Forkhead box O and the control of cellular oxidative stress

Forkhead box O en de controle van cellulaire oxidatieve stress

(met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College van Promoties
in het openbaar te verdedigen op
donderdag 25 november 2004 des ochtends te 10:30 uur

door

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geboren op 29 juli 1976 te Zevenaar

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ISBN: 90-393-3853-1

Print: Febodruk BV, Enschede
Cover: M. Essers

The research described in this thesis was supported by a grant from the Dutch Cancer Society (KWF). Publication of this thesis was financially supported by University Medical Center Utrecht (UMCU) and Tebu-Bio.

Table of contents

Abbreviations

4OHT	4-hydroxy-tamoxifen
ALL	acute lymphoid leukemia
APC	adenomatous polyposis coli
AR	androgen receptor
ARM	alveolar rhabdomyosarcomas
ASK	apoptosis signal-regulating kinase
β-TRCP	β-transducin repeat-containing protein
cAMP	cyclic adenosine 3',5'-monophosphate
C/EBPβ	CAAT/enhancer-binding protein β
CK1	casein kinase 1
CBP	CREB binding protein
CDK	cyclin dependent kinase
CK1	casein kinase 1
CKI	CDK inhibitor
CREB	cAMP responsive element binding protein
Crm1	chromosome region maintenance 1
CtBP	C-terminal binding protein
DAF	dauer formation
DBE	DAF-16 binding element
DLK	dual leucine zipper-bearing kinase
Dsh	Dishevelled
DYRK1a	dual-specificity tyrosine-phosphorylated and regulated kinase 1a
EGF	epidermal growth factor
Epo	erythropoietin
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
Exp7	exportin 7
FasL	Fas ligand
FHRE	FOXO responsive element
FOX	Forkhead box
GADD45	DNA damage-inducible gene 45
GEF	guanine nucleotide exchange factor
G6Pase	glucose 6 phosphatase
GR	glucocorticoid receptor
GSK3	glycogen synthase kinase 3
GST	glutathione S transferase
GTP	guanosine triphosphate
HNF	hepatic nuclear factor
H_2O_2	hydrogen peroxide
IGF1	insulin-like growth factor-1
IKK	IkB kinase
IL	interleukin
InsR	insulin receptor
IRE	insulin response element
JIP	JNK interacting protein
LEF/TCF	lymphoid enhancer factor/T cell factor
Lgs	Legless
LiCl	lithium chloride

LMB	leptomycin B
LPL	lipoprotein lipase
LZK	leucine zipper-bearing kinase
MAPK	mitogen activated protein kinase
MEF	mouse embryo fibroblast
MEK	MAP or ERK kinase
MKK	mitogen-activated protein kinase kinase
MLK	mixed lineage kinase
MLL	myeloid lineage leukemia
MnSOD	manganese superoxide dismutase
NADH	nicotinamide adenine dinucleotide
NES	nuclear export signal
NFκB	nuclear factor κB
NLS	nuclear localization signal
Pak-1	p21-activated kinase-1
PAX	paired homeobox
PBS	phosphate buffered saline
PDK-1	PI(3,4,5)P3-dependent kinase-1
PDK4	pyruvate dehydrogenase kinase 4
PEPCK	phosphoenolpyruvate carboxy kinase
PH	pleckstrin homology
PI3K	phosphatidylinositol-3OH-kinase
PI(3)P	phosphatidylinositol (3) phosphate
PI(3,4)P2	phosphatidylinositol (3,4) diphosphate
PI(3,4,5)P3	phosphatidylinositol (3,4,5) triphosphate
PKB	protein kinase B
PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
Pygo	Pygopus
Rb	retinoblastoma tumor suppressor protein
PTEN	phosphatase and tensin homologue on chromosome 10
RalGDS	Ral guanine nucleotide dissociation stimulator
RAR	retinoic acid receptor
RBD	Ras-binding domain
Rlf	RalGDS-like factor
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SAPK/JNK	stress-activated kinase/c-Jun NH2-terminal kinase
SCF	Skp1-Cul1-F-box-protein
SGK	serum- and glucocorticoid-induced kinase
SIR	silent information regulator
SWI/SNF	mating type switching/sucrose non-fermenting
TAK1	TGFb activated kinase
TGFβ	transforming growth factor β
THR	thyroid hormone receptor
TKO	pRB-/- p107-/- p130-/- triple knockout
TNF	tumor necrosis factor
TPL2	tumor progression locus 2
TRAIL	TNF-related apoptosis-inducing ligand

Chapter 1

General introduction:
Forkhead box O transcription factors

Forkhead transcription factors of the FOXO subfamily have been identified as direct targets of phosphoinositide 3-kinase-mediated signal transduction. FOXO1, FOXO3a, and FOXO4 are directly phosphorylated by protein kinase B/Akt, resulting in nuclear export and inhibition of transcription. Several other kinases have been identified, which regulate phosphorylation of FOXOs as well, and processes like acetylation and ubiquitination may play a role in the regulation of FOXO activity. FOXO transcription factors are emerging as a shared component among pathways regulating a multitude of biological processes including, cell cycle, apoptosis, DNA repair, differentiation, metabolism, and protection from oxidative stress. These data indicate FOXO as a meeting point within the cell, integrating different signalling pathways and regulating cell fate.

Forkhead transcription factors

The super family of Forkhead transcription factors consists of over 100 different members with orthologues expressed in species ranging from yeast to humans (reviewed in Kaufmann and Knochel, 1996). About 40 different Forkhead transcription factors have been identified in mammalian cell. Members of the Forkhead family are characterized by the presence of a conserved 110 amino acid DNA binding, or Forkhead domain, which was first identified by comparison of the DNA binding domain of the *Drosophila* homeotic Forkhead protein and the hepatic nuclear factor-3 (HNF-3) (Lai et al., 1991; Weigel and Jackle, 1990). The 110 amino acids of the Forkhead domain form a typical butterfly-shaped structure made up of three tightly packed N-terminal α-helices forming the thorax of the butterfly, three β-sheets, and two loop regions at the C-terminal end that shape the wings of the butterfly (Clark et al., 1993; Jin et al., 1999; Marsden et al., 1998; van Dongen et al., 2000; Weigelt et al., 2001). Therefore, the Forkhead domain is also referred to as winged helix motif. DNA binding of Forkhead proteins depends on the interaction of the third helix in the Forkhead box (H3) with DNA bases of its recognition sequence within the major grove of double-stranded DNA (Clark et al., 1993). Residues in the two loop regions at the carboxy-terminal end of the Forkhead domain make additional contact with the DNA-binding element, and these interactions may contribute to binding-site selectivity of the different Forkhead proteins (Clark et al., 1993; Overdier et al., 1994). Comparison of binding-site selection of a variety of Forkhead proteins has led to the identification of a core region of recognition motif of 7 bp: T-[G/A]-T-T-[G/T]-[G/A]-[C/T] (Kaufmann et al., 1994; Kaufmann et al., 1995; Overdier et al., 1994; Pierrou et al., 1994). This core region is essential for the binding of Forkhead proteins. Bases immediately flanking the core region contribute to the binding specificity of the different members (Hellqvist et al., 1996; Kaufmann et al., 1995; Overdier et al., 1994; Pierrou et al., 1994).

Genetic studies have demonstrated that members of the family of Forkhead transcription factors play important roles in cell proliferation and differentiation, both during embryonic development and in the adult (Carlsson and Mahlapuu, 2002). In addition to these diverse roles in normal development, several Forkhead proteins have also been suggested to play a role in neoplasia. First, the *v-qin* viral

oncogene, which was derived from the avian retrovirus ASV31, is essential for the ASV31 induced formation of sarcomas in young chicken (Li and Vogt, 1993). The *v-qin* gene, as well as its cellular counterpart *c-qin*, was found to encode a Forkhead transcription factor that is highly homologous to FoxG1 (BF-1). Consistent with the role of *qin* in regulating cellular proliferation, mice homozygous for a null allele of *Foxg1* (BF-1) die perinatally, with significantly reduced cerebral hemispheres (Xuan et al., 1995). Second, Forkhead proteins have been shown to be involved in chromosomal translocations associated with several forms of human cancer. In pediatric alveolar rhabdomyosarcomas (ARMs), PAX3 and PAX7, members of the PAX family of paired class homeodomain transcription factors, undergo protein fusions with FOXO1 (Barr et al., 1993; Galili et al., 1993; Shapiro et al., 1993; Davis et al., 1994). This fusion results in a gain of function mutation (Fredericks et al., 1995; Bennicelli et al., 1996). The transformation capacity of the fusion protein depends on the integrity of the third α-helix of the PAX3 homeodomain and a small region of the FOXO1 transactivation domain (Lam et al., 1999). The DNA-binding capacity of the fusion protein is identical to the binding capacity of the PAX3 protein, since the complete DNA-binding domain of PAX3 is still present in the fusion protein (Barr, 2001). However it is dispensable for the transformation capacity (Lam et al., 1999). Another example of the involvement of Forkhead transcription factors in chromosomal translocations comes from human leukemia, where the majority of chromosomal translocations disrupt the mixed lineage leukemia (MLL) transcription factor gene, fusing it to a variety of different partner genes. In two cases these partner genes are members of the Forkhead family. The t(6;11) translocations results in the generation of a fusion of MLL with FOXO2 (AF6q21) (Hillion et al., 1997). Similarly, the t(X;11) chromosomal translocation results in a fusion of MLL with another Forkhead gene, FOXO4 (Borkhardt et al., 1997; Parry et al., 1994). Both translocations result in fusion proteins containing the amino-terminal part of MLL fused in frame to the carboxy-terminus of the Forkhead protein, including the DNA-binding helix of the Forkhead box as well as two transactivation domains of FOXO. The strong transactivation domain CR3 is sufficient for the transcriptional activation of the fusion protein and binding of the protein to cofactors like CBP (So and Cleary, 2002; So and Cleary, 2003). However, for the transformation capacity of the MLL-FOXO protein the less potent second transactivation domain CR2 is also required (So and Cleary, 2002; So and Cleary, 2003). Interestingly, it was recently shown that the MLL-FOXO fusion could competitively interfere with transcription and apoptosis induced by wild-type FOXOs (So and Cleary, 2002; So and Cleary, 2003). This dominant negative effect on FOXO activity in cells could be a way of the MLL-FOXO fusion protein to contribute to malignant transformation.

Since the initial identification of FOXO1 as a translocation partner of PAX3 in alveolar rhabdomyosarcomas, related FOXO subfamily members have been identified in several different organisms including *Caenorhabditis elegans* (DAF-16) (Lin et al., 1997; Ogg et al., 1997), *Drosphila Melannogaster* (dFOXO) (Junger et al., 2003), mouse (FoxO1, FoxO3, FoxO4, and FoxO6) (Biggs et al., 2001; Jacobs et al., 2003) and human (FOXO1, FOXO3a, and FOXO4) (Anderson et al., 1998; Borkhardt et al., 1997; Galili et al., 1993; Hillion et al., 1997).

Regulation of FOXO transcription factors through phosphorylation

The first evidence for the protein kinase B (PKB)/Akt signalling pathway to control the FOXO transcription factors came from analysis in the nematode *Caenorhabditis elegans*. Under conditions that are unfavorable for the growth and survival, the worms can enter a stage of developmental arrest, referred to as dauer stage (Thomas, 1993). This developmental stage is associated with reduced metabolic activity and increased resistance to oxidative stress, and is essential for long-term survival of the worms. Genetic analysis demonstrated that the Forkhead homologue DAF-16 promotes entry into the dauer stage, enhances longevity of the worm and is involved in controlling metabolism and the defense against oxidative stress (Honda and Honda, 1999; Lin et al., 1997; Ogg et al., 1997). This activity of DAF-16 is negatively regulated by DAF-2, the insulin receptor-like protein (Kimura et al., 1997). Further

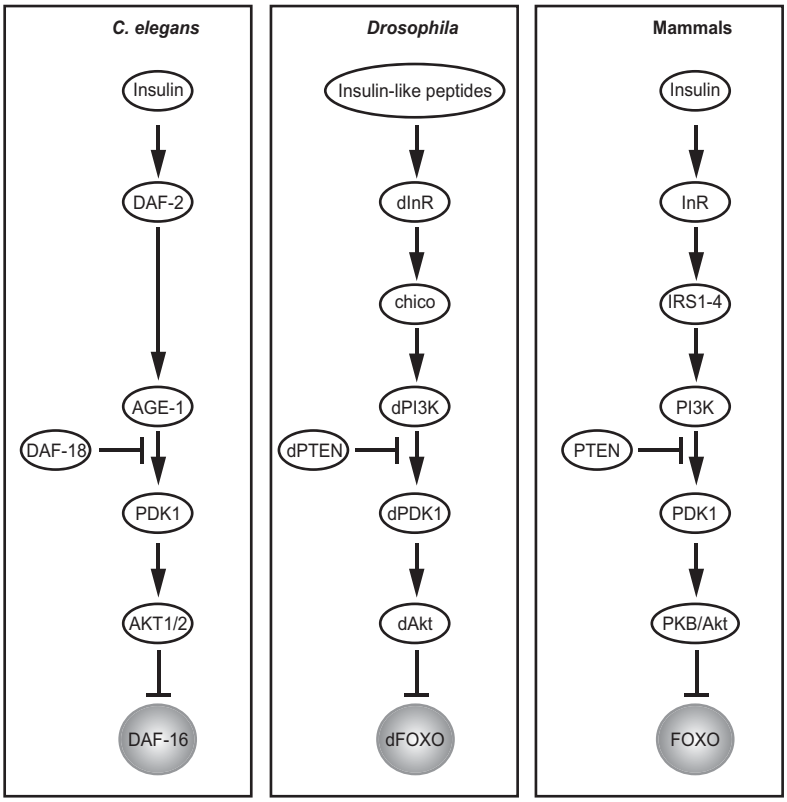


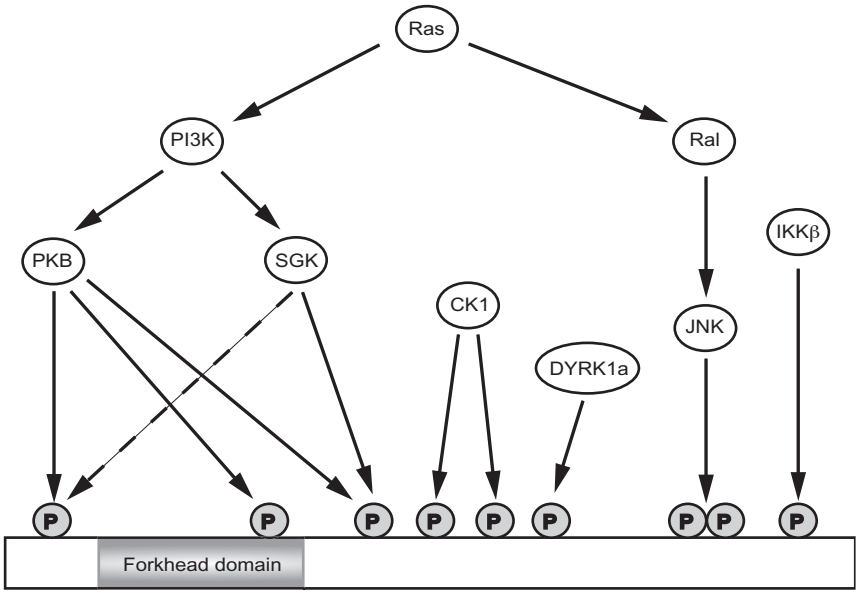
Figure 1. The insulin-PI3K-PKB-FOXO pathway is conserved between mammals, *Caenorhabditis elegans*, and *Drosophila melanogaster*

In these three organisms, the insulin receptor (DAF-2) activates PKB (AKT) via PI(3)K (AGE-1) and PDK1. Conversely, PTEN (DAF-18) inactivates the pathway. Activation of PKB (AKT) leads to inactivation of FOXO (DAF-16), via direct phosphorylation by PKB.

epistatic analyses have established that PI3-kinase (AGE-1), PTEN (DAF-18), PDK1 (PDK-1) and PKB-related kinases (AKT-1/2) are all involved in this regulation of DAF-16 by DAF-2 (Lin et al., 1997; Ogg et al., 1997; Ogg and Ruvkun, 1998; Paradis et al., 1999; Paradis and Ruvkun, 1998) (figure 1). These studies in *C. elegans* provided evidence that a PI3-kinase-activated signal transduction pathway negatively regulates the DAF-16 Forkhead transcription factor, thereby controlling processes like longevity, dauer formation, metabolism and oxidative stress protection.

