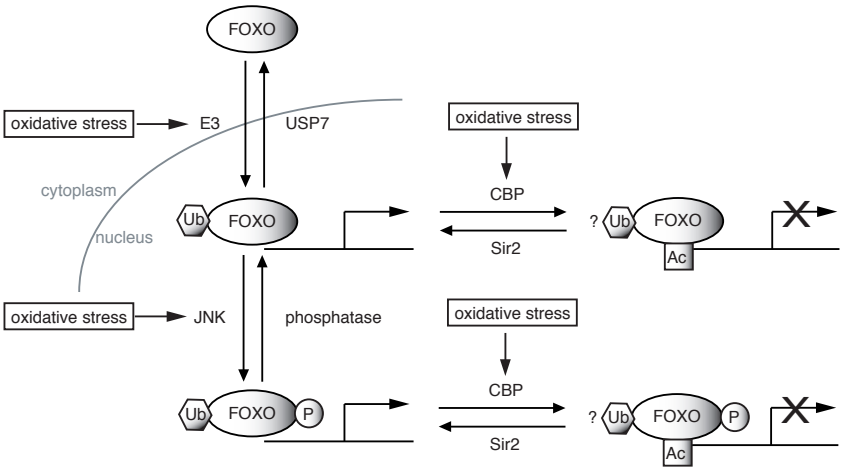


Figure 2 Proposed sequence of events that occur on FOXOs when cells encounter oxidative stress. First, FOXOs are monoubiquitinated by an as yet unknown E3. This monoubiquitination increases the fraction of FOXO present in the nucleus. Subsequently, FOXOs are phosphorylated by JNK, possibly to recruit transcriptional coactivators. As a negative feedback, FOXOs can be acetylated by CBP. Alternatively, deubiquitination of FOXO by USP7 or polyubiquitination could inhibit FOXO activity. By deacetylating FOXO, Sir2 could prolong FOXO activity. Whether modifications persist when other modifications are attached is unclear, especially in case of acetylation and ubiquitination, which potentially occur at the same residue(s). As polyubiquitination is a late event and might take place at monoubiquitinated FOXO, monoubiquitination and JNK phosphorylation probably coexist. Ub=ubiquitin-moiety, Ac=acetyl-moiety, P=phosphate-moiety

monoubiquitination of FOXO could contribute to studies focusing on ways to activate FOXO transcription factors.
The deubiquitinating enzyme USP7 removes the ubiquitin moiety from FOXO and inhibits FOXO activity (Chapter 4). If the function of USP7 would be to remove polyubiquitin chains from FOXO, as it does for p53, Mdm2 and hMdmx, USP7 would stabilise FOXO and consequently stimulate FOXO activity. However, USP7 does not change FOXO half-life, nor does oxidative stress (as mentioned above), and USP7 inhibits FOXO activity (Chapter 4). Therefore, we conclude that the main role of USP7 is to remove monoubiquitin from FOXO. Indeed, ubiquitin-mediated degradation does not seem to be an important regulatory mechanism for FOXO activity as FOXO half-life is approximately 10 hours[51,52]. Notably, Sir2 alike, USP7 regulates the activity of both FOXO and p53[54]. However, whereas Sir2 stimulates FOXO and inhibits p53 activity, USP7 does the opposite (Figure 1). This reciprocal regulation could provide a mechanistic explanation for the trade-off between longevity and cancer incidence. Sir2 enhances FOXO activity and extends lifespan at the expense of increasing the chance of getting cancer by concomitantly inhibiting p53. By inhibiting FOXO and stimulating p53 activity, USP7 would act as a tumour-suppressor protein and a factor decreasing lifespan. In support of this model are data showing that Sir2 enhances cell survival[5556] and that USP7 causes cell death[57]. One mechanism that increases Sir2 activity and increases lifespan is caloric restriction. Seemingly in contrast to our model, caloric restriction at the same time lowers cancer incidence. This can be explained by the fact that caloric restriction has numerous effects, including lowering the risk of getting cancer as well as for example cardiovascular diseases (reviewed in Dirks and Leeuwenburgh, 2006[58]). So, lowering dietary intake may seem beneficial in several aspects. However, side-effects of caloric restriction in humans, such as hypotension, infertility, and osteoporosis should prevent people from changing their diet to a truly restrictive regimen.

FOXO interaction partners

The interaction partners of FOXOs discussed above belong to the FOXO-modifying enzymes. However, FOXO transcription factors also interact with other transcription factors, like PPAR[59], Smads[60] and the androgen receptor[61]. A screen to identify novel transcriptional partners of FOXOs, besides confirming known interaction partners such as PPAR and Smad3/4, shows that ICSBP and NFATc interact with FOXO (Chapter 6). ICSBP contains a region related to Smads and this domain binds proteins that contain PEST sequences[62,63]. The N-terminus of FOXO may contain such a PEST sequence and interestingly Smads bind to FOXOs as well. The interaction of FOXO with ICSBP is enhanced by oxidative stress, so it is tempting to speculate that this interaction depends on a posttranslational modification of FOXO. Alternatively, nuclear localization as a result of oxidative stress could enhance FOXO binding to ICSBP. Interestingly, the interaction between FOXO, ICSBP and NFATc enhances expression of the interleukin 12 gene (Chapter 6), implicating FOXOs in control of T_H1 cell activation by acting in antigen presenting cells. This adds up to the previous notion that FOXO3 regulates T cell homeostasis by inhibiting NFκB signalling and/or inducing apoptosis in these cells by regulating expression of Bim, Bcl6 and/or FasL (reviewed in Coffer and Burgering, 2004[64]).