Protein kinase B (PKB)/Akt is a serine/threonine kinase, which is regulated by the PI3-kinase (PI3K) signalling pathway. Cytokines and growth factors trigger the activation of PI3K, which results in the recruitment of PKB/Akt to the plasma membrane, where it is activated by the 3-phosphoinositide-dependent protein kinase-1 (PDK-1) (Fruman et al., 1998; Vanhaesebroeck and Alessi, 2000). This pathway is inhibited by the lipid phosphatase PTEN, a tumor suppressor gene (Cantley and Neel, 1999). Analysis of the DAF-16 sequence reveals several consensus sequences for phosphorylation by PKB/Akt (R-X-R-X-X-[S/T]) (Alessi et al., 1996), suggesting that DAF-16 and perhaps other Forkhead transcription factors may be direct substrates of PKB/Akt. In human FOXO transcription factors, three of the consensus sequences for PKB/Akt phosphorylation are conserved: an N-terminal and C-terminal site and one located within the Forkhead domain. Several groups have independently shown that PKB/Akt can indeed directly phosphorylate FOXOs *in vitro* at these three conserved residues equivalent to Thr24, Ser256, and Ser319 of FOXO1 (figure 2) (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999). Moreover, exposing cells to growth factors that activate the PI3K/PKB pathway causes phosphorylation of the FOXOs on multiple sites *in vivo*, including those sites that are phosphorylated by PKB/Akt *in vitro* (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999). Expression of active mutants of PKB/Akt is also sufficient to induce *in vivo* phosphorylation of the three conserved sites (Brunet et al., 1999; Kops et al., 1999), although the stoichiometry of the *in vitro* and *in vivo* phosphorylation of the different sites of the different FOXOs by PKB/Akt varies. Phosphorylation of FOXO by PKB/Akt results in all cases in a reduction of the transcriptional activity of the transcription factor and induces the nuclear exclusion of the protein (Brunet et al., 1999; Kops et al., 1999). Thus, like in *C. elegans*, the PI3K/PKB pathway negatively regulates mammalian FOXO homologues, indicating that this pathway is evolutionary conserved between worms and mammals (figure 1).

Growth factor stimulation of cells also induces phosphorylation of FOXO on other sites than the PKB/Akt sites. Therefore, other kinases have been suggested to phosphorylate FOXOs. Serum- and glucocorticoid-induced kinase (SGK) is a kinase closely related to PKB/Akt, and is activated by a variety of growth and survival factors (Kobayashi and Cohen, 1999; Park et al., 1999). The activation of SGK is dependent on PI3K activity and supposedly involves the phosphorylation by PDK1 (Kobayashi and Cohen, 1999; Park et al., 1999). However, unlike PKB/Akt, SGK does not have a pleckstrin homology domain and appears not to be recruited to the plasma membrane prior to its activation (Buse et al., 1999). Instead, SGK translocates directly to the nucleus following activation by PI3K (Buse et al., 1999). *In vitro* kinase experiments showed that SGK could phosphorylate the two PKB/Akt sites Thr32 and Ser315 of



FOXO1	T24	S256	S319	322	325	329	?	?
FOXO3a	T32	S253	S315	318?	321?	325?	?	Ser644
FOXO4	T28	S193	S258	261?	264?	268	T447/451	?

Figure 2. Regulation of phosphorylation of FOXO transcription factors
The specific amino acid number of the phosphorylation sites for each FOXO member is indicated. ?: the residue of the phosphorylation site in the FOXO member is unknown and no clear, conserved sites can be assigned. ? following the residue number: a conserved residue is present, but phosphorylation of this site in this FOXO member by the indicated kinase has not been demonstrated.

FOXO3a *in vitro* (Brunet et al., 2001). However *in vivo* SGK displays a preference for the Ser315 site (figure 2) (Brunet et al., 2001). Phosphorylation of FOXO3a by SGK also results in an inactivation and nuclear exclusion of FOXO (Brunet et al., 2001). Although the efficacy of SGK and PKB/Akt with which they phosphorylate the specific sites on FOXO3a differs, these data do suggest that SGK and PKB/Akt act together to induce complete phosphorylation of FOXO3a on Thr32, Ser253 and Ser315 after growth factor stimulation, thereby inactivating the transcription factor. Recently, it was shown in *C. elegans* that activated Akt-1, Akt-2 and SGK form a kinase complex to antagonize DAF-16 by direct phosphorylation (Hertweck et al., 2004). Although part of one complex, the kinases seem to be involved in different processes regulated by Daf-16. Akt-1/2 was shown to be the major kinase involved in Daf-16 regulation during dauer formation, whereas SGK is involved lifespan regulation and stress response (Hertweck et al., 2004). Whether PKB/Akt and SGK are involved in different aspects of mammalian FOXO signalling is not clear.
Dual-specificity tyrosine-phosphorylated and regulated kinase 1a (DYRK1a) is

another kinase identified to be involved in phosphorylation of FOXOs. DYRK1a was shown to induce phosphorylation of Ser329 in FOXO1, both *in vitro* and *in vivo* (figure 2) (Woods et al., 2001). Mutation of this site to an alanine enhances the transcriptional activity and nuclear localization of FOXO1, indicating that phosphorylation of this site is also negatively regulating FOXO (Woods et al., 2001). However, DYRK1a appears to be a constitutively active kinase in cells, so the relevance of the DYRK1a induced phosphorylation of Ser329 remains unclear.
In addition to these phosphorylation events, PKB/Akt or SGK induced phosphorylation of Ser319 in FOXO1 creates a recognition motif for yet another kinase: casein kinase 1 (CK1). Phosphorylation of Ser319 allows CK1 to phosphorylate FOXO1 on Ser 322 (Rena et al., 2002). The phosphorylated Ser322 in turn primes the CKI induced phosphorylation of Ser 325 (figure 2) (Rena et al., 2002). Mutation of Ser319 to an alanine prevents the phosphorylation of both CK1 residues (Rena et al., 2002). Phosphorylation of these CK1 sides together with Ser319 and Ser 329 creates a stretch of phosphorylated serines, proposed to have a role in nuclear export of FOXO (see below).
Finally, the small GTPase Ras can also affect FOXO function, as was described for FOXO4 (Kops et al., 1999). Activated Ras can stimulate the exchange factor RalGEF for another small GTPase, Ral. Binding of Ras to RalGEF results in the activation of Ral (Bos, 1998). Activation of this Ras/Ral pathway results in the phosphorylation of Thr447 and Thr451 in the C-terminus of FOXO4 (figure 2) (De Ruiter et al., 2001). This Ral induced phosphorylation is dependent on the stress kinase JNK (Chapter 4) and results in enhanced activity of FOXO4 (De Ruiter et al., 2001; Chapter 4). However, when activation of Ral is combined with activation of PKB/Akt, for example by introducing oncogenic Ras into cells, Ral appears to act synergistically with PKB/Akt to inhibit FOXO4 activity (De Ruiter et al., 2001). At present, it is unknown whether this regulation by Ral is specific for FOXO4 or also applies to FOXO1 and FOXO3a.

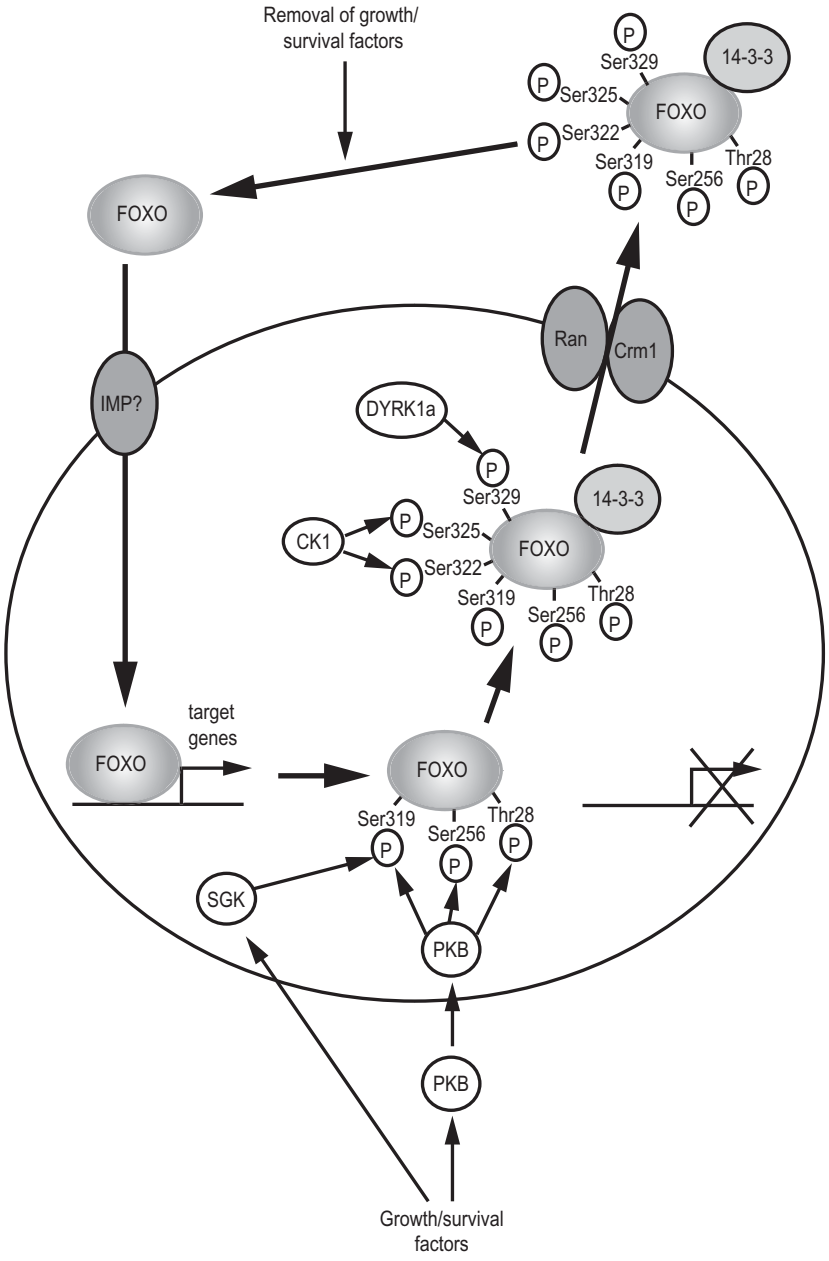
Nuclear import/export of FOXO transcription factors
Consistent with the negative regulation of DAF-16 by the DAF-2 pathway in *C. elegans*, phosphorylation of FOXO transcription factors by PKB/Akt causes inhibition of their transcriptional activity, as a consequence of a subcellular redistribution of the FOXOs (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 1999). PKB/Akt induced FOXO phosphorylation results in retention of FOXO in the cytoplasm, while inhibition of the PKB/Akt pathway causes FOXO to localize almost exclusively in the nucleus. Moreover, FOXO proteins in which the three PKB/Akt phosphorylation sites were mutated to alanine failed to relocalize to the cytoplasm upon activation of PKB/Akt (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 1999). The same effect on translocation of FOXOs was seen in *C. elegans*, where inhibition of DAF-2 results in nuclear localization of DAF-16 (Henderson and Johnson, 2001).
Shuttling proteins between the cytoplasm and the nucleus is a highly regulated process and requires both importin and exportin proteins (Komeili and O'Shea, 2001). The importins and exportins bind to specific sequences in their target proteins, termed nuclear localization signal (NLS) and nuclear export signal (NES) respectively. The

association of the importins and exportins to these signal sequences is controlled by the small GTPase Ran (Dasso, 2002). FOXO import and export were shown to be dependent on this active transport system (figure 3). Treatment of cells with Leptomycin B (LMB), a herbal fungicide that specifically inhibits the export receptor Crm1, induced accumulation of FOXO proteins in the nucleus (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 2002). Moreover, it was described that FOXO4 can bind to Crm1 (Brownawell et al., 2001), although others claim that FOXO1 directly binds to Ran (Rena et al., 2002). Deletion of the putative NES in FOXO4 clearly affects nuclear export of FOXO4 (Brunet et al., 2002). However, binding of Crm1 to FOXO is not dependent on the phosphorylation status of FOXO, and mutating the PKB/Akt phosphorylation sites in FOXO4 does not prevent nuclear export of the FOXO factor (Brownawell et al., 2001). This indicates that PKB/Akt-induced FOXO shuttling must be controlled through regulation of the nuclear import rather than nuclear export.

All FOXO proteins contain a sequence that conforms an atypical NLS that surrounds the PKB phosphorylation site within the Forkhead domain (Ser193 in FOXO4). This NLS consists of three arginine residues, present on the C-terminus of the Forkhead domain, and three lysine residues located downstream of the arginines and Ser193 (Brownawell et al., 2001; Brunet et al., 2002; Zhang et al., 2002). The basic region of the NLS is essential for its function, since mutating these residues results in reduced import of FOXO4. Phosphorylation of the PKB/Akt phosphorylation site Ser193 introduces a negative charge, which might influence the NLS. Indeed, mutation of Ser193 to an alanine confirmed that phosphorylation of the PKB/Akt site inhibits the function of the NLS, shifting FOXO localization to the cytoplasm (figure 3) (Brownawell et al., 2001). The primary sequence of the FOXO4 NLS shows that it is not a classical NLS and none of the classical importins were found to bind FOXO4 (Brownawell et al., 2001). At present, the importin responsible for the nuclear import of FOXO is still unknown (figure 3).

PKB/Akt-mediated phosphorylation of FOXO in the nucleus not only inhibits the function of the NLS, it also creates docking sites for 14-3-3 proteins (Brownawell et al., 2001; Brunet et al., 1999; Rena et al., 2001). 14-3-3 proteins are abundant, ubiquitously expressed molecules that preferentially bind to specific motifs containing phosphorylated serine and threonine residues. Phosphorylated FOXO binds to 14-3-3 proteins in the nucleus immediately before FOXOs relocalize to the cytoplasm. Phosphorylation of the N-terminal PKB/Akt motif (Thr32 in FOXO3a) and the PKB/Akt phosphorylation site within the Forkhead domain and NLS (Ser253 in FOXO3a)

Figure 3. Regulation of nuclear import/export of FOXO transcription factors
FOXO transcription factors are continuously shuttling between the nucleus and the cytoplasm. Addition of growth / survival factors results in activation of PKB and SGK, which induces the phosphorylation of FOXO. This phosphorylation of FOXO results in release of FOXO from the DNA, and binding to 14-3-3 proteins. In addition, phosphorylation of Ser319 is required to prime FOXOs for further phosphorylation by CK1 and DYRK1a. These sequential phosphorylation events create an acidic patch that stimulates binding to the Crm1/Ran-containing protein complex that mediates the nuclear export of FOXOs. Upon removal of growth/survival factors, FOXO is dephosphorylated, 14-3-3 is released, and FOXO



is transported back into the nucleus where it can bind FOXO-responsive elements and induce or repress expression of its target genes. Import requires α- and β-importins, but the identity of the specific subunits involved in import of FOXOs has not been resolved.

generates 14-3-3 binding sites (Brownawell et al., 2001; Rena et al., 2001; Obsil et al., 2003). Mutating these PKB/Akt site to alanines disrupts the 14-3-3 binding and inhibits nuclear export (Brownawell et al., 2001; Cahill et al., 2001; Obsil et al., 2003; Rena et al., 2001). Once in the cytoplasm, FOXOs remain phosphorylated and complexed to 14-3-3 proteins, thereby preventing nuclear import (figure 3). 14-3-3 proteins may not directly mediate nuclear transport themselves. Binding of 14-3-3 to FOXOs has been shown to occur via the c-terminal α-helix, which has been proposed to be a nuclear export signal (NES) (Brunet et al., 2002). Recently however, Obsil et al showed that binding of 14-3-3 to phosphorylated FOXO occurs on two binding sites (Obsil et al., 2003). These two phosphorylated binding motifs are needed for the complete inhibition of FOXO4 binding to its target DNA by a 14-3-3 dimer (Obsil et al., 2003). These observations indicate that 14-3-3 inhibits the binding of FOXO to DNA, rather than inducing nuclear export. Nuclear export of 14-3-3 was suggested to be CRM1 dependent (Brunet et al., 2002; van Hemert et al., 2004). However, recent data argue against this: 14-3-3 directly binds to Exportin 7 (Exp7), a novel nuclear export mechanism component, and not to Crm1 (Mingot et al., 2004). Export of 14-3-3 is inhibited when Exp7 is blocked, and stimulated by exogenous Exp7 (Mingot et al., 2004). An explanation for these different observations could lie in the use of LMB to block Crm1 dependent export. Selective inactivation of Crm1 by LMB will probably also influence the nucleocytoplasmic gradient of RanGTP, thereby indirectly inhibit nuclear transport pathways that operate in parallel to Crm1. Thus, whether binding of 14-3-3 to FOXO will facilitate Crm1 dependent export of FOXO is highly disputed, and further experiments will be needed to elucidate the mechanism of 14-3-3 binding and export of FOXOs.

Affecting subcellular localization may not be the only way phosphorylation influences the transcriptional activity of FOXOs. The phosphorylation of FOXOs has also been suggested to directly inhibit DNA binding of FOXO (Cahill et al., 2001; Zhang et al., 2002). This might imply that phosphorylation by PKB/Akt would be primarily required to release FOXOs from DNA rather than to induce their cytoplasmic retention. Also, phosphorylation by CK1 and DYRK1a may be involved in controlling FOXO localization (figure 3). Phosphorylation of FOXO1 on the two CK1-specific sites has been proposed to accelerate nuclear export and disrupt nuclear retention of FOXO (Rena et al., 2002). Phosphorylation of the DYRK1a site was proposed to have the same effect (Woods et al., 2001). This indicates that phosphorylation of this stretch of serines by PKB/Akt, CK1 and DYRK1a forms an acidic patch, facilitating nuclear export.

Transcriptional regulation of FOXO factors

Subcellular localization of FOXOs clearly affects the transcriptional activity of the protein. However, there are more mechanisms involved in regulation of FOXO activity. FOXO mediated expression of downstream target genes can be mediated via direct binding of FOXO to FOXO responsive elements (FHRE) in the promoter of the target gene. However, FOXOs also have the capacity to regulate downstream targets independent of DNA binding. This can be explained by the interaction of FOXO with accessory proteins or cofactors. In a recent study, it was indeed

demonstrated, using a comprehensive gene array analysis, that FOXOs can activate two different subsets of genes: (1) genes that require FOXO DNA binding, and (2) genes regulated independently of FOXO DNA binding (Ramaswamy et al., 2002). This thus suggests that FOXOs interact with other transcription factors, thereby modulating their activity.

Indeed, several different binding partners of FOXO have been identified. FOXOs were shown to interact with both steroid and nonsteroid nuclear receptors. These receptors include the estrogen receptor (ER) (Schuur et al., 2001; Zhao et al., 2001), the progesterone receptor (PR) (Zhao et al., 2001), the androgen receptor (AR) (Li et al., 2003), the thyroid hormone receptor (THR) (Zhao et al., 2001), the glucocorticoid receptor (GR) (Zhao et al., 2001), the retinoic acid receptor (RAR), the peroxisome proliferator activated receptor (PPAR), and the hepatocyte nuclear factor-4 (HNF-4) (Hirota et al., 2003). Binding of FOXOs to these receptors probably occurs through the interaction with an LxxLL domain located in the far C-terminal part of FOXO (Leo et al., 2000). The interaction between nuclear receptors and FOXOs can be dependent or independent of nuclear receptor ligand binding, and FOXO differentially regulated the transactivation mediated by the different receptors, either as a coactivator or corepressor. The mechanism and outcome of the interaction appeared to be dependent on the receptor and cell type.

FOXO1 can also interact with CAAT/Enhancer-binding proteinβ (C/EBPβ), a transcription factor, which plays a role in differentiation (Christian et al., 2002). The interaction is dependent on DNA binding of C/EBPβ and binding of FOXO1 further promoted C/EBPβ activity (Christian et al., 2002).

Finally, FOXO can interact with the transcriptional coactivators p300 and CREB binding protein (CBP) (Mahmud et al., 2002; Nasrin et al., 2000). p300 and CBP are acetyl transferases that modulate the activity of their targets by acetylation. Indeed, binding of p300 or CBP induces acetylation of FOXOs (Brunet et al., 2004; Daitoku et al., 2004; Mahmud et al., 2002; Motta et al., 2004; van der Horst et al., 2004). Binding of p300 and acetylation of FOXO3a are both induced under serum free conditions. Addition of survival factors induces deacetylation of FOXO (Mahmud et al., 2002). Recently, several independent papers have demonstrated that deacetylation of FOXO is mediated by hSir2^{SIRT1} (Brunet et al., 2004; Daitoku et al., 2004; Motta et al., 2004; van der Horst et al., 2004). hSir2^{SIRT1} is an evolutionary conserved histone deacetylase, which activity is dependent on NAD (nicotinamide adenine dinucleotide). Interestingly, genetic analysis in *C. elegans* demonstrated that the *C. elegans* homologue SIR2 extends the lifespan of the worm by negatively regulating the DAF2/DAF16 signalling pathway (Tissenbaum and Guarente, 2001). Both this genetic evidence and the data showing deacetylation of FOXOs by hSir2^{SIRT1} indicate that FOXOs are downstream targets of hSir2^{SIRT1}. The functional consequences of this regulation of FOXOs by hSir2^{SIRT1} are less clear. While one group reported that FOXO deacetylation decreases expression of FOXO target genes (Motta et al., 2004), others propose that deacetylation of FOXO leads to increased expression of FOXO target genes (Brunet et al., 2004; Daitoku et al., 2004; van der Horst et al., 2004). More work will be required to understand the effect of acetylation/deacetylation on FOXO function.

Degradation of FOXOs

Various cellular proteins, including transcription factors, are tightly regulated by proteolysis through the proteasome in response to external signalling molecules. In haematopoietic cells, activation of PKB/Akt results in a decrease of FOXO protein levels (Plas and Thompson, 2003). Treatment of these cells with a proteasome inhibitor restores the FOXO levels, indicating that the protein levels of FOXO are regulated by PKB/Akt in a proteasome dependent manner (Plas and Thompson, 2003). Moreover, the PI3K inhibitor LY294002 can prevent the accumulation of ubiquitinated PKB/Akt substrates (Plas and Thompson, 2003). Also in other cells, insulin treatment resulted in a proteasome dependent degradation of FOXO (Matsuzaki et al., 2003). Insulin induced polyubiquitination of FOXO, which was dependent on PKB/Akt induced phosphorylation and cytoplasmic retention of FOXO (Matsuzaki et al., 2003). However, the mechanism via which FOXOs are ubiquitinated is unknown, and it will be of interest to identify the E3 ubiquitin ligase responsible for the ubiquitination of FOXOs.

FOXO mediated regulation of cell cycle progression

Overexpression of FOXOs causes a strong inhibition of cell proliferation in a large variety of cell lines (Collado et al., 2000; Kops et al., 2002b; Medema et al., 2000; Nakamura et al., 2000). Even in transformed cell lines, such as Ras-transformed or PTEN deficient cells, FOXO expression reduces proliferation rates (Medema et al., 2000; Nakamura et al., 2000). Thus, FOXOs have the opposite effect of PKB, which induces growth stimulation in cells. In most cells growth inhibition by FOXO is associated with an arrest in cell cycle progression (Medema et al., 2000). However, in other cell types, such as haematopoietic and neuronal cells, activation of FOXO can result in the induction of apoptosis (Brunet et al., 1999; Dijkers et al., 2000b; Stahl et al., 2002). The effect on cell cycle progression will be discussed first.

Cell cycle progression is a tightly controlled process. Cells require extracellular proliferative signals directly after mitosis for continued proliferation. However, in the absence of proliferative signals, the cell will either die or arrest, depending on the cell type. Proper cell cycle progression is controlled by cyclins and cyclin dependent kinases (CDKs). Two classes of proteins negatively regulate cell cycle progression, namely the retinoblastoma (Rb) family and the CDK inhibitors (CKIs). Several reports have demonstrated that the FOXOs induce a cell cycle arrest in the G_1 phase of the cell cycle, and that this arrest is dependent on the increased expression of the CKI $p27^{kip1}$ (Medema et al., 2000; Nakamura et al., 2000). FOXO regulates $p27^{kip1}$ levels through direct transcriptional activation of the $p27^{kip1}$ gene, as was shown by a strong activation of the $p27^{kip1}$ promoter activity (figure 4) (Dijkers et al., 2000b; Medema et al., 2000). The induction of the $p27^{kip1}$ promoter activity was confirmed by an increase in both mRNA and protein levels of $p27^{kip1}$ in cells expressing FOXO (Dijkers et al., 2000b; Medema et al., 2000). Increased $p27^{kip1}$ levels will lead to an increased inhibition of cyclin/CDK complexes by $p27^{kip1}$, and thus negatively affects cell cycle progression (Dijkers et al., 2000b; Medema et al., 2000). The effect of FOXOs on $p27^{kip1}$ levels might not always be via transcriptional regulation, since it has been reported that in some cell lines, regulation of $p27^{kip1}$ by FOXOs appears to

occur predominantly post-translationally, through stabilization of the $p27^{kip1}$ protein (Nakamura et al., 2000). In addition, FOXO induced cell cycle arrest may also require a p27-independent mechanism, since there is still some residual inhibition of the cell cycle in response to FOXO in p27-/- MEFs.
Other important regulators of the G_1-S transition are the D-type cyclins. D-type cyclins are required for phosphorylation and inactivation of Rb, and thus influence progression in G_1 (Sherr, 1995). Activation of FOXOs resulted in a decrease of cyclin D1 and D2 (figure 4) (Ramaswamy et al., 2002; Schmidt et al., 2002). Whether FOXOs also influence the levels of the third D-type cyclin, cyclin D3, is at present unknown. FOXO regulates cyclin D1/D2 at the transcriptional level, resulting in a decrease of mRNA and protein levels (Ramaswamy et al., 2002; Schmidt et al., 2002). The mechanism through which FOXO influences the transcription of cyclin D is not clear yet. Although the cyclin D1 and D2 promoters are repressed by FOXOs, no bona fide FOXO DNA-binding elements are present in the cyclin D promoters (Schmidt et al., 2002). Furthermore, some data suggest that the repression indeed does not involve

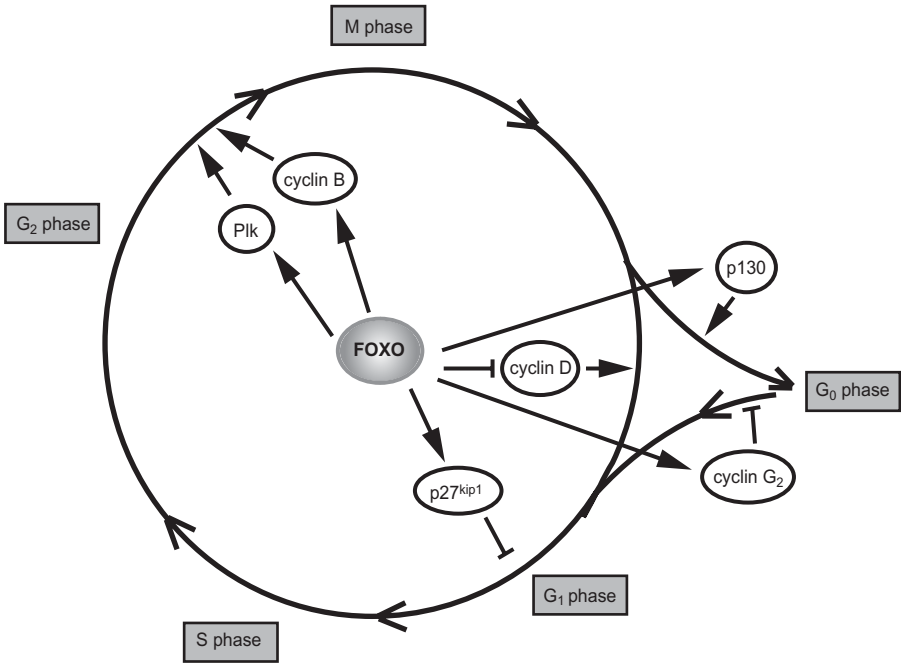


Figure 4. FOXO mediated cell cycle regulation
FOXOs induce a cell cycle arrest in the G_1 phase, through direct regulation of p27kip1 and cyclin D levels. The FOXO-induced G_1 arrest is reversible, and FOXO arrested cells show characteristic markers of quiescence. Both p130 and cyclinG2, two regulators of quiescence, are direct targets of FOXOs. Thus, FOXOs might function as a switch, controlling cell cycle entry and exit of cells. During the G_2 phase of the cell cycle, FOXOs are dephosphorylated and partially localized in the nucleus, where FOXOs induce transcriptional up regulation of Plk and cyclin B.

direct binding of FOXO to the cyclin D promoter (Ramaswamy et al., 2002). Our own data (Chapter 3) suggest that one mechanism by which FOXOs may repress cyclin D expression is through competing with TCF for β-catenin binding. However, it is disputed whether cyclin D1 is a bona fide TCF target gene. In addition, this mechanism may only be operational under conditions in which β-catenin is stabilized (e.g. APC-/- cells). Thus, regulation of cyclin D might involve another transcriptional regulator more genrally involved in cyclin D regulation, capable of recruiting FOXO to the cyclin D promoter. Recently, it was proposed that there is a coordinate regulation of the cyclin D promoter by FOXOs and the c-myc proto-oncogene (Bouchard et al., 2004). Myc recruits TFIIH, P-TEFb and Mediator to the cyclin D2 promoter, while inactivation of FOXO by PI3K controls the formation of the preinitiation complex and loading of RNA polymerase II (Bouchard et al., 2004). Activation of FOXO leads to the inhibition of induction of Myc target genes like cyclin D2, Myc-induced cell proliferation and transformation by Myc and Ras (Bouchard et al., 2004). Thus, the inhibition of the cyclin D promoter by FOXO might be an important way of inhibiting Myc-induced proliferation and transformation.

Cyclin D expression is induced as quiescent cells are stimulated to reenter the cell cycle. On the other hand, $p27^{kip1}$ expression is reduced during cell cycle entry, allowing full activation of the G1 cyclin/CDK complexes, resulting in cell cycle progression. Since these two important regulators of cell cycle entry are both regulated by FOXOs, the question was raised whether the FOXO induced cell cycle arrest in G_1 might represent a cell cycle exit and entry into quiescence. Indeed, the FOXO-induced arrest is reversible, an important marker for quiescent cells, compared to senescent cells (Kops et al., 2002b). In addition, also other characteristic markers of quiescence were observed in FOXO arrested cells. One of these markers of quiescence is p130, an Rb-related pocket protein. Protein levels of p130 increase in quiescence and this is accompanied by hypophosphorylation of the p130 protein (Grana et al., 1998). Activation of FOXO, and thus induction of quiescence, indeed resulted in an increase in mRNA and protein levels of p130 (figure 4) (Kops et al., 2002b). Along with the effect on p130, also protein synthesis was inhibited in FOXO arrested cells, and G_0 specific E_2F complexes appeared (Kops et al., 2002b). Recently, cyclin G2 was identified as a novel target for FOXOs during quiescence, although the relevance of cyclin G2 regulation by FOXO remains to be determined (figure 4) (Martinez-Gac et al., 2004). The findings on cell cycle regulation by FOXO indicate that FOXOs function as a switch, controlling cell cycle entry and exit of cells.