Perspectives

In conclusion, we have shown that the second messenger cAMP by inhibiting the PI3K/PKB signalling pathway, activates FOXO transcription factors to arrest proliferation of mammalian cells. Oxidative stress, by enhancing monoubiquitination of FOXO and concomitantly inducing FOXO nuclear retention, also activates FOXO transcriptional activity. Conversely, the deubiquitinating enzyme USP7/HAUSP inhibits FOXO activity by removing the ubiquitin moiety. Acetylation of FOXO by the acetyltransferase CBP is also induced by oxidative stress, albeit at later timepoints than monoubiquitination. In contrast to monoubiquitination, acetylation of FOXO inhibits its transcriptional activity. The NAD-dependent deacetylase Sir2 in turn counteracts this inhibition by removing the acetyl-group, resulting in cell cycle arrest and increased stress resistance. Similarly, likely by acting upstream of Sir2, the nicotinamidase PNC1 enhances stress resistance in C.elegans. Figure 2 shows a model that illustrates the order of events upon oxidative stress signalling towards FOXOs. This model, which also includes JNK-mediated phosphorylation of FOXO, is based on the observed timing of events. This model, furthermore, implies that both FOXO acetylation and deubiquitination could act as a negative feedback loop. Knowing under which conditions the one or the other takes place could provide a valuable tool to exogenously modulate FOXO activity and as such benefit from FOXO-mediated cell cycle arrest, apoptosis and/or enhanced stress resistance. Also, in cells where cAMP inhibits proliferation, activation of cAMP signalling could fulfil a similar role.

References

1. Burgering, B. M. & Kops, G. J. Cell cycle and death control: long live Forkheads. *Trends Biochem Sci* **27**, 352-60. (2002).
2. Burgering, B. M. & Bos, J. L. Regulation of Ras-mediated signalling: more than one way to skin a cat. *Trends Biochem Sci* **20**, 18-22 (1995).
3. Dumaz, N., Light, Y. & Marais, R. Cyclic AMP blocks cell growth through Raf-1-dependent and Raf-1-independent mechanisms. *Mol Cell Biol* **22**, 3717-28 (2002).
4. McKenzie, F. R. & Pouyssegur, J. cAMP-mediated growth inhibition in fibroblasts is not mediated via mitogen-activated protein (MAP) kinase (ERK) inhibition. cAMP-dependent protein kinase induces a temporal shift in growth factor-stimulated MAP kinases. *J Biol Chem* **271**, 13476-83 (1996).
5. Kuiperij, H. B. et al. Activation of FoxO transcription factors contributes to the antiproliferative effect of cAMP. *Oncogene* **24**, 2087-95 (2005).
6. Tissenbaum, H. A. & Guarente, L. Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature* **410**, 227-30. (2001).
7. Brunet, A. et al. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* **303**, 2011-5 (2004).
8. van der Horst, A. et al. FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2(SIRT1). *J Biol Chem* **279**, 28873-9 (2004).
9. Wang, L., Liu, F. & Adamo, M. L. Cyclic AMP inhibits extracellular signal-regulated kinase and phosphatidylinositol 3-kinase/Akt pathways by inhibiting Rap1. *J Biol Chem* **276**, 37242-9 (2001).
10. Lee, H. T. & Kay, E. P. Regulatory role of cAMP on expression of Cdk4 and p27(Kip1) by inhibiting phosphatidylinositol 3-kinase in corneal endothelial cells. *Invest Ophthalmol Vis Sci* **44**, 3816-25 (2003).
11. Kim, S., Jee, K., Kim, D., Koh, H. & Chung, J. Cyclic AMP inhibits Akt activity by blocking the membrane localization of PDK1. *J Biol Chem* **276**, 12864-70 (2001).