Furthermore, FOXOs also have an important role in the control of cell cycle completion. In cells arrested in the G_2 phase of the cell cycle FOXO3a phosphorylation is low, and FOXO3a is partially relocalized in the nucleus, resulting in an increased activity (Alvarez et al., 2001). The mechanism by which FOXO3a regulates cytokinesis and exit from mitosis include FOXO induced transcriptional up regulation of cyclin B and polo-like kinase (plk) genes in G_2 phase (figure 4) (Alvarez et al., 2001). These results propose a model whereby FOXOs must be inactivated to initiate cell cycle entry. However, exit from the mitotic program depends on activation of FOXO.

FOXO-induced apoptosis

In a number of cell types, particularly in cells of the haematopoietic system and neurons, FOXO members have been described to regulate apoptosis, rather than cell cycle progression. Apoptosis is a tightly regulated process and a critical element for the maintenance of self-tolerance in the immune system. The innate immune system must be able to adapt to sudden changes in the production of blood cells, such as during an infection. Therefore, the homeostasis of the different haematopoietic subpopulations is controlled by a continued balance between stem cell renewal and differentiation, using apoptosis as an important tool. In haematopoietic cells, survival factors induce PI3K/PKB mediated phosphorylation and thus inhibition of FOXOs. For instance, IL-2 and IL-3, which play important roles in the proliferation and survival of lymphocytes, inactivate FOXO via PKB (Dijkers et al., 2000b; Stahl et al., 2002). Cytokine withdrawal of these lymphocytes, on the other hand, induces activation of FOXO, leading to apoptosis (Dijkers et al., 2000b; Stahl et al., 2002). In these cells, the induction of apoptosis by FOXO is preceded by a decrease of cells entering the cell cycle (Dijkers et al., 2000b; Stahl et al., 2002). The downstream target of FOXO in regulating cell cycle progression, $p27^{kip1}$, is also up regulated in FOXO induced apoptotic lymphocytes (Dijkers et al., 2000b; Stahl et al., 2002).

The first candidate target gene identified to be involved in FOXO induced apoptosis was the Fas ligand (figure 5). The Fas ligand promoter was shown to be responsive to FOXO activation (Brunet et al., 1999). Moreover, it was demonstrated that FOXO induced apoptosis in cerebellar granule cells and Jurkat T cells is dependent on signalling via the Fas receptor (Brunet et al., 1999). However, this appears to be a cell-type dependent effect since Fas-independent mechanisms of FOXO induced apoptosis have been described in other cell lines (Dijkers et al., 2002).

In a pre B cell line, BaF3, FOXO induced apoptosis occurs independent of death-receptor signalling (Dijkers et al., 2002; Dijkers et al., 2000a). Instead, FOXO activation induces the expression of Bim, a pro-apoptotic Bcl-2 family member (figure 5) (Dijkers et al., 2002; Dijkers et al., 2000a). Cytokine withdrawal in these cells results in an up regulation of Bim expression, followed by apoptosis of these cells (Dijkers et al., 2002; Dijkers et al., 2000a; Stahl et al., 2002). Bim transcription, both in haematopoietic cells and in neurons, is directly regulated by FOXO activation (Dijkers et al., 2002; Dijkers et al., 2000a; Gilley et al., 2003; Stahl et al., 2002). Bim has been implicated in modulating lymphocyte homeostasis. Bim-/- mice develop autoimmune kidney disease, accumulation of lymphoid and myeloid cells and show perturbed T cell development (Bouillet et al., 1999). These findings suggest that FOXOs can regulate T cell selection via the regulation of the pro-apoptotic Bim protein.

Another target of FOXOs in the induction of apoptosis is Bcl-6, a transcriptional repressor. Expression of Bcl-6 was induced by FOXO activation, via direct binding of FOXO to specific binding sites in the Bcl-6 promoter (figure 5) (Tang et al., 2002). Increases in Bcl-6 levels will result in the downregulation of protein levels of the anti-apoptotic protein Bcl-X_L (Tang et al., 2002). This suggests that FOXOs can induce apoptosis via Bcl6-mediated repression of Bcl-X_L. However, it is likely that through Bcl-6 other genes besides Bcl-X_L are regulated by FOXO not all of which are

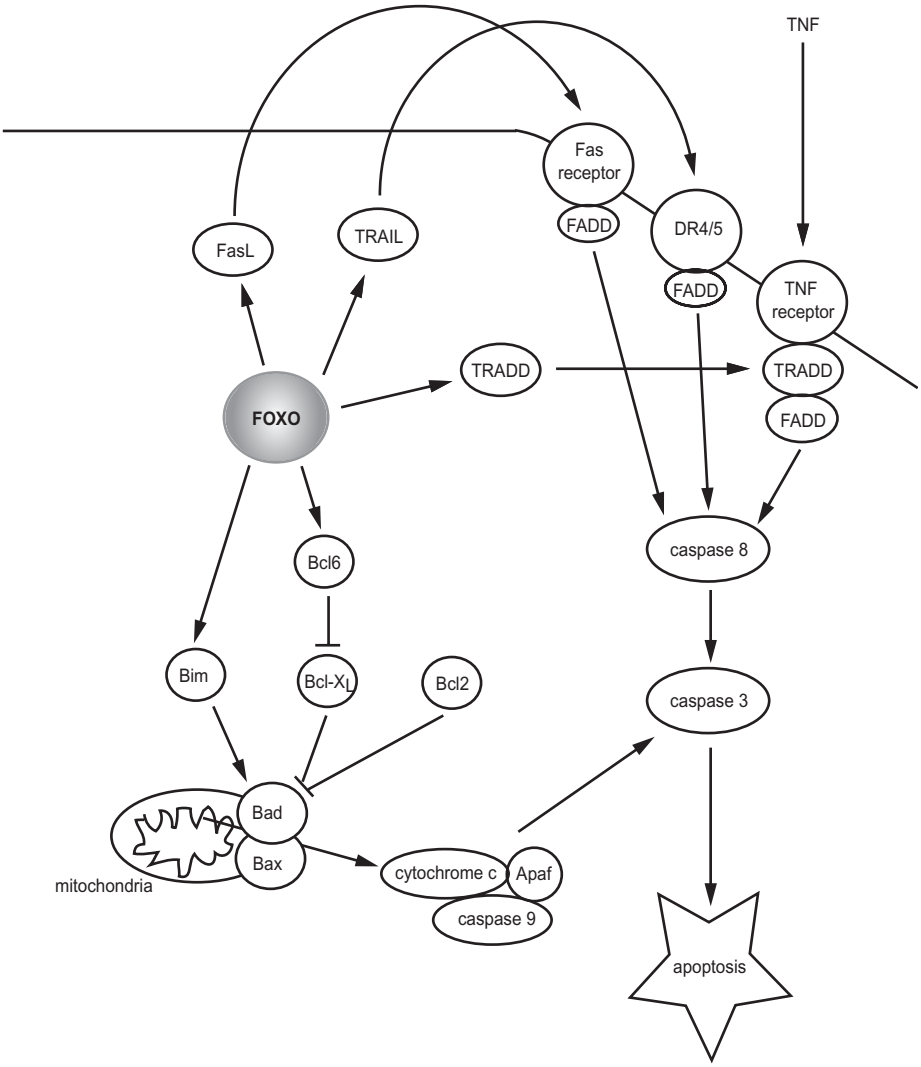


Figure 5. FOXO-induced apoptosis
FOXO can induce apoptosis through both death receptor-dependent and –independent pathways. Up-regulation of FasL results in cross-linking and activation of Fas, with subsequent caspase-8 activation and resultant apoptosis. Moreover, expression of tumor necrosis factor (TNF) receptor-associated death domain (TRADD) and TNF-related apoptosis-inducing ligand (TRAIL) are also induced by FOXO, contributing to enhanced death receptor signalling and caspase-8 activation. In addition, direct transcriptional regulation of the pro-apoptotic Bcl-2 family member Bim by FOXO results in mitochondrial permeability and thus cytochrome c release, resulting in caspase-3 activation. Apart from up regulation of Bim, FOXOs also repress transcription of Bcl-XL, through the induction of Bcl6. These pathways may act independently or cooperate in a cell type-specific fashion.

necessarily involved in the control of apoptosis.
Adenoviral overexpression of FOXO in prostate cancer cell lines resulted in apoptosis and induced the expression of several genes involved in the FOXO induced apoptosis (Modur et al., 2002). One of these FOXO targets was TRAIL, a pro-apoptotic member of the tumor necrosis factor family (Modur et al., 2002). TRAIL was shown to be a direct transcriptional target of FOXO, although the role of TRAIL in FOXO induced apoptosis remains to be determined (figure 5) (Modur et al., 2002). Interestingly, expression of the tumor necrosis factor receptor-associated death domain (TRADD) gene is also under direct control of FOXO (figure 5) (Rodukai et al., 2002). The induction of TRADD by FOXO was shown to be important in chemotherapeutic drug induced apoptosis (Rodukai et al., 2002).
As listed here, several different pro-apoptotic genes are regulated by FOXOs and most of them have been demonstrated to be involved in the FOXO induced apoptosis. At present it is not clear why multiple pro-apoptotic targets of FOXO exist. Future experiments will have to address the relevance of the different proteins in the FOXO induced apoptosis.

Oxidative stress and lifespan
In *C. elegans*, enhanced lifespan and resistance to cellular stress are two processes that are tightly linked. Worms deficient in PI3K/PKB signalling have an increased lifespan, correlated with increased resistance to oxidative stress. Also in other animal model systems, oxidative stress regulation has been shown to correlate with the process of longevity and aging. These observations form the basis for the free-radical theory of aging, which states that the production of endogenously generated ROS contributes to the pathophysiology of aging and limits potential lifespan (Harman, 1956).
Recently, several groups have demonstrated that in mammalian cell systems FOXOs are also involved in protecting cells against oxidative stress. FOXO3a can protect quiescent cells from oxidative stress by directly controlling expression of manganese superoxide dismutase (MnSOD) (Kops et al., 2002a). MnSOD is an antioxidant enzyme, which is localized in the mitochondria and can convert oxygen radicals into H_2O_2 (figure 6). FOXO3a increases MnSOD levels in quiescent cells by directly inducing transcription of the MnSOD gene (Kops et al., 2002a). Another antioxidant enzyme directly regulated by FOXO is catalase (Nemoto and Finkel, 2002). Catalase is predominantly localized in peroxisomes and it converts H_2O_2 into H_2O and O_2, thereby protecting cells from oxidative stress (figure 6). The catalase promoter contains several putative FOXO consensus binding sequences. However, the exact binding site of FOXO in the catalase promoter remains to be determined. Taken together, these data demonstrate that FOXOs regulate the expression of several enzymes that are important in protecting cells from oxidative stress.
Furthermore, it was demonstrated that there might be a link between FOXOs and the p66shc. To date, the homozygous deletion of the p66shc locus is one of the few genetic alterations demonstrated to increase life span in mammals (Migliaccio et al., 1999). Mouse embryonic fibroblasts derived from these p66shc-/- mice, also showed increased resistance against oxidative stress (Migliaccio et al., 1999; Nemoto and

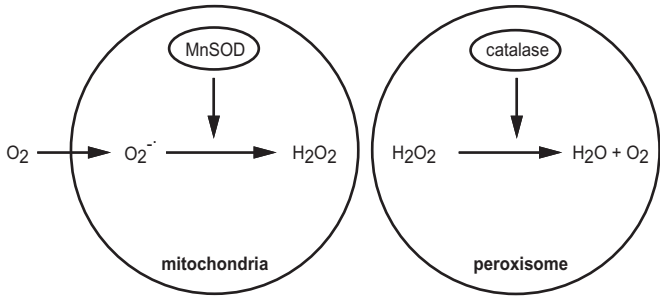


Figure 6. Role of MnSOD and catalase in the respiratory chain
The antioxidant enzyme MnSOD is localized in the mitochondria, where it can convert oxygen radicals into H_2O_2. Another antioxidant enzyme, catalase, is localized in peroxisomes, and converts H_2O_2 into H_2O and O_2. Both MnSOD and catalase are direct FOXO targets.

Finkel, 2002). The increased resistance correlated with a reduced ability of H_2O_2 to activate PKB and thus inactivate FOXO3a (Nemoto and Finkel, 2002). This would imply that oxidative stress under normal conditions causes inhibition of FOXO, which is the opposite effect of its role in protection against oxidative stress. However, the doses of H_2O_2 necessary to induce PKB activation are high (500 μM or more) compared to the doses of oxidative stress when FOXOs can give protection.
In response to oxidative stress FOXOs not only induce cells to arrest in the G_1 phase, but also induce a delay in G_2-M (Tran et al., 2002). FOXO3a is localized in the nucleus in cells passing through the G_2 phase, and by binding to DNA, FOXO3a induces DNA repair (Tran et al., 2002). By high-density cDNA microarray the growth arrest and DNA damage response gene GADD45 was identified as a target gene of FOXO3a in the induction of DNA repair (Tran et al., 2002). FOXO3a directly binds to several binding sequences within the GADD45 promoter, thereby inducing GADD45 expression (Tran et al., 2002). These data indicate that FOXOs not only play a role in the resistance of cells against oxidative stress by inducing antioxidant enzymes, but FOXOs also play a role in regulating DNA repair.

Metabolic functions of FOXOs

By now, it is well established that caloric restriction leads to lifespan extension in many species, linking longevity to metabolism. Since FOXOs are important regulators of longevity, and insulin is one of the key enzymes in regulating glucose metabolism, this suggests there might be a link between FOXOs and insulin induced glucose metabolism. A first indication was the observation that caloric restriction or glucocorticoid treatment increases FOXO levels in skeletal muscle (Furuyama et al., 2003; Furuyama et al., 2002; Imae et al., 2003). Re-feeding the mice reverses the starvation-induced increase in FOXO levels (Furuyama et al., 2003; Furuyama et al., 2002; Imae et al., 2003). To further elucidate the role of FOXOs in metabolism, loss- and gain-of-function experiments in transgenic and knockout mice were performed. Mice lacking the three FOXO isoforms display remarkably different

phenotypes (Hosaka et al., 2004). FOXO1 homozygous null mutants are embryonic lethal due to defective angiogenesis (Furuyama et al., 2004; Hosaka et al., 2004). Haploinsufficiency of FOXO1, however, protects against insulin resistance caused by defective insulin signalling (Nakae et al., 2002). It also rescues diabetes due to pancreatic β-cell failure caused by ablation of the insulin receptor substrate 2 (Irs2) (Kitamura et al., 2002). Moreover, overexpression of a constitutively active FOXO1 mutant induces diabetes, presumably as a result of increased glucose production (Nakae et al., 2002). FOXO4 knockout mice do not have any obvious abnormality (Hosaka et al., 2004). Although FOXO3a knockout mice reveal haematological abnormalities and a decreased glucose uptake in glucose tolerance tests, the most remarkable phenotype is premature ovarian failure (Castrillon et al., 2003; Hosaka et al., 2004). This is the result of accelerated differentiation and consequent depletion of primary ovarian follicles (Castrillon et al., 2003). The knockout studies thus point to FOXO1 as the FOXO isoform involved in glucose metabolism.
The underlying mechanism for FOXO1 function in metabolism involves the ability of FOXO1 to regulate expression of genes involved in insulin sensitivity. Pyruvate dehydrogenase kinase 4 (PDK4) is involved in regulating glucose consumption in skeletal muscles, liver and kidney. Caloric restriction induced increase in PDK4 levels is dependent on the presence of FOXO1, which directly binds to the PDK4 promoter (Furuyama et al., 2003). Also the levels of lipoprotein lipase (LPL), a protein involved in lipid usage in muscles, are increased by FOXO (Furuyama et al., 2003). Phosphoenolpyruvate carboxy kinase (PEPCK) and glucose 6 phosphatase (G6Pase), two key enzymes in gluconeogenesis and glyconeogenesis, are induced by FOXO as well (Hall et al., 2000; Nakae et al., 2001; Schmoll et al., 2000). In the liver, FOXO1 acts in concert with the Pparγ coactivator Pgc1α to promote transcription of genes that increase glucose production (Puigserver et al., 2003).
Taken together, these data provide a model in which FOXO1 is a key player in the insulin control of glucose metabolism, and thus may be involved in the development of diabetes. This is underlined by the effect of FOXO1 haploinsufficiency to rescue genetic (Kitamura et al., 2002; Nakae et al., 2002) as well as environmental forms of diabetes (Nakae et al., 2003).

The role of FOXO in cellular differentiation
FOXO3a knockout mice exhibit an ovarian phenotype of global follicular activation leading to oocyte death, early depletion of functional ovarian follicles and secondary infertility (Castrillon et al., 2003). These observations indicate that FOXO3a has antidifferentiative effects, at least on follicular activation. Recently, a number of other reports showed, both in cultured cells and in genetically modified mice, that FOXOs have effects on differentiation in several different cell types. During differentiation of T cell progenitors in the thymus, for example, FOXO1 is a crucial factor for the cell survival and cycling of these differentiating thymocytes (Leenders et al., 2000). Expression of a dominant negative FOXO1 reduced total thymocyte numbers, whereas an increase in cycling T cell progenitors was observed (Leenders et al., 2000). Differentiation of erythroid progenitors is depended on the presence of erythropoietin (Epo) (Von Lindern et al., 2001). Activation of FOXO3a in erythroid progenitors

induces accelerated differentiation and reduced the number of cell divisions (Bakker et al., 2004). A downstream target of FOXO3a in erythroid differentiation is the B cell translocation gene 1 (BTG1), which controls protein methylation via the control of protein arginine methyl transferase 1 (Bakker et al., 2004). Genetic analysis showed that FOXO1 is an effector of Irs2 signalling in pancreatic β cells (Kitamura et al., 2002). FOXO1 inhibits β cell proliferation by increasing $p27^{kip1}$ levels in the β cells, and inhibiting the expression of pancreatic transcription factor pancreas/duodenum homeobox gene-1 (Pdx1) (Kitamura et al., 2002). In adipocytes, an active FOXO1 mutant inhibits differentiation via a complex mechanism, involving direct induction of p21 levels in adipocytes (Nakae et al., 2003). On the other hand, FOXO1 haploinsuffiency in these adipocytes prevents insulin resistance and diabetes induced by a high-fat diet (Nakae et al., 2003). Taken together, these data demonstrate a role for FOXOs in several differentiation programs of cells.

The last years, FOXO function has been well studied in muscle development and function. During myoblast differentiation, PI3K signalling leads to myoblast fusion and activation of the terminal differentiation program. This requires transient exclusion of FOXO from the nucleus. In C2C12 mouse myoblasts a constitutively nuclear FOXO1 inhibits myoblast differentiation, while a loss of function of FOXO1 enhanced myoblast differentiation (Hribal et al., 2003). However, once the differentiation program has started, active FOXO1 can increase myotube formation in primary mouse myoblasts (Bois and Grosveld, 2003). This might seem in contrast with the data from C2C12 myoblasts. Nonetheless, these data do emphasize that FOXO activity is tightly controlled during the different steps of differentiation. The importance of tight regulation of FOXO during myoblast differentiation is underlined by the fact that uncontrolled FOXO activity as a result of chromosomal translocations can lead to alveolar rhabdomyosarcomas (Xia et al., 2002).

Besides controlling myoblast differentiation, FOXOs have recently been linked to muscle atrophy. Inhibition of PI3K/PKB reduces the size of myotubes in culture (Rommel et al., 2001). Moreover, mice deficient in PKBα/β have very little muscle mass (Peng et al., 2003). On the other hand, in the atrophic muscles, FOXO1 is dephosphorylated and located in the nucleus (Sandri et al., 2004; Stitt et al., 2004). Alterations in muscle mass as in muscle atrophy are accompanied by the induction of two ubiquin ligases, Atrogin-1 (MAFbx) and MuRF1. Studies in atrophic muscles now show that these two ubiquin ligases are direct targets of FOXO (Sandri et al., 2004; Stitt et al., 2004). Thus, activation of FOXO was shown to be necessary and sufficient for muscle atrophy.

FOXO and Cancer

As mentioned, the first suggestion for a role of FOXOs in tumorigenesis came from the involvement of FOXOs in the two chromosomal translocations with MLL and Pax3/7, in leukemia and alveolar rhabdomyosarcomas respectively. In addition to these chromosomal translocations, there is growing evidence for role of FOXOs in other forms of cancer. In PTEN-deficient cells, FOXOs are inactive and restoration of PTEN induces either apoptosis or cell cycle arrest via up regulation of $p27^{kip1}$. In prostate cancer, with a high incidence of PTEN mutations, overexpression of FOXOs

resulted in apoptosis through direct binding to TRAIL (Modur et al., 2002). These data suggest that any decrease in PTEN will decrease FOXO activity, and thus negatively regulate FOXO target genes in apoptosis or cell cycle regulation. Inactivation of FOXO will shift the balance between cell proliferation and apoptosis and contribute to tumorigenesis by promoting cell growth and survival.
In breast cancers, which are paclitaxel-sensitive, paclitaxel appears to upregulate FOXO3a, which subsequently increases BIM expression and thus decreasing cell survival and contributing to the tumor response of paclitaxel (Birkenkamp and Coffer, 2003). On the other hand, estrogen treatment of mammary cancer cells induces stimulation of p21-activated kinase-1 (Pak-1) and estrogen receptor α (Birkenkamp and Coffer, 2003). This promotes cell survival by inducing phosphorylation and nuclear exclusion of FOXO1 in a Pak-1 dependent manner (Birkenkamp and Coffer, 2003).
In a subset of primary breast tumors, where no PKB phosphorylation was detected, FOXO3a was excluded from the nucleus, suggesting there is a PKB independent FOXO regulatory mechanism in these tumors (Hu et al., 2004). These same set of tumors exhibited high levels of IKKβ, a major catalytic subunit of IKK (Hu et al., 2004). IKK is important for regulation of several cytokine dependent pathways, including the NFκB pathway, which is involved in cell proliferation, prevention of apoptosis and increasing angiogenic and metastatic potential. Cytoplasmic localization of FOXO3a and expression of IKKβ and phosphorylated PKB in breast tumors is inversely associated with patient survival (Hu et al., 2004). The same correlation was seen in other tumor types. Further investigation showed that FOXO3a is a direct target of IKKβ. Phosphorylation by IKKβ on Ser644 repressed FOXO3a transactivation activity, and promoted nuclear exclusion (Hu et al., 2004). This IKKβ inactivation of FOXO3a proceeded through ubiquitination and degradation of FOXO3a by the proteasome, which was dependent on the Ser644 phosphorylation (Hu et al., 2004). On the other hand, overexpression of FOXO3a can override IKKβ stimulation and suppress cell cycle progression, proliferation and tumorigenesis (Hu et al., 2004), indicating that, in this setting, FOXO3a acts as a tumor suppressor.
In *C. elegans*, the DAF-16 induced longevity phenotype both depends on DAF-2 (insulin/IGF-1 receptor) and DAF-4/7 (TGFβ) signalling pathways. Recently, a link between FOXO and TGFβ signalling in mammalian cells has been described. In response to TGFβ, FOXO binds specifically and directly to Smad3 and Smad4 and induces $p21^{cip1}$ activity by direct binding to the $p21^{cip1}$ promoter (Seoane et al., 2004). This role of FOXOs in the TGFβ pathway can be inhibited by activation of the PI3K/PKB pathway (Seoane et al., 2004). On the other hand, this activation of this growth inhibitory gene $p21^{cip1}$ is positively regulated by another FOX transcription factor, FOXG1. FOXG1 can bind the FOXO-Smad complex and thus prevents induction of $p21^{cip1}$ (Seoane et al., 2004). Analysis of both cell lines and tumor samples from gliomas and glioblastomas showed that these tumors have increased levels of FOXG1 and PI3K/PKB activity, resulting in the suppression of $p21^{cip1}$ (Seoane et al., 2004). Thus, by inhibition of $p21^{cip1}$ both pathways play a role in promoting cell survival and proliferation, important features for successful tumor growth and metastasis. Interestingly, $p21^{cip1}$ is also a prominent FOXO target during pre-

adipocyte differentiation (Nakae et al., 2003), suggesting again a link between two processes regulated by FOXO: metabolism and carcinogenesis.
Taken together, these studies suggest that inactivation of FOXO appears to be an important step in carcinogenic transformation. This also argues that FOXOs can be classified as tumor suppressors, and might be interesting targets for cancer-related drugs discovery.

Outline of this thesis

In *C. elegans*, longevity and resistance to cellular stresses are two processes that are tightly linked, and both controlled by DAF-16. Overexpression of DAF-16 in the worm will lead to an increased lifespan, correlated with increased resistance to oxidative stress. Recent data show that in mammalian systems FOXOs are also involved in inducing a cell cycle arrest and quiescence, and on the other side protection against stress. By regulating MnSOD and catalase, two anti-oxidant enzymes important in the defense to oxygen radicals, FOXOs protect cells from oxidative stress.
This thesis describes our effort to understand in further detail the role of mammalian FOXOs in the control of cellular oxidative stress. It focuses on the possibility that FOXOs not only repress cellular oxidative stress, but that cellular oxidative stress itself also signals to FOXOs. FOXOs can directly bind to regions within promoters of target genes. However, as discussed above, a variety of cofactors for FOXO induced effects on transcription exist, for example p300 and several nuclear receptors. Yeast-two-hybrid screening with β-catenin (courtesy of N. Barker, Hubrecht Laboratory, Utrecht) revealed FOXOs as potential binding partners. The results described in **Chapter 2** show that binding of β-catenin to FOXO occurs in cells and that this binding enhances FOXO transcriptional activity. Binding of β-catenin to FOXOs is induced under conditions of increased cellular oxidative stress. In addition to these biochemical data in mammalian cells, we also show evolutionary conservation of this binding in *C. elegans*, where DAF-16 induced protection against oxidative stress is partially dependent on BAR-1 (β-catenin). These data implicate β-catenin binding to FOXO as part of the mechanism by which cellular oxidative stress signals to FOXO (**Chapter 2**).
Binding of β-catenin to FOXO is also shown to influence the function of β-catenin as coactivator for the transcription factor TCF. The TCF/ β-catenin complex is activated upon Wnt signalling and it regulates transcription of several downstream target genes. β-catenin levels in a cell are tightly controlled by the APC/GSK3/Axin complex. In the absence of Wnt signalling, this complex targets β-catenin for degradation by inducing its phosphorylation. Binding of FOXO to β-catenin results in inhibition of TCF/β-catenin transcriptional activity and it is shown that this depends on the ability of FOXO to bind β-catenin combined with the ability to shuttle β-catenin out of the nucleus. This binding and relocalization of β-catenin to the cytosol by FOXO results in an apparent increase in the stability of β-catenin, whereas actual β-catenin/TCF transcriptional activity is reduced. Importantly, cellular oxidative stress also reduces TCF/β-catenin transcriptional activity, consistent with its ability to increase binding of β-catenin to FOXO (**Chapter 3**).

Phosphorylation of FOXOs is regulated by several kinases. Activation of the Ras/Ral pathway also results in phosphorylation of FOXO, as was described for FOXO4. Activation of Ral results in phosphorylation of Thr447 and Thr451 in the C-terminus of FOXO4. In **Chapter 4** we show that phosphorylation of these sites is increased upon oxidative stress. This H_2O_2 induced, Ral mediated phosphorylation of FOXO4 is dependent on the stress kinase JNK, which directly phosphorylates FOXO4 on Thr447 and Thr451. Finally, oxidative stress induces nuclear localization of FOXO4 and an increase in FOXO4 activity through Thr447 and Thr451 phosphorylation. These data provide evidence for a feedback loop from oxidative stress to FOXO transcription factors, involving the small GTPase Ral and JNK.
In the **Addendum** of **Chapter 4** different responses of distinct cell types to different forms of stress will be discussed. We show that FOXOs have a different ability to reduce JNK activation in cells in response to oxidative stress and stress induced by UV. Inhibition of JNK activation induced by oxidative stress is dependent on MnSOD, since FOXOs can no longer inhibit c-Jun phosphorylation after H_2O_2 treatment in SOD-/- MEFs. However, FOXOs still reduce c-Jun phosphorylation in response to UV treatment in SOD-/- MEFs. Experiments in TKO MEFs, which lack the Rb family and thus are incapable of going into cell cycle arrest, indicate a cell cycle arrest dependent increase in stress by H_2O_2, whereas UV induced stress is likely independent of the ability to go into cell cycle arrest.
Finally, the possible consequences of the findings described in this thesis for the function of FOXOs and their role in biological processes like aging and stress protection will be discussed in **Chapter 5**.

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Chapter 2

Functional interaction between β-catenin and FOXO transcription factors in oxidative stress signalling

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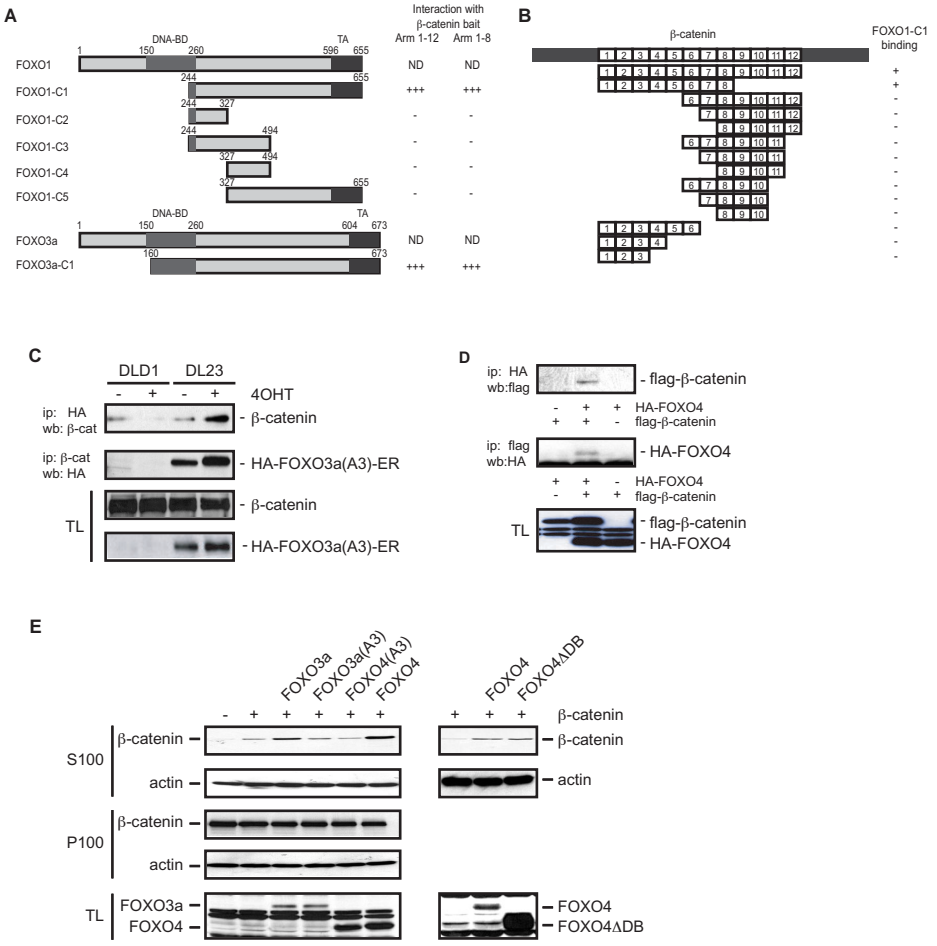
Submitted

β-catenin is a multifunctional protein that regulates gene transcription within the Wingless (Wnt) signalling pathway by direct binding to members of the TCF/Lef-1 family of transcription factors (Huelsken and Behrens, 2002). Here we demonstrate a novel functional interaction between β-catenin and members of the FOXO family of transcription factors, which play a key role in insulin and oxidative stress signalling. β-catenin binds directly to FOXO and enhances FOXO transcriptional activity. Binding of β-catenin to FOXO is increased under conditions of oxidative stress. Genetic analysis in *Caenorhabditis elegans* demonstrates that this interaction is evolutionarily conserved. The β-catenin homologue BAR-1 is required for DAF-16/FOXO dependent dauer formation, lifespan regulation, oxidative stress resistance and the expression of the DAF-16 target gene SOD-3 following oxidative stress. Taken together, these data demonstrate a novel role for β-catenin in regulating FOXO function, which is particularly important under conditions of oxidative stress.