12. Sewing, A. et al. Human cyclin D1 encodes a labile nuclear protein whose synthesis is directly induced by growth factors and suppressed by cyclic AMP. *J Cell Sci* **104 (Pt 2)**, 545-55 (1993).
13. van Oirschot, B. A., Stahl, M., Lens, S. M. & Medema, R. H. Protein kinase A regulates expression of p27(kip1) and cyclin D3 to suppress proliferation of leukemic T cell lines. *J Biol Chem* **276**, 33854-60 (2001).
14. L'Allemain, G., Lavoie, J. N., Rivard, N., Baldin, V. & Pouyssegur, J. Cyclin D1 expression is a major target of the cAMP-induced inhibition of cell cycle entry in fibroblasts. *Oncogene* **14**, 1981-90 (1997).
15. Schmidt, M. et al. Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. *Mol Cell Biol* **22**, 7842-52 (2002).
16. Medema, R. H., Kops, G. J., Bos, J. L. & Burgering, B. M. AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1. *Nature* **404**, 782-7. (2000).
17. Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R. & Pouyssegur, J. Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. *J Biol Chem* **271**, 20608-16 (1996).
18. Aktas, H., Cai, H. & Cooper, G. M. Ras links growth factor signaling to the cell cycle machinery via regulation of cyclin D1 and the Cdk inhibitor p27KIP1. *Mol Cell Biol* **17**, 3850-7 (1997).
19. Lee, R. J. et al. pp60(v-src) induction of cyclin D1 requires collaborative interactions between the extracellular signal-regulated kinase, p38, and Jun kinase pathways. A role for cAMP response element-binding protein and activating transcription factor-2 in pp60(v-src) signaling in breast cancer cells. *J Biol Chem* **274**, 7341-50 (1999).
20. Chen, F., Kim, E., Wang, C. C. & Harrison, L. E. Ciglitazone-induced p27 gene transcriptional activity is mediated through Sp1 and is negatively regulated by the MAPK signaling pathway. *Cell Signal* **17**, 1572-7 (2005).
21. Delmas, C. et al. The p42/p44 mitogen-activated protein kinase activation triggers p27Kip1 degradation independently of CDK2/cyclin E in NIH 3T3 cells. *J Biol Chem* **276**, 34958-65 (2001).
22. Cook, S. J. & McCormick, F. Inhibition by cAMP of Ras-dependent activation of Raf. *Science* **262**, 1069-72 (1993).
23. Burgering, B. M., Pronk, G. J., van Weeren, P. C., Chardin, P. & Bos, J. L. cAMP antagonizes p21ras-directed activation of extracellular signal-regulated kinase 2 and phosphorylation of mSos nucleotide exchange factor. *Embo J* **12**, 4211-20 (1993).
24. Wu, J. et al. Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. *Science* **262**, 1065-9 (1993).
25. Stork, P. J. & Schmitt, J. M. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. *Trends Cell Biol* **12**, 258-66 (2002).
26. Greer, E. L. & Brunet, A. FOXO transcription factors at the interface between longevity and tumor suppression. *Oncogene* **24**, 7410-25 (2005).
27. Kobayashi, Y. et al. SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative stress. *Int J Mol Med* **16**, 237-43 (2005).
28. Gan, L. et al. FoxO-dependent and -independent mechanisms mediate SirT1 effects on IGFBP-1 gene expression. *Biochem Biophys Res Commun* **337**, 1092-6 (2005).
29. Frescas, D., Valenti, L. & Accili, D. Nuclear trapping of the forkhead transcription factor FoxO1 via Sirt-dependent deacetylation promotes expression of glucogenetic genes. *J Biol Chem* **280**, 20589-95 (2005).
30. Daitoku, H. et al. Silent information regulator 2 potentiates Foxo1-mediated transcription through its deacetylase activity. *Proc Natl Acad Sci U S A* **101**, 10042-7 (2004).
31. Motta, M. C. et al. Mammalian SIRT1 represses forkhead transcription factors. *Cell* **116**, 551-63 (2004).

32. Yang, Y., Hou, H., Haller, E. M., Nicosia, S. V. & Bai, W. Suppression of FOXO1 activity by FHL2 through SIRT1-mediated deacetylation. *Embo J* **24**, 1021-32 (2005).
33. Giannakou, M. E. & Partridge, L. The interaction between FOXO and SIRT1: tipping the balance towards survival. *Trends Cell Biol* **14**, 408-12 (2004).
34. Bouras, T. et al. SIRT1 deacetylation and repression of p300 involves lysine residues 1020/1024 within the cell cycle regulatory domain 1. *J Biol Chem* **280**, 10264-76 (2005).
35. Matsuzaki, H. et al. Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. *Proc Natl Acad Sci U S A* **102**, 11278-83 (2005).
36. Perrot, V. & Rechler, M. M. The coactivator p300 directly acetylates the forkhead transcription factor Foxo1 and stimulates Foxo1-induced transcription. *Mol Endocrinol* **19**, 2283-98 (2005).
37. Fukuoka, M. et al. Negative regulation of forkhead transcription factor AFX (Foxo4) by CBP-induced acetylation. *Int J Mol Med* **12**, 503-8. (2003).
38. Sakaguchi, K. et al. DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes Dev* **12**, 2831-41 (1998).
39. Bereshchenko, O. R., Gu, W. & Dalla-Favera, R. Acetylation inactivates the transcriptional repressor BCL6. *Nat Genet* **32**, 606-13 (2002).
40. Anderson, R. M., Bitterman, K. J., Wood, J. G., Medvedik, O. & Sinclair, D. A. Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. *Nature* **423**, 181-5 (2003).
41. Gallo, C. M., Smith, D. L., Jr. & Smith, J. S. Nicotinamide clearance by Pnc1 directly regulates Sir2-mediated silencing and longevity. *Mol Cell Biol* **24**, 1301-12 (2004).
42. Klass, M. R. Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. *Mech Ageing Dev* **6**, 413-29 (1977).
43. Wood, J. G. et al. Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* **430**, 686-9 (2004).
44. Rongvaux, A. et al. Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. *Eur J Immunol* **32**, 3225-34 (2002).
45. Revollo, J. R., Grimm, A. A. & Imai, S. The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. *J Biol Chem* **279**, 50754-63 (2004).
46. Bode, A. M. & Dong, Z. Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer* **4**, 793-805 (2004).
47. Plafker, S. M., Plafker, K. S., Weissman, A. M. & Macara, I. G. Ubiquitin charging of human class III ubiquitin-conjugating enzymes triggers their nuclear import. *J Cell Biol* **167**, 649-59 (2004).
48. Watanabe, K. et al. Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination. *Embo J* **23**, 3886-96 (2004).
49. Montes de Oca, R. et al. Regulated interaction of the Fanconi anemia protein, FANCD2, with chromatin. *Blood* **105**, 1003-9 (2005).
50. Huang, H. et al. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. *Proc Natl Acad Sci U S A* **102**, 1649-54 (2005).
51. Matsuzaki, H., Daitoku, H., Hatta, M., Tanaka, K. & Fukamizu, A. Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation. *Proc Natl Acad Sci U S A* **100**, 11285-90 (2003).
52. Plas, D. R. & Thompson, C. B. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. *J Biol Chem* **278**, 12361-6 (2003).
53. Aoki, M., Jiang, H. & Vogt, P. K. Proteasomal degradation of the FoxO1 transcriptional regulator in cells transformed by the P3k and Akt oncoproteins. *Proc Natl Acad Sci U S A* **101**, 13613-7 (2004).