Forkhead box O (FOXO) transcription factors are regulated by the phosphoinositide 3-kinase (PI-3K)/protein kinase B (PKB/c-Akt) signalling pathway (Burgering and Kops, 2002). Phosphorylation of FOXO by PKB results in nuclear exclusion and inhibition of transcriptional activity. A yeast-two-hybrid screen using β-catenin as a bait resulted in the isolation of several clones of FOXO3a and FOXO1 as interacting prey (figure 1A), suggesting a direct interaction between β-catenin and members of the FOXO class of Forkhead transcription factors. First, we characterized binding between FOXO and β-catenin in more detail. Deletion analysis of the FOXO1 prey obtained from the yeast-two-hybrid screen showed that binding to β-catenin requires the entire C-terminal domain of FOXO1, starting at the end of the DNA binding region (figure 1A). In addition, several truncated β-catenin constructs were tested for binding in a yeast-two-hybrid assay (figure 1B) and this showed binding of FOXO to armadillo repeats 1-7 or 1-8. Importantly, binding of FOXO to the armadillo repeats of β-catenin was specific, as FOXO did not bind either APC-1 or APC-2 armadillo repeats (data not shown).

To determine whether FOXO3a and β-catenin interact in mammalian cells, we analyzed whether in DL23 cells, DLD-l cells expressing a conditionally active HA-FOXO3a(A3)-ER fusion, β-catenin and HA-FOXO3a(A3)-ER co-immunoprecipitate (figure 1C). We observed binding between endogenous β-catenin and HA-FOXO3a(A3)-ER. The HA-FOXO3a(A3)-ER is retained in the cytosol in the absence

Figure 1. Binding of β-catenin to FOXO family members
A. Schematic representation of yeast-two-hybrid results. A yeast-two-hybrid screen was performed with two independent β-catenin baits; the first containing armadillo repeats 1-8 and the second containing all 12 armadillo repeats of β-catenin. Several clones of FoxO1 were isolated using the β-catenin (Arm1-12) bait and a FoxO3a clone isolated in the screen performed with the β-catenin (Arm1-8) bait (original clones denoted as C1). Deletion mutants of FOXO1-C1 used to further analyze binding between FOXO and β-catenin are indicated (denoted as C2-C5). **B.** Schematic representation of β-catenin deletion analysis. The FOXO1-C1 construct (see figure 1A) was tested as bait, with the indicated deletion constructs of β-catenin as a prey. **C.** Co-immunoprecipitation between β-catenin and FOXO3a. DL23 cells, DLD1



cells expressing a conditionally active HA-FOXO3a(A3)-ER fusion, were treated with 500 nM 4-OHT for 24 h. The HA-FOXO3a(A3)-ER fusion was immunoprecipitated using 12CA5 monoclonal antibody and binding of endogenous β-catenin to HA-FOXO3a(A3)-ER was analyzed by immunoblotting (upper panel). DLD1 cells were taken along as a control. Immunoprecipitates of endogenous β-catenin were analyzed for the presence of HA-FOXO3a-ER by immunoblotting and probing with anti-ER. As can be seen we observe background signal of β-catenin, when immunoblotting for β-catenin. We attribute this to the high expression of β-catenin in these cells, as this is not observed in the reciprocal experiment (lower panel). **D.** Co-immunoprecipitation between β-catenin and FOXO4. HEK293T cells, transfected with HA-FOXO4 and flag-β-catenin as indicated were lysed and either FOXO4 (upper panel) or β-catenin (lower panel) were immunoprecipitated. Binding of β-catenin and FOXO4 was analyzed by immunoblotting. **E.** Expression of FOXO4 stabilizes β-catenin. A14 cells, untransfected (-) and transfected with flag-β-catenin either in the absence or presence of the indicated HA-FOXO constructs, were lysed and separated in a soluble (S100) and membrane (P100) fraction. HA-FOXO4(A3) and HA-FOXO3(A3) both have mutated PKB phosphorylation sites. HA-FOXO4ΔDB lacks the DNA binding element. Both fractions were tested for β-catenin expression by immunoblotting. Expression of actin served as a loading control. Lower panel shows expression of the transfected FOXO constructs. For this, total cell lysates (TL) were analyzed by immunoblotting with 12CA5.

of the ER-ligand 4-hydroxytamoxifen (4-OHT), because of structural constraints. Treatment with 4-hydroxy-tamoxifen (4-OHT), induces the ER moiety and thus the entire fusion to assume its proper tertiary structure and this induces also increased β-catenin binding. The activated HA-FOXO3a(A3)-ER fusion protein relocalizes to the nucleus because the sites for PKB phosphorylation are mutated (A3) (Medema et al., 2000). This indicates that the interaction with β-catenin can occur both in the cytosol and the nucleus. The FOXO subfamily of Forkhead transcription factors consists of FOXO1 (FKHR), FOXO3a (FKHR-L1) and FOXO4 (AFX). FOXO4 was not isolated from the yeast-two-hybrid screen. We therefore tested a possible interaction between FOXO4 and β-catenin by transient expression of HA-FOXO4 and flag-β-catenin in HEK293T cells. This showed that also FOXO4 and β-catenin could interact (figure 1D).

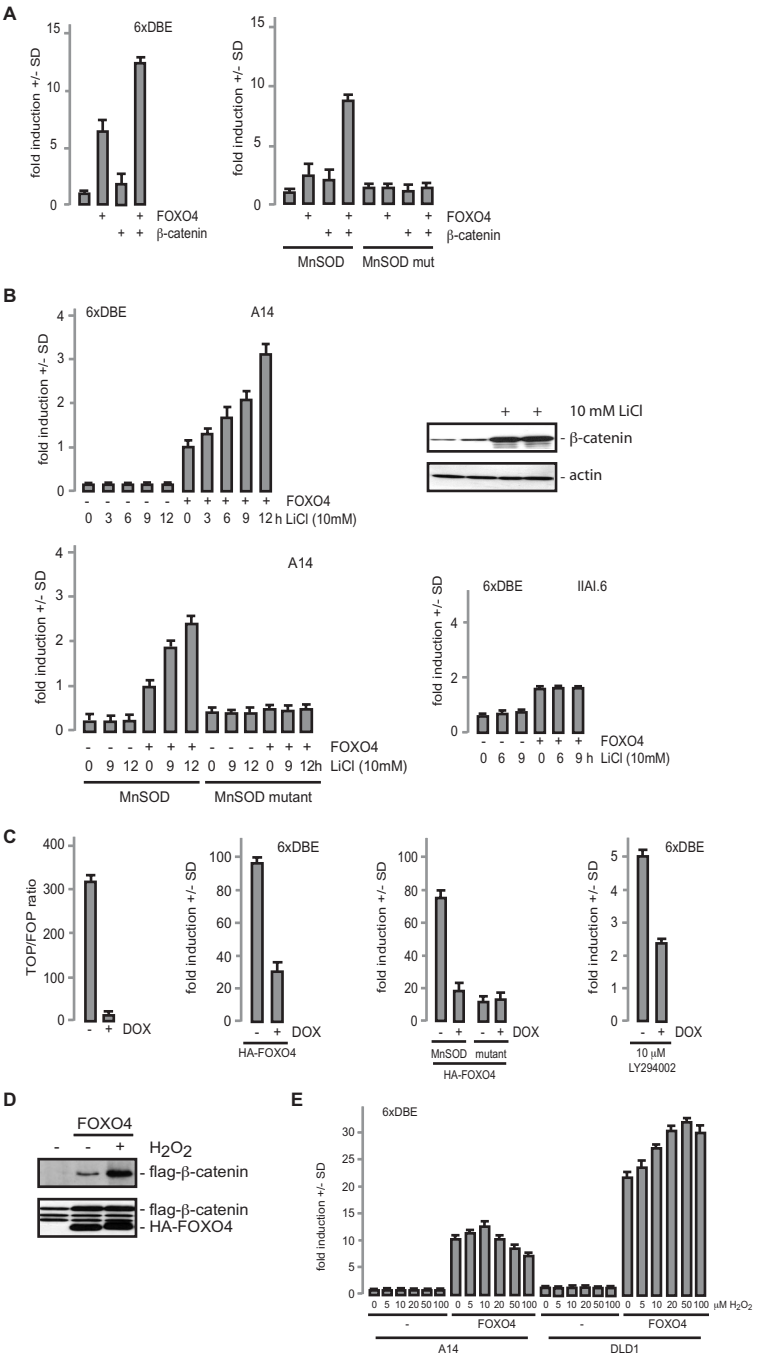
Within the cytosol, free unbound β-catenin is rapidly degraded. Protection of β-catenin from degradation, for example through inhibition of glycogen synthase kinase-3 (GSK-3) activity, results in elevated cytosolic levels of stabilized β-catenin, and this is thought to be an essential requirement for β-catenin to translocate to the nucleus and function as a transcriptional co-activator for TCF/Lef-1. Binding of β-catenin to other proteins, such as E-cadherin, can also protect β-catenin from degradation (Sadot et al., 1998). Thus, to obtain independent evidence for an interaction between FOXO and β-catenin, we tested whether this interaction within the cytosolic compartment would protect β-catenin from degradation and consequently would increase the level of cytosolic β-catenin. Co-expression of β-catenin and FOXO resulted in an increased level of stabilized cytosolic β-catenin (S100 fraction) (figure 1E). This increase was not seen in the membrane (E-cadherin) bound fraction (P100 fraction), indicating that the effect of FOXO expression was specific with respect to the pool of β-catenin. In addition, mutants of FOXO in which all PKB phosphorylation sites were mutated to alanines (A3), displayed reduced ability to increase the level of cytosolic β-catenin. These mutants do bind β-catenin as measured by means of co-immunoprecipitation on total cell lysates (figure 1C and data not shown), but as these mutants are localized within the nucleus, they cannot efficiently stabilize cytoplasmic β-catenin. In addition, the increase of the cytosolic β-catenin level is not due to a transcriptional effect of FOXO on β-catenin expression, as a similar increase was observed with a FOXO mutant that lacks the DNA binding domain (FOXO4-ΔDB). This mutant has retained the entire C-terminal part and therefore still interacts with β-catenin (data not shown), but has lost its ability to activate transcription. Thus, in summary, these results show that β-catenin and FOXO can interact in vivo and that this interaction can protect β-catenin from degradation within the cytosolic compartment.

Next, we investigated whether binding of β-catenin to FOXO serves to regulate FOXO transcriptional activity. Therefore, we analyzed FOXO transcriptional activity in cells lacking detectable β-catenin expression (IIAI.6 cells, figure 2A) (van de Wetering et al., 1997; Korinek, 1997). In these cells, over-expression of FOXO4 can induce transcription of several different FOXO reporter constructs (MnSOD-luc; 6xDBE-luc) (p27-luc, data not shown), but transcriptional activity is clearly enhanced when β-catenin is co-expressed. Inhibition of GSK-3 activity, through treatment of cells with LiCl, increases β-catenin stabilization in cells expressing β-catenin. Treatment of

A14 cells (NIH3T3 overexpressing human insulin receptor) (Burgering and Coffer, 1995) with LiCl enhanced FOXO transcriptional activity (figure 2B). This is likely due to LiCl stabilizing β-catenin, as LiCl treatment of IIAI.6 cells did not affect FOXO transcriptional activity (figure 2B). Thus, we conclude that β-catenin, through direct binding of FOXO, appears to function as a positive modulator of FOXO activity. To further analyze whether endogenous β-catenin enhances FOXO transcriptional activity, we made use of LS174T cells expressing siRNA against β-catenin under control of a tetracycline-inducible promoter (van de Wetering, 2003). As expected, induction of β-catenin siRNA reduced TCF-dependent signalling (van de Wetering, 2003) (figure 2C). Importantly, induction of β-catenin siRNA also reduced FOXO dependent signalling. Transcription induced by FOXO4 over-expression, but also by endogenous FOXO activation through PI-3K inhibition by LY294002 treatment was significantly reduced by siRNA against β-catenin (figure 2C). This effect of β-catenin on FOXO transcriptional activity is in apparent contrast to the regulation of FOXO by the insulin/PI-3K/PKB pathway, which results in inhibition of FOXO activity (Burgering and Kops, 2002). It seems therefore likely that in this respect, β-catenin does not function in insulin signalling, but rather as part of a pathway that activates FOXO.

In *C. elegans*, a variety of stresses, including oxidative stress, stimulate translocation of the FOXO homologue DAF-16 from the cytosol to the nucleus and thereby activate DAF-16 function (Henderson and Johnson, 2001). Also in mammalian cells, oxidative stress can induce nuclear translocation of FOXO and activate FOXO transcriptional activity (Brunet et al., 2004; Essers *et al*., Chapter 4). We therefore tested whether β-catenin binding to FOXO is affected by oxidative stress. Indeed, treating cells with H_2O_2 to induce oxidative stress resulted in increased binding of ectopically expressed β-catenin to FOXO (figure 2D), whereas insulin treatment of cells, which reduces FOXO activity, did not affect β-catenin binding to FOXO (data not shown). To further analyze the consequence of the H_2O_2-induced binding of β-catenin to FOXO, we compared H_2O_2-induced FOXO transcriptional activity between cells that differ with respect to β-catenin expression (figure 2E). In normal NIH3T3 cells, H_2O_2 induces a differential response, with low concentrations of H_2O_2 (<20 μM on average; n=5) increasing FOXO transcriptional activity and higher concentrations reducing FOXO transcriptional activity. In cells expressing stabilized β-catenin (DLD1) H_2O_2-induced FOXO transcriptional activity is enhanced and occurs over a wider concentration range, as H_2O_2-induced repression of FOXO transcriptional activity requires higher concentrations of H_2O_2 (>100 μM on average; n=3). These results show that binding of β-catenin to FOXO activates FOXO transcriptional activity under conditions of cellular oxidative stress.

Next, we analyzed whether endogenously expressed FOXO and β-catenin could interact and whether this interaction is sensitive to H_2O_2. DLD1 cells express endogenous FOXO3a, and indeed FOXO3a and β-catenin could be co-immunoprecipitated from these cells. In addition, the FOXO3a/β-catenin interaction slightly increased following treatment of cells with H_2O_2 (figure 2F). We have previously shown that ectopic expression of FOXO results in increased transcription of the $p27^{kip1}$ gene and a consequent arrest in the G1 phase of the cell cycle (Medema et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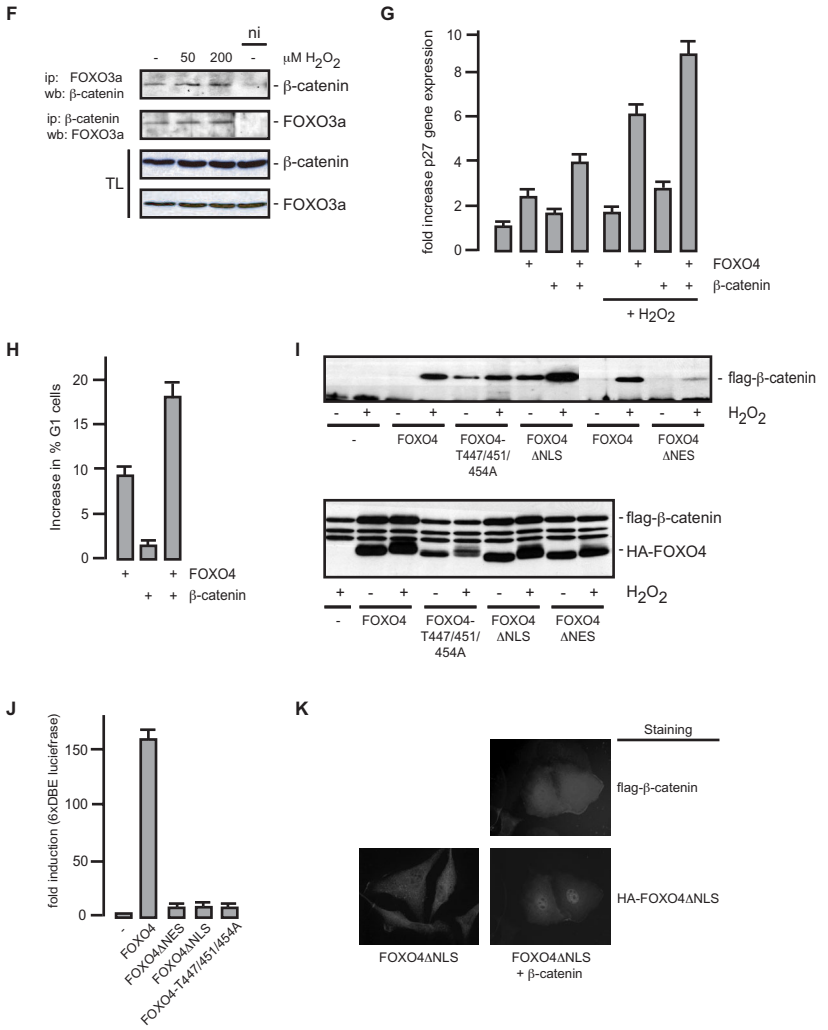

Figure 2. β-catenin binding to FOXO enhances FOXO transcriptional activity
A. β-catenin enhances FOXO transcriptional activity. IIAI.6 cells, which lack detectable β-catenin expression, were transfected with either 6xDBE-luciferase (bearing six canonical FOXO binding sites) (Furuyama et al., 2000), pSODLUC-3340 (bearing the FOXO binding sites within the SOD promoter) (Kim et al., 1999) or pSODLUC-3340 mutant (carrying point mutations in the first and second DBE) (Kops et al., 2002a) and Tk-renilla as internal control, and with or without HA-FOXO4 and flag-β-catenin as indicated. 48h after electroporation transcriptional activity was measured. Data represent the average of three independent experiments. **B.** Stabilization of β-catenin by LiCl treatment enhances FOXO transcriptional activity. A14 cells (upper graph) or IIAI.6 cells (lower graph) transfected with pSODLUC-3304 or pSODLUC-3340 mutant with or without FOXO4, were treated with 10 mM LiCl for indicated times, and transcriptional activity was measured. Similar results were obtained in A14 cells using the 6xDBE-luciferase. Data represent the average of three independent experiments. Western blot panel demonstrates stabilization of β-catenin following LiCl treatment (12hr LiCl treatment) in A14 cells. **C.** Induction of β-catenin siRNA reduces FOXO dependent signalling. LS174T cells expressing siRNA against β-catenin under control of a tetracycline inducible promoter were transfected with pFopglow, pTopglow, 6xDBE-

luciferase, pSODLUC-3340 or pSODLUC-3340 mutant and Tk-renilla as internal control, and with or without HA-FOXO4. Cells were treated with tetracycline for 72 hours and transcriptional activity was measured. LY294002 treatment (10 μM) was for the last 16 hours. 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. Data represent the average of three independent experiments. FOXO4 expression was analyzed on western blot. **D.** Oxidative stress increases the β-catenin FOXO interaction. HEK293T cells were transfected with flag-β-catenin and HA-FOXO4 as indicated. After 1h treatment with 200 μM H_2O_2 (+) HA-FOXO4 was immunoprecipitated and binding of β-catenin was analyzed on blot. Similar results were obtained using 100 or 400 μM H_2O_2. The lower panel shows expression of the different constructs. **E.** H_2O_2-induced FOXO transcriptional activity in cells that differ with respect to β-catenin expression. A14 cells or DLD1 cells transfected with 6xDBE-luciferase with or without FOXO4, were treated with indicated concentrations of H_2O_2 for 16h, and transcriptional activity was measured. Data represent the average of three independent experiments. **F.** Co-immunoprecipitation between endogenous β-catenin and FOXO3a. DLD1 cells were left untreated or treated with 50 or 200 μM H_2O_2 where after either endogenous FOXO3a (upper panel) or β-catenin (lower panel) were immunoprecipitated. Binding of the other endogenous protein was analyzed by immunoblotting. H_2O_2 treatment results in a 2.1 fold increase in binding when FOXO3a is immunoprecipitated (first panel), and a 1.6 fold increase in binding when β-catenin is immunoprecipitated (second panel). These numbers represent the average of four independent experiments. **G.** A14 cells, transfected with HA-FOXO4 and/or flag-β-catenin, were treated with 100 μM H_2O_2 for 16h or left untreated. RNA was isolated and Q-PCRs were performed for $p27^{kip1}$. The fold induction is indicated. Data represent the average of three independent experiments. **H.** Cell cycle profiles of transfected A14 cells expressing HA-FOXO4 and/or flag-β-catenin. The increase in the percentage of G1 cells is indicated. Data represent the average of five independent experiments. **I.** 293T cells were transfected with flag-β-catenin together with different HA-FOXO4 constructs. Cells were treated with 100 μM H_2O_2 for 1h, where after FOXO4 was immunoprecipitated. Binding of flag-β-catenin to the FOXO4 mutants was analyzed on blot. **J.** DLD1 cells were transfected with the different FOXO4 constructs together with 6xDBE-luciferase and Tk-renilla. 36h after transfection luciferase activity was measured. Data represent the average of three independent experiments. Expression of the different FOXO4 constructs was analyzed on western blot (not shown). **K.** NCI-H28 cells, lacking endogenous β-catenin, were transfected with HA-FOXO4ΔNES with or without flag-β-catenin. Cells were fixed and stained for HA-FOXO4 and flag-β-catenin.

al., 2000). Therefore, we tested whether β-catenin expression would affect FOXO-induced $p27^{kip1}$ gene expression and FOXO-induced G1 cell cycle arrest. Indeed, consistent with the reporter assays, β-catenin enhanced FOXO-induced $p27^{kip1}$ gene expression and this was further enhanced after treatment of cells with H_2O_2 (figure 2G). Also, β-catenin expression consistently enhanced FOXO-induced G1 cell cycle arrest (figure 2H). Taken together, the data demonstrate that β-catenin binding increases FOXO activity and function, especially following conditions of increased cellular oxidative stress.

To gain insight into the mechanism whereby β-catenin increases FOXO transcriptional activity we tested a series of FOXO4 mutants for their ability to bind β-catenin following treatment of cells with H_2O_2 (figure 2I). Interestingly, deleting the putative nuclear export sequence (ΔNES) resulted in reduced β-catenin binding, whereas deleting the nuclear localization signal (ΔNLS) resulted in the opposite, increased basal as well as H_2O_2-induced β-catenin binding. In addition, we observed that a mutant in which Thr447, Thr451 and Thr454 are mutated to alanine also displayed increased β-catenin binding. Previously, we have shown that a Ras/Ral signalling pathway regulates phosphorylation of these threonine residues (De Ruiter et al., 2001). In addition, this mutant displays normal insulin-induced cytosolic relocalization, but enhanced kinetics of nuclear entry following serum-withdrawal (De Ruiter et al., 2001; De Ruiter, 2002). Thus, the differential binding of β-catenin to these mutants and the apparent role of the mutated regions in FOXO shuttling, would suggest a role for β-catenin in the regulation of nucleo-cytoplasmic shuttling of FOXO. In addition, all these mutants are severely impaired in transcriptional activity even in

cells in which β-catenin is highly expressed (DLD1, figure 2J). This would exclude a role for β-catenin as transcriptional co-activator, since these mutants are not impaired in their DNA binding. To further obtain evidence for a role of β-catenin in FOXO shuttling, we analyzed the subcellular localization of the FOXO mutants in β-catenin-negative cells. Both ΔNES and Thr447/451/454A mutant are nuclear in the presence or absence of ectopic β-catenin (data not shown). The ΔNLS is cytosolic in the absence of β-catenin, but clearly shows nuclear translocation in the presence of β-catenin (figure 2K). Because both the ΔNES and the Thr447/451/454A mutant harbor an intact NLS, these data indicate that β-catenin regulates shuttling independent of the NLS. This would be consistent with previous findings that the NLS is regulated by PKB-mediated phosphorylation (Brownawell et al., 2001). Taken together, we interpret these data to suggest that in contrast to its function in TCF/Lef-1 regulation, β-catenin does not act as a transcriptional co-activator for FOXO, but that the binding of β-catenin to FOXO may regulate FOXO nucleo-cytoplasmic shuttling under conditions of oxidative stress.

The regulation of FOXO by insulin//PI-3K/PKB signalling is evolutionary conserved. In *C. elegans*, the FOXO homologue DAF-16 regulates entry into the dauer diapause, an alternative larval stage that is induced by starvation, high temperature and dauer pheromone (reviewed in Tissenbaum and Guarente, 2002). We found that animals that contain a null mutation in the canonical β-catenin gene *bar-1* are defective in starvation-induced dauer development (not shown). Therefore, we investigated if there is a functional interaction between DAF-16 and BAR-1. As shown in figure 3A, BAR-1 can be co-immunoprecipitated with DAF-16 when both proteins are expressed in mammalian cells, demonstrating that the physical interaction between FOXO and β-catenin is evolutionarily conserved. Next, we tested if BAR-1 is required for DAF-16 induced dauer development. To activate DAF-16 signalling, we used a temperature sensitive allele of *daf-2*/insulin-like receptor, that induces nuclear translocation of DAF-16 and dauer development at the restrictive temperature (Gems, 1998; Henderson and Johnson, 2001). At 25°C both *daf-2(m41)* and *daf-2(m41); bar-1(ga80)* double mutants showed 100% dauer development (n>200), demonstrating that BAR-1 is not essential for executing the dauer fate. At the intermediate temperatures of 20°C and 18°C, however, there is a significantly lower induction of dauer formation in the *daf-2(m41); bar-1(ga80)* double mutant as compared to *daf-2(m41)* alone (figure 3B). This shows that BAR-1 is required for DAF-16 signalling when DAF-16 activity is limited. Furthermore, over-expression of BAR-1 significantly increases dauer induction by *daf-2(m41)*. This increase in dauer formation is strongly reduced when *daf-16* is inhibited by RNAi (figure 3C), demonstrating that over-expression of BAR-1 enhances DAF-16 signalling. This is in agreement with our observation that over-expression of β-catenin enhances FOXO dependent reporter activity in mammalian cells (figure 2A and 2B). In addition to the regulation of dauer development, DAF-16 activity also influences lifespan (reviewed in Tissenbaum and Guarente, 2002). Since *daf-16(mu86)* mutants show a shorter average lifespan than wild type animals (Lin et al., 2001), we asked if BAR-1 is required for normal lifespan regulation as well. As is shown in figure 3D, *bar-1(ga80)* mutants show a reduction in mean lifespan that is similar to the reduction observed in *daf-16(mu86)*, demonstrating that BAR-

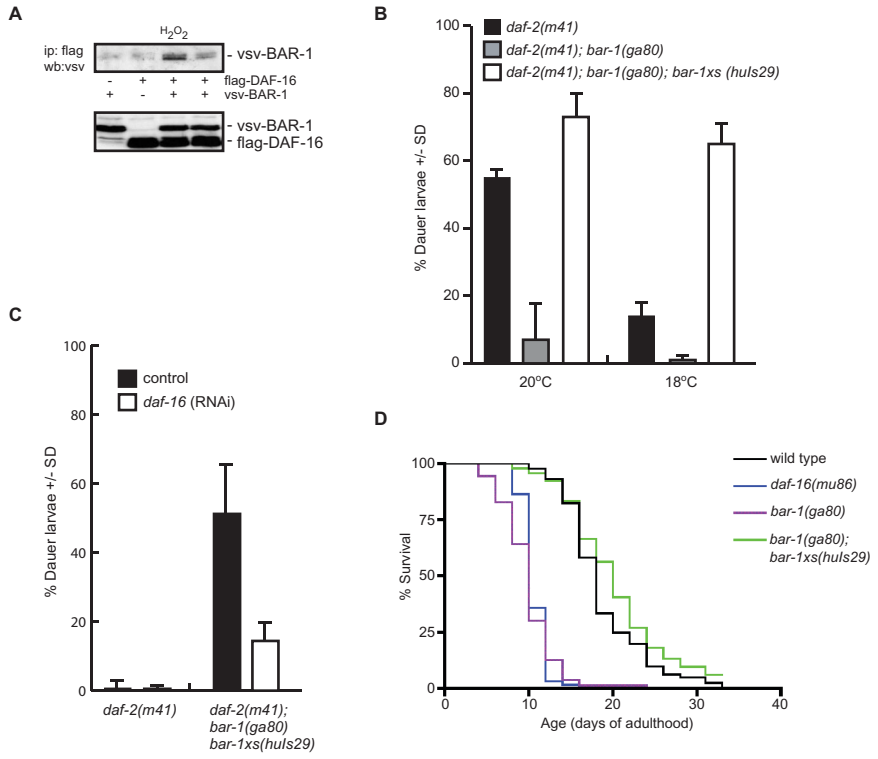

Figure 3. Genetic interaction between BAR-1 and DAF-16
A. Co-immunoprecipitation of BAR-1 and DAF-16. HEK293T cells were transfected with vsv-BAR-1 and flag-DAF-16. After 1h treatment with 200 μM H_2O_2 DAF-16 was immunoprecipitated and co-precipitation of BAR-1 was analyzed on blot. **B.** BAR-1 is required for DAF-16 induced dauer development. The percentage of dauer larvae induced in *daf-2(m41)*/insulin-like receptor mutants and in combinations of *daf-2(m41)* with *bar-1(ga80)* and the BAR-1 overexpressing transgene *huIs29* (*bar-1xs*) was determined at 20ºC and 18ºC. Dauer induction is significantly reduced in the absence of *bar-1* ($p<0.001$, Student's t-test), but is enhanced when BAR-1 is overexpressed ($p<0.001$ at 18ºC). In each case n>200 and data represent the average of four independent experiments. **C.** The enhanced dauer formation induced by BAR-1 over-expression is dependent on DAF-16 activity. Animals were grown at 18ºC on control bacteria or on a bacterial strain expressing *daf-16* dsRNA to induce *daf-16* specific RNAi. The dauer inducing effect of BAR-1 over-expression is strongly reduced when DAF-16 function is inhibited by RNAi ($p<0.001$). In each case n>150 and data represent the average of five independent experiments. **D.** Loss-of *bar-1* reduces lifespan. Survival curves show that *bar-1(ga80)* and *daf-16(mu86)* mutants have a shorter mean lifespan (10 days, n=100) than wild type and BAR-1 overexpressing animals (18 and 20 days, respectively, n=100) ($p<0.0001$, Kaplan-Meier survival analysis).