54. Li, M. et al. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. *Nature* **416**, 648-53 (2002).
55. Yeung, F. et al. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *Embo J* **23**, 2369-80 (2004).
56. Cohen, H. Y. et al. Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. *Science* **305**, 390-2 (2004).
57. Yoo, K. J. et al. Expression and functional analyses of mHAUSP regulating apoptosis of cervical adenocarcinoma cells. *Int J Oncol* **27**, 97-104 (2005).
58. Dirks, A. J. & Leeuwenburgh, C. Caloric restriction in humans: Potential pitfalls and health concerns. *Mech Ageing Dev* **127**, 1-7 (2006).
59. Dowell, P., Otto, T. C., Adi, S. & Lane, M. D. Convergence of peroxisome proliferator-activated receptor gamma and Foxo1 signaling pathways. *J Biol Chem* **278**, 45485-91 (2003).
60. Seoane, J., Le, H. V., Shen, L., Anderson, S. A. & Massague, J. Integration of Smad and forkhead pathways in the control of neuroepithelial and glioblastoma cell proliferation. *Cell* **117**, 211-23 (2004).
61. Li, P. et al. AKT-independent protection of prostate cancer cells from apoptosis mediated through complex formation between the androgen receptor and FKHR. *Mol Cell Biol* **23**, 104-18 (2003).
62. Massague, J. & Wotton, D. Transcriptional control by the TGF-beta/Smad signaling system. *Embo J* **19**, 1745-54 (2000).
63. Marecki, S. & Fenton, M. J. PU.1/Interferon Regulatory Factor interactions: mechanisms of transcriptional regulation. *Cell Biochem Biophys* **33**, 127-48 (2000).
64. Coffer, P. J. & Burgering, B. M. Forkhead-box transcription factors and their role in the immune system. *Nat Rev Immunol* **4**, 889-99 (2004).

Summary

FOXO transcription factors play an important role in essential biological processes such as differentiation, proliferation, apoptosis, metabolism and stress resistance. FOXOs were originally identified as transcription factors that act downstream of insulin/growth factor signalling through PI3K and PKB. In fact, at the time of the start of the study described in this thesis the only modification that had been described to regulate FOXO transcriptional activity is phosphorylation. However, many proteins can also be regulated by other posttranslational modifications like acetylation and ubiquitination. In case of transcription factors, acetylation can both positively and negatively regulate their activity. The same holds true for ubiquitination: monoubiquitination of transcription factors generally enhances transcriptional activity whereas polyubiquitination (and subsequent degradation) inhibits their activity. These posttranslational modifications can be induced by certain types of cellular stress and interestingly in this respect FOXO transcription factors and oxidative stress seem to be intimately linked. On the one hand, FOXOs increase cellular resistance to oxidative stress and in turn oxidative stress induces phosphorylation of FOXOs, mediated by the stress-kinase JNK. Before the study described in this thesis was initiated our understanding of the effects of oxidative stress on FOXO activity and FOXO regulation by posttranslational modifications was little. Therefore, we set out to further our knowledge in these directions and focused on both the cellular and organismal level.

Chapter 2 was inspired by a study in C.elegans by Tissenbaum and Guarente (2001) demonstrating that the longevity protein Sir2 extends lifespan of the nematode in a manner that is dependent on FOXO activity. In chapter 2 we provide a possible explanation for this increase in C.elegans lifespan by Sir2 through FOXO. We show that mammalian Sir2, also called SIRT1, directly binds to and deacetylates FOXO4. Thereby Sir2 activates FOXO transcriptional activity both towards cell cycle regulation and stress resistance. Furthermore, we show that FOXOs are acetylated in response to oxidative stress by the acetyltransferase CBP. As predicted from the stimulatory effect of Sir2 on FOXO activity, CBP inhibits FOXO transcriptional activity.

Nicotinamide, a product of the Sir2-mediated deacetylation reaction, inhibits Sir2 activity and hence may decrease organismal lifespan. In **Chapter 3** we describe that the addition of exogenous nicotinamide indeed shortens the lifespan of C.elegans. Physiologically, nicotinamide is converted by a so-called NAD^+-salvage pathway present in organisms from bacteria to humans. In C.elegans, the nicotinamidase PNC-1 may accomplish this conversion resulting in an increased worm lifespan. We found that RNAi-mediated knockdown of PNC-1 indeed decreases worm lifespan. On the contrary, transgenic overexpression of PNC-1 enhanced the worm's oxidative stress resistance, which has been implicated in increasing lifespan.

In **Chapter 4** we show that, besides stimulating FOXO acetylation, oxidative stress induces monoubiquitination of FOXO4. This monoubiquitination results in nuclear retention and increased transcriptional activity of FOXO4. The ubiquitin-moiety is cleaved from FOXO4 by the DUB USP7. By deubiquitinating FOXO, USP7 inhibits FOXO activity, probably best illustrated by our finding that RNAi-mediated knockdown of USP7 enhances $p27^{kip1}$ expression in a FOXO-dependent manner. As expected from the observed monoubiquitination neither oxidative stress nor USP7 affects FOXO4 half-life, which is about 9 hours.

cAMP has long been appreciated as a second messenger that can induce cell cycle arrest. Several models, concerning both MAPK and PI3K/PKB signalling, have been proposed to

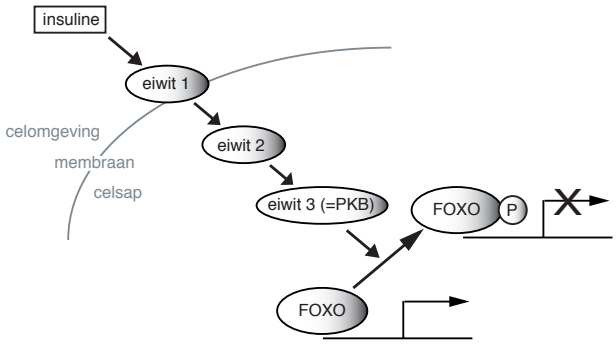
explain this phenomenon. In **Chapter 5** we demonstrate that in MEFs, cAMP acts through the PI3K/PKB/FOXO axis to induce growth arrest. cAMP inhibits PKB phosphorylation, thus activation, resulting in reduced phosphorylation of FOXO. As such cAMP enhances FOXO localization to the nucleus and subsequent induction of $p27^{kip1}$ and inhibition of cyclin D expression. Regulation of these two proteins probably is responsible for the cell cycle arrest induced by cAMP.

To exert their diverse array of effects, FOXOs interact with other proteins. In **Chapter 6** we describe a screen to discover novel FOXO interacting transcription factors. Besides confirming previously reported FOXO interactors such as Smad3/4 an PPARγ, we identified interferon consensus sequence binding protein (ICSBP), nuclear factor of activated T-cells (NFATc), nuclear respiratory factor 1 (NRF1), Transcriptional Intermediary Factor 1 (TIF1), Sp1 and Homeobox D9/10 (HOXD9/10) as possible FOXO interactors. By co-immunoprecipitations we confirmed binding of ICSBP to FOXO4, which is enhanced both by oxidative stress and NFATc, another possible novel interactor of FOXO. NFATc and ICSBP have been reported to stimulate expression of IL-12, a cytokine that regulates T_H1 cell proliferation, and we demonstrate that FOXO4 acts synergistically with NFATc and ICSBP to induce expression of this cytokine.

In conclusion, this thesis describes the regulation of FOXO activity by posttranslational modifications. We show that FOXO phosphorylation through PKB is inhibited by cAMP and that monoubiquitination and acetylation of FOXO are induced by oxidative stress, probably in this order. Furthermore, we demonstrate that monoubiquitination stimulates and that acetylation inhibits FOXO transcriptional activity. These findings add up to our knowledge about pathways that keep FOXO activity in check, with potentials for therapeutic interventions in for example diabetes. Moreover, our findings that FOXO activity is regulated both by Sir2 and USP7 along with previous reports that p53 is regulated by the same two proteins but in the opposite direction, led us to propose an intriguing model for a trade-off between aging and cancer: activation of Sir2 enhances FOXO activity and as such may enhance lifespan, but at the same time increases cancer risk by inhibiting p53. Reversely, USP7 decreases cancer risk by stimulating p53 activity, but at the same time limits lifespan by inhibiting FOXO.

Samenvatting (voor de leek)

Grotere organismen, zoals de mens, bestaan uit organen zoals het hart, de lever en de hersenen en systemen zoals het bloedvatsysteem. Elk orgaan is op zijn beurt opgebouwd uit verschillende weefsels. Bindweefsel is zo'n weefsel. Een ander voorbeeld is epitheel, ook wel dekweefsel genoemd; dit bedekt bijvoorbeeld de mondholte. Weefsels bestaan dan weer uit cellen. Zo vormen bindweefselcellen bindweefsel en epitheelcellen epitheel. Uit bovenstaande kan worden opgemaakt dat organen uit verschillende typen cellen zijn opgebouwd. Veelal dienen de bindweefselcellen ertoe een netwerk te vormen waarin/waarop de andere cellen vastzitten. Die andere cellen zijn meer orgaan-specifieke cellen, zoals hartspiercellen, insuline-producerende cellen etcetera. Voor organismen is het erg belangrijk om cellen te verversen als die slechter worden. Dit kan gebeuren door celdeling te laten plaatsvinden van gezonde cellen, wat overigens niet zomaar gebeurt, maar op aangeven van bijvoorbeeld hormonen. Hierbij ontstaan uit één cel twee cellen. U zult begrijpen dat een storing in deze celdeling kan leiden tot kanker.
De cel is het kleinste deel van ons lichaam dat zelfstandig kan leven. Ter vergelijking: er zijn organismen die maar uit één cel bestaan: bacteriën en gist bijvoorbeeld. Een cel bestaat hoofdzakelijk uit suikers, vetten en eiwitten, waarvan de eiwitten het belangrijkst zijn voor de regulatie van allerlei processen in de cel, zoals bijvoorbeeld het "verbranden" van suiker of de genetische code (=DNA) aflezen. Deze onderdelen van een cel bevinden zich veelal in het celsap. Dit celsap wordt omsloten door een membraan. Dit is een soort vlies met onder andere communicatiemiddelen met de buitenwereld. In het binnenste van een cel bevindt zich een bibliotheek (=kern) met genetische informatie (=DNA) die nodig is om de cel zelf en dus het organisme als geheel te laten functioneren. Zoals hierboven al even genoemd, kunnen cellen met elkaar communiceren met behulp van hormonen. Laten we bijvoorbeeld weer een hormoon nemen dat de cel ertoe dwingt te delen zodat er twee cellen ontstaan. Dit hormoon bindt dan aan de buitenkant van de cel (membraan) wat ertoe leidt dat er een teken/signaal de cel in gaat dat dat specifieke hormoon is gebonden. Dit signaal wordt via bepaalde eiwitten doorgegeven en heeft als resultaat dat een eiwit in de kern van de cel (dit eiwit heet een transcriptiefactor) bepaalde delen van het DNA minder gaat aflezen. Hierdoor worden er bepaalde eiwitten, die celdeling remmen, niet meer gevormd en gaat de cel delen.



Figuur 1 Schematische weergave van de route die door insuline wordt aangezet en die leidt tot het aanbrengen van een fosforgroep (aangegeven met de letter P) op FOXO door eiwit 3, dat PKB heet. Zo'n fosforgroep op FOXO remt de mogelijkheid van FOXO om DNA af te lezen en dus celdeling te remmen. Deze remming is aangegeven door een kruis door de pijl die de afleesmogelijkheid weergeeft.

In dit proefschrift staan de zogenaamde FOXO transcriptiefactoren (FOXOs) centraal en het zojuist genoemde hormoon zou insuline kunnen zijn en de transcriptiefactor FOXO. Oorspronkelijk zijn FOXOs namelijk beschreven als transcriptiefactoren wier activiteit geremd wordt door insuline en als zodanig spelen FOXOs een rol in suikerziekte/diabetes. FOXOs hebben verder een belangrijke rol in processen zoals celdeling en celdood. FOXOs kunnen in bepaalde cellen de celdeling remmen en in andere cellen celdood veroorzaken.
Een transcriptiefactor is niet altijd actief, maar zoals aangegeven in het voorbeeld wordt zijn activiteit gereguleerd/beïnvloed door signalen van buiten de cel, zoals hormonen. Die regulatie gebeurt doordat eiwitten op de desbetreffende transcriptiefactor een kleine verandering (modificatie) aanbrengen. Voorbeelden hiervan zijn fosforylering, acetylering en ubiquitinering, waarbij er respectievelijk een fosfor-, acetyl-, of ubiquitinegroep op de transcriptiefactor wordt gezet met behulp van in de cel zelf aanwezige groepen. Ter illustratie: het laatste eiwit in de bovengenoemde insuline-route zet een fosforgroep op FOXO en remt daarmee de DNA-afleesactiviteit van FOXO (Figuur 1). Naast diabetes, lijken FOXOs ook betrokken bij het krijgen van kanker. In veel tumoren is er namelijk iets mis met de route naar FOXO en dit zou belangrijk kunnen zijn voor het krijgen van een tumor. FOXO kan dan namelijk niet meer de celdeling remmen of celdood geven. Begrip van de regulatie van FOXOs kan dus van belang zijn voor de behandeling van diabetes en kanker. In dit proefschrift wordt daarom ons onderzoek naar de regulatie van de zogenaamde FOXO transcriptiefactoren door bovengenoemde modificaties (acetylering, fosforylering en ubiquitinering) beschreven.
In **Hoofdstuk 2** beschrijven we dat de activiteit van FOXO ook geremd wordt door acetylering. Het resultaat is dat een cel sneller deelt. Deze acetylering wordt verricht door een eiwit dat CBP heet en treedt op als de cel last heeft van stress, in dit geval een stress die ontstaat bij bijvoorbeeld het verbranden van glucose en die schade kan geven aan bouwstenen van de cel. Dus waar insuline de activiteit van FOXO remt door fosforylering van FOXO te geven, remt stress FOXO activiteit door acetylering te bewerkstelligen. De acetyl-groep kan na een tijdje ook weer verwijderd worden van FOXO om diens effect ongedaan te maken. Het eiwit dat verantwoordeljik is voor deze verwijdering heet Sir2. Van dit eiwit was al bekend dat het de levensduur van een heel klein proefdier, namelijk het wormpje C.elegans, verlengt en voor deze verlenging FOXO nodig heeft. Wij denken nu dat ook in mensen Sir2 levensduur kan verlengen via het verwijderen van de acetyl-groep van FOXO.
De activiteit van Sir2 kan geremd worden door een stof die Nam (=nicotinamide) heet. Deze stof zou als het goed is verkorting van levensduur moeten geven, omdat Sir2 verlenging van levensduur veroorzaakt. Wij hebben dit getest in het wormpje C.elegans en zien inderdaad dat dit wormpje korter leeft als het wordt behandeld met Nam **(Hoofdstuk 3)**. Veel organismen hebben een manier om dit Nam af te breken. Deze afbraak gebeurt in C.elegans door een eiwit dat PNC-1 heet. Ook als wij in dit wormpje de aanmaak van PNC-1 eiwit verhinderen en zo de hoeveelheid Nam verhogen, zien wij dat het wormpje korter leeft, wat er op wijst dat de regulatie van Sir2, en wellicht FOXO activiteit, belangrijk is voor de bepaling van de levensduur.
In Hoofdstuk 2 hebben wij beschreven dat een stress-situatie acetylering van FOXO kan geven. In **Hoofdstuk 4** beschrijven wij dat dezelfde vorm van stress ook ubiquitinering van FOXO kan veroorzaken. Wij vinden dat deze verandering eerder plaatsvindt dan acetylering en de activiteit van FOXO stimuleert. Ons idee is nu dat in een stress-situatie ubiquitinering van FOXO eerst optreedt om FOXO te activeren en dat daarna acetylering plaatsvindt om

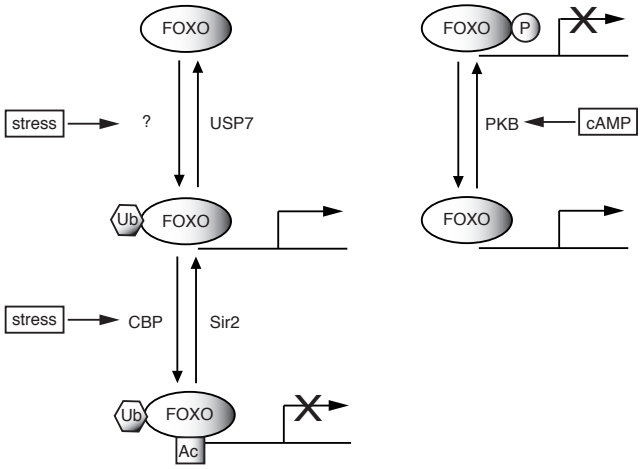
FOXO weer te remmen. Net zoals de acetylering, kan de ubiquitinering ongedaan gemaakt worden door het verwijderen van de ubiquitine-groep. Dit wordt gedaan door het eiwit USP7. Om FOXO te remmen kan er dus een acetyl-groep op worden vastgemaakt, maar ook kan de ubiquitine-groep worden verwijderd. Wij weten momenteel nog niet wanneer welke van de twee plaatsvindt.

In **Hoofdstuk 5** beschrijven we ons onderzoek naar de fosforylering van FOXO. Zoals boven al genoemd, heeft insuline als effect fosforylering van FOXO. In Hoofdstuk 5 laten wij zien dat het stofje cAMP, dat bijvoorbeeld gevormd wordt als een bepaald hormoon bij een cel aankomt, de route tussen insuline en FOXO remt, wat resulteert in minder fosforylering van FOXO en dus actiever FOXO. Het effect van actiever FOXO is het remmen van de celdeling.

De regulatie van FOXOs door de bovengenoemde veranderingen staan niet op zichzelf. Het effect van de veranderingen kan bijvoorbeeld zijn dat FOXOs makkelijker DNA kunnen aflezen of aan een ander eiwit (=bindingspartner) gaan zitten en daardoor geremd worden.

In **Hoofdstuk 6** beschrijven we een studie naar mogelijke bindingspartners van FOXO. We vonden twee eiwitten waarvan we al wisten dat ze aan FOXO kunnen binden, wat bevestigde dat we de studie goed uitgevoerd hadden. Verder vonden we een aantal mogelijke nieuwe bindingspartners. Voor ons waren ICSBP en NFATc de meest interessante. We laten zien dat FOXO samen met ICSBP en NFATc zorgt voor de productie van het eiwit IL-12 dat belangrijk is om infecties te bestrijden. Dit zou een heel interessante nieuwe rol voor FOXOs zijn. We gaan dit dan ook verder onderzoeken.

Samengevat beschrijft dit proefschrift de regulatie van FOXO activiteit door veranderingen/ modificaties aan FOXO zelf. We laten zien dat cAMP FOXO activiteit stimuleert door



Figuur 2 Schematische weergave van de veranderingen die op FOXOs plaatsvinden tijdens stress en de verandering die door cAMP. wordt geregeld, inclusief de verantwoordelijke eiwitten. Stress (*links*) veroorzaakt eerst ubiquitinering (aangegeven met "Ub") van FOXO wat de activiteit van FOXO ten goede komt. Vervolgens kan CBP een acetyl-groep ("Ac") op FOXO zetten. Deze verandering geeft verlaging van FOXO activiteit. Deze remming is aangegeven door een kruis door de pijl die de afleesmogelijkheid weergeeft. cAMP (*rechts*) remt de activiteit van PKB en voorkomt daardoor dat er een fosforgroep op FOXO wordt gezet. Deze remming verhoogt de activiteit van FOXO om DNA af te lezen en dus celdeling te remmen.

fosforylering van FOXO te verhinderen en daardoor celdeling remt. Verder beschrijven we dat stress ubiquitinering en acetylering van FOXO veroorzaakt met een respectievelijk activerend en remmend effect op FOXO. Voorts laten wij zien welke eiwitten betrokken zijn bij het aanbrengen en/of verwijderen van deze modificaties. Dit alles staat schematisch weergegeven in Figuur 2. Deze vindingen verruimen ons inzicht in de regulatie van FOXO transcriptiefactoren en zouden kunnen leiden tot nieuwe inzichten voor de ontwikkeling van therapieën voor diabetes en kanker.

Curriculum Vitae

Armando van der Horst werd geboren op 16 april 1978 te Harderwijk. In 1996 behaalde hij het VWO diploma aan het Christelijk College Nassau-Veluwe eveneens te Harderwijk. In datzelfde jaar begon hij met de studie Medische Biologie aan de Universiteit Utrecht. Zijn propedeutisch examen werd afgelegd in 1997. In de laatste fase van de studie deed hij onderzoekservaring op in het Universitair Medisch Centrum Utrecht bij de afdeling Pathologie onder begeleiding van Drs. F.J. Bijlsma en Dr. R.A. de Weger en bij de afdeling Celbiologie onder supervisie van Drs. J. Gent en Prof. Dr. G.J. Strous. Voorts heeft hij een onderzoeksstage gedaan op het Anthony Nolan Research Institute te Londen onder begeleiding van Dr. I.A. Dodi, Dr. M.G. Tilanus en Prof. Dr. J.A. Madrigal. Het doctoraaldiploma werd behaald in juni 2001. Het promotie-onderzoek, zoals beschreven in dit proefschrift, is verricht in de periode van september 2001 tot en met februari 2006 onder begeleiding van Prof. Dr. Ir. B.M.T. Burgering bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum Utrecht.

List of publications

Bijlsma FJ, **van der Horst AA**, Tilanus MG, Rozemuller E, de Jonge N, Gmelig-Meyling FH, de Weger RA (2002). No association between transforming growth factor beta gene polymorphism and acute allograft rejection after cardiac transplantation. *Transpl Immunol*. **10**:43-7

Schmidt M, Fernandez de Mattos S, **van der Horst A**, Klompmaker R, Kops GJ, Lam EW, Burgering BM, Medema RH (2002). Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D. *Mol Cell Biol*. **22**:7842-52

Van der Horst A, Tertoolen, LGJ, de Vries-Smits LMM, Frye RA, Medema RH, Burgering BMT (2004). FOXO4 is acetylated upon peroxide stress and deacetylated by the longevity protein hSir2^{SIRT1}. *J Biol Chem*. **279**:28873-9

Kuiperij HB, **van der Horst A**, Raaijmakers J, Weijzen S, Medema RH, Bos JL, Burgering BM, Zwartkruis FJ (2005). Activation of FoxO transcription factors contributes to the antiproliferative effect of cAMP. *Oncogene* **24**:2087-95

Van der Horst A, de Vries-Smits AMM, Brenkman AB, van Triest MH, Broek N, Colland F, Maurice MM, and Burgering BMT. FOXO4 transcriptional activity is regulated by monoubiquitination and USP7/HAUSP. *Submitted*

Van der Horst A, Schavemaker JM, Pellis-van Berkel W, Zwartkruis FJT, Burgering BMT. The Caenorhabditis elegans nicotinamidase PNC-1 extends lifespan. *In preparation*

Van der Horst A, Hummel M, Van den Heuvel APJ, Burgering BMT. FOXO4 functionally interacts with ICSBP. *In preparation*

Dankwoord

Tja, het waren 4.5 fantastische jaren (en daar komt nog een half jaartje bij!). Deze zijn mede mogelijk gemaakt door (in willekeurige volgorde) mijn collega's, vrienden en familie en daarom wil dan ook graag nog 't een en 't ander zeggen.

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