1 is also required for the function of DAF-16 in determining lifespan. As mentioned above, in mammalian cells (Brunet et al., 2004) as well as in *C. elegans*, oxidative stress induces nuclear localization of DAF-16 and activation of DAF-16 target genes (Henderson and Johnson, 2001). We observed that similar to FOXO and β-catenin, the binding between DAF-16 and BAR-1 is enhanced by oxidative stress (figure 3A). Therefore, we investigated if BAR-1 is required for oxidative stress-induced DAF-16

signalling. A known FOXO target gene that is upregulated after oxidative stress is manganese superoxide dismutase (MnSOD). Of the three SOD genes of *C. elegans*, *sod-3* is most similar to human MnSOD and only *sod-3* expression is regulated by DAF-16 (Honda and Honda, 1999). We observed that a *sod-3::gfp* reporter gene is expressed at low levels in the pharynx and in the most anterior and posterior intestinal cells (figure 4A). This low-level expression is DAF-16 independent, as the expression is unchanged in a *daf-16* null mutant. As expected, *sod-3::gfp* expression is strongly upregulated when animals are exposed to oxidative stress. As shown

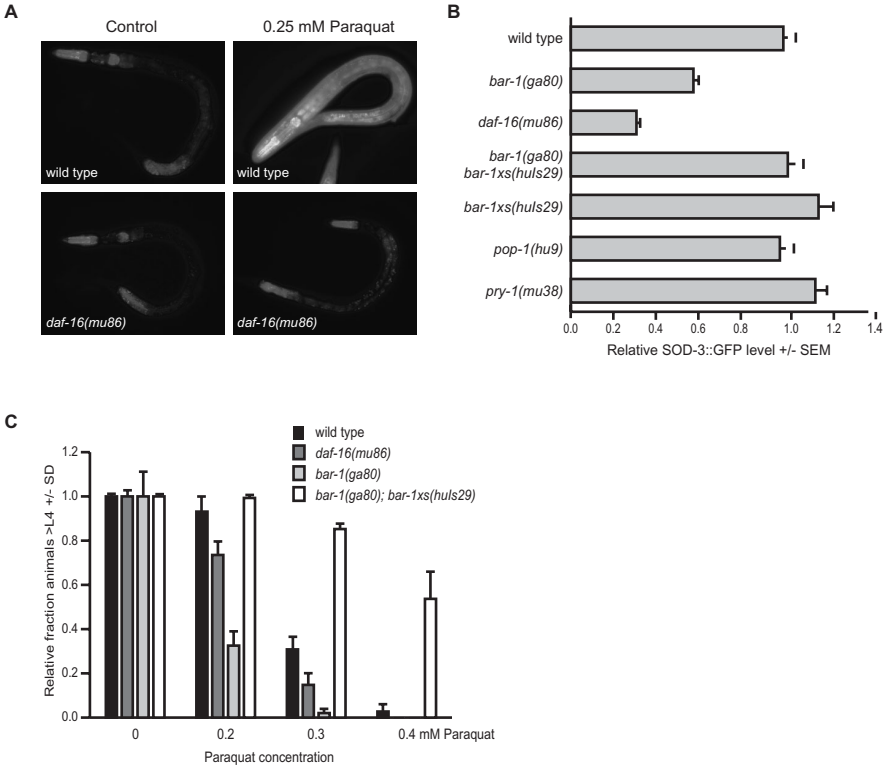

Figure 4. BAR-1 is required for the oxidative stress induced expression of the manganese superoxide dismutase gene *sod-3*

A. Expression pattern of a *sod-3::gfp* reporter gene under normal and oxidative stress conditions. The induction of *sod-3::gfp* expression after exposure to 0.25 mM Paraquat is dependent on DAF-16 activity. Expression in the pharynx and anterior and posterior intestinal cells is DAF-16 independent. **B.** BAR-1 is required for oxidative stress induced DAF-16 signalling. The paraquat induced expression of *sod-3::gfp* is significantly reduced in *bar-1(ga80)* mutants ($p<0.001$), but is enhanced in *pry-1(mu38)* mutants ($p<0.001$) and when BAR-1 is overexpressed (*huls29*) ($p<0.005$). In each case, data represent the average of *sod-3::gfp* expression levels measured in at least 25 animals. **C.** BAR-1 is required for the response to oxidative stress. *bar-1(ga80)* and *daf-16(mu86)* mutants are more sensitive to paraquat induced oxidative stress, whereas animals that overexpress BAR-1 are more resistant. Data represent the average of three independent experiments, each with approximately 50 to 100 animals.

in figure 4A, there is a strong increase in *sod-3::gfp* expression when animals are grown in the presence of 0.25 mM paraquat, which induces the formation of reactive oxygen species (Ishii et al., 1990). The paraquat induced upregulation of *sod-3::gfp* expression is DAF-16 dependent, as there is no significant increase in *sod-3::gfp* expression levels in *daf-16(mu86)* (figure 3C). To quantify *sod-3::gfp* expression, we measured the SOD-3::GFP level in intestinal cells of the mid body, a region which shows the lowest DAF-16 independent *sod-3::gfp* expression. Next, we tested if BAR-1 is required for the paraquat-induced expression of *sod-3*. As is shown in figure 4B, there is a significant reduction in *sod-3::gfp* expression levels in *bar-1(ga80)*, a defect that is rescued by over-expression of wild type BAR-1. This shows that BAR-1 is required for oxidative stress induced DAF-16 signalling. Indeed, over-expression of BAR-1 (in a wild type *bar-1* background) and mutation of *pry-1*/Axin (Korswagen et al., 2002), which also increases BAR-1 levels, leads to a modest, but significant increase in *sod-3::gfp* expression (figure 4B). As expected, this increase in *sod-3* expression is *daf-16* dependent (figure S1). To exclude the possibility that BAR-1 may affect *sod-3::gfp* expression through an interaction with POP-1/TCF, we tested if *pop-1(hu9)*, which contains a mutation that specifically disrupts the function of POP-1 in canonical Wnt signalling (Korswagen et al., 2002), has an effect on the paraquat induced *sod-3::gfp* expression. As shown in figure 4B, there is no difference in *sod-3::gfp* expression levels between wild type and *pop-1(hu9)*.

The regulation of anti-oxidant enzymes such as SOD-3 by DAF-16 signalling is required for the defense against oxidative damage. As a consequence, loss-of *daf-16* results in an enhanced sensitivity to oxidative stress (Yanase et al., 2002). Since BAR-1 is required for the DAF-16 dependent expression of *sod-3*, we tested if *bar-1(ga80)* mutants are also more sensitive to oxidative stress. When we analyzed growth on different concentrations of paraquat, we observed that *daf-16(mu86)* and especially *bar-1(ga80)* mutants are more sensitive to oxidative stress (figure 4C). Furthermore, over-expression of BAR-1 resulted in a significant resistance to oxidative stress. Taken together, these results demonstrate that BAR-1 is required for the DAF-16 mediated response to oxidative stress. This is consistent with our finding that in mammalian cells the binding of β-catenin to FOXO increases the activity and

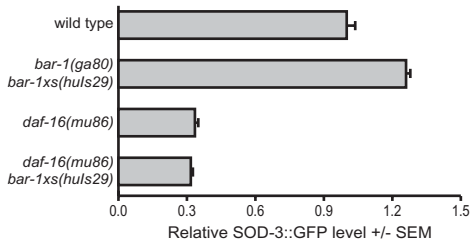

Figure S1. The enhanced expression of *sod-3::gfp* induced by over-expression of BAR-1 is *daf-16* dependent
There is no significant difference in *sod-3::gfp* expression between *bar-1xs(huIs29); daf-16(mu86)* and *daf-16(mu86)*. In each case, data represent the average of *sod-3::gfp* expression levels measured in at least 25 animals.

function of FOXO under conditions of oxidative stress (figure 2E and G).
Here we have demonstrated by biochemical and genetic analysis a functional interaction between β-catenin and members of the FOXO subfamily of Forkhead transcription factors. These data reveal a novel and evolutionary conserved function for β-catenin, which is independent of TCF/Lef-1. Similar to its role in TCF/Lef-1 signalling, β-catenin enhances transcriptional activity of FOXO. We have previously shown that FOXOs inhibit cell cycle progression and here we show that this function is stimulated by β-catenin. In contrast, β-catenin/TCF interaction stimulates cell cycle progression, and deregulated β-catenin/TCF signalling is thought to be a major cause for the development of cancer. Over-expression of constitutively active FOXO can inhibit cellular transformation induced by aberrant Ras signalling and PI-3K signalling (Medema et al., 2000). Also, in DLD1 cells, which express a truncated form of the adenomatous polyposis coli (APC) tumor suppressor and therefore display increased TCF/β-catenin signalling, expression of FOXO inhibits cell proliferation (Kops et al., 2002b). Thus, β-catenin appears to fulfill a critical function to balance positive (TCF/Lef-1) and negative regulation (FOXO) of cell cycle progression. This importance is further illustrated by the recent observation that loss of $p27^{kip1}$, a critical FOXO target for regulating cell cycle arrest, strongly cooperates with loss of APC, but not mutated Smad, in colon carcinoma progression (Philipp-Staheli et al., 2002). It is clear that different modes of signalling input, including insulin signalling and oxidative stress as shown here, may shift the balance between TCF/Lef-1 and FOXO. DAF-16 (FOXO) and oxidative stress have all been implicated in aging, and in model organisms such as *C. elegans* there appears indeed a strong correlation between DAF-16 function, oxidative stress regulation and aging. Consistent with this, we observe that BAR-1 affects DAF-16-dependent stress resistance and longevity in *C. elegans*. Given the known role of β-catenin in the development of cancer, our results also suggest that a shift in the balance from β-catenin binding FOXO to β-catenin binding to TCF/Lef-1 may link aging and the development of certain types of cancer.

Acknowledgements

We would like to thank Hans Bos, Hans Clevers, Fried Zwartkruis, and Ronald Plasterk for critical reading of the manuscript, David Weinkove for initial *C. elegans* stress assays, Rene Medema for initial cell cycle analysis, Marc van de Wetering for providing pcDNA3-flag-β-catenin and IIAI.6 cells, Marc Vidal for providing the *daf-16* RNAi clone, Maria-Alexander Bridges for providing flag-DAF-16, Johan Van Es for providing hAPC1 and hAPC2 two-hybrid constructs, Laura van 't Veer for pMD4, Leon Tertoolen for help with digital imaging, the *C. elegans* Genetics Center (University of Minnesota, Minneapolis) for strains and the Dutch Cancer Foundation (ME) and the Center for Biomedical Genetics (HCK) for financial support.

Material and Methods

Cell culture, transfection, electroporation
DL23 cells, DLD1 cells expressing a conditionally active FOXO3a(A3)-ER fusion, were created as described (Burgering and Kops, 2002). DL23 cells were treated with 500 nM of 4-OHT to activate the fusion protein. LS174T cells expressing siRNA against β-catenin under control of a tetracycline inducible promoter (van de Wetering, 2003) and IIAI.6 cells were a gift from M. van de Wetering (van de Wetering et al., 1997; Korinek, 1997). DLD1 human colon carcinoma cells, DL23 cells, NCI-H28 malignant mesothelioma cells and IIAI.6 cells were maintained in RPMI-1640 supplemented with L-glutamine, penicillin/streptomycin and 10% FCS. A14 cells (mouse NIH3T3 cells overexpressing the human insulin receptor) (Burgering and Coffer, 1995) and HEK293T cells were maintained in Dulbecco's Modified Eagle Medium supplemented with L-glutamine, penicillin/streptomycin and 10% FCS. A14 cells were transiently transfected using the calcium phosphate method. HEK293T cells, NCI-H28 cells and LS174T cells expressing siRNA against β-catenin were transiently transfected using FuGENE6 reagent according to the manufacturer (Roche). IIAI.6 cells were transiently transfected by electroporation using a total of 7.5 μg of plasmid DNA. Cells (2.5 x 10^6 cells in 0.25 ml 20% RPMI 1640 medium) were pulsed at 260 V and 950 μF with plasmid DNA as indicated. Subsequently, the cells were kept on 10% medium and used 48h after transfection. Total amounts of transfected DNA were equalized using pBluescript KSII+.

Plasmids
The following constructs have been described before: pMT2-HA-FOXO4 (Kops et al., 1999); pMT2-HA-FOXO4.A3 (Medema et al., 2000); pMT2-HA-FOXO4-ΔDB (Medema et al., 2000); pMT2-HA-FOXO4-ΔNES; pMT2-HA-FOXO4-ΔNLS (Brownawell et al., 2001); pMT2-HA-FOXO4-Thr447/451/454A (De Ruiter et al., 2001); pECE-FOXO3a (Brunet et al., 1999); pECE-FOXO3a.A3 (Brunet et al., 1999); pcDNA3.1-flag-DAF-16a1 (Nasrin et al., 2000); pcDNA3-vsv-BAR-1 (Korswagen et al., 2000); 6xDBE luciferase (Furuyama et al., 2000); pSODLUC-3340 and pSODLUC-3340 mutant (Kim et al., 1999); pFopglow and pTopglow (van de Wetering, 2003); pcDNA3-flag-β-catenin was a gift of M. van de Wetering. pRL-Tk (Tk renilla luciferase) was purchased from Promega. All β-catenin yeast-two-hybrid bait constructs were generated by cloning the indicated regions in-frame with the DNA-binding domain of yeast Gal4 in the pMD4 vector. FoxO1-C1-C5 pACT2 constructs were generated by high-fidelity PCR. All constructs were checked by sequencing.

Antibodies
Monoclonal 12CA5 for HA-tagged proteins was produced using a hybridoma cell line. Mouse monoclonal β-catenin antibody, goat polyclonal antibody actin and rabbit polyclonal antibody FOXO3a (H144), mouse monoclonal flag M2 antibody, and mouse monoclonal VSV-G (P5D4) antibody were obtained from Transduction Laboratories, Santa Cruz Biotechnology, Sigma and Pharmingen, respectively.

Immunoprecipitation and western blots
Non-confluent cells were lysed in RIPA buffer (50 mM Tris pH 7.5, 1% Triton X-100, 0.5% deoxycholate, 10 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 mM sodium vanadate, 1 μg/ml leupeptin and 0.1 μg/ml aprotinin), and lysates were cleared for 10 minutes at 14.000 rpm at 4°C. Lysates were incubated for 2 hours at 4°C with either 1 μl 12CA5, or VSV-G antibody, 0.3 μl of flag M2 antibody, or 7.5 μl β-catenin antibody and 50 μl pre-washed protein-A beads. The immunoprecipitations were washed four times with RIPA buffer, cleared for all liquid, and 25 μl of 1x Laemmli sample buffer was added. Samples were subjected to SDS-PAGE and transferred to PVDF membrane (Perkin Elmer). Western blot analysis was performed under standard conditions and using the indicated antibodies.

Luciferase reporter assays
A14 cells, DLD1 cells, and LS174T cells expressing siRNA against β-catenin were transiently transfected with a reporter construct bearing six canonical FOXO binding sites (6xDBE-luciferase). Cells were cotransfected with HA-FOXO4 or a control plasmid. Luciferase counts were normalized using Tk-renilla-luciferase. After treatment with 10 mM LiCl cells were washed twice with PBS, lysed in passive lysis buffer (PLB) and luciferase activity was analyzed using a luminometer and a dual-luciferase assay kit according to the manufacturer (Promega).
The IIAI.6 cells transfections were performed by electroporation of 2.5 x 10^6 cells with 100 ng TK-Renilla, 1 μg 6xDBEluciferase or pSODLUC-3340, bearing the FOXO binding site within the SOD promoter, and 2.5 μg of either pMT2-HA-FOXO4 or flag-β-catenin or both. Luciferase activities were determined using the Dual Luciferase Reporter (DLR) system. Reporter luciferase activity was normalized relative to Renilla-luciferase activity. Transfections were performed in duplicate.

S100/P100 fractionation
Cell fractionation for analysis of the cytosolic pool of β-catenin was essentially done as previously described (Ding et al., 2000) . Cells were harvested in Tris buffer (20 mM Tris pH 7.5, 50 mM NaF, and 1 mM EDTA), put on ice for 20 minutes, and homogenized through a 23 G 1 1/4 Microlance syringe. Nuclear and membrane components were separated by a sequential two times centrifugation step at 14000 rpm for 10 minutes at 4°C. The supernatant was collected as the soluble fraction (S100) and 5x sample buffer was added. The pellet fraction was taken up in 1x sample buffer (P100).

Real time PCR analysis
The expression of the endogenous $p27^{kip1}$ gene in A14 cells was determined by reverse transcription of total RNA followed by real time PCR analysis. Total RNA was isolated from A14 cells using the RNAZol procedure (TELTEST, Inc). Single-stranded cDNA was synthesised from the total RNA using AMV reverse transcriptase (Promega). Real time PCR was performed on an ABI thermo-cycler using Sybr green (ABI) with the following primer set: p27 (forward): CTGGGTTAGCGGAGCACTGT and p27 (reverse): GGAAAACAAAACGCTTCTTCTTAG.

Cell cycle distribution
To analyze cell cycle redistribution, A14 cells were transfected with the appropriate expression plasmids. Thirty-six hours after transfection, DNA profiles were obtained by flow cytometry (Medema et al., 1995) and analyzed using ModFit software (Becton Dickinson).

Immunofluorescence
NCI-H28 cells were cultured on coverslips, transfected with 1 μg of pMT2-HA-FOXO4 NES with or without 1 μg flag-β-catenin with the total DNA of 2 μg equalized by empty vector, and fixed in 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS, and non-specific binding was blocked with 0.5% BSA in PBS for 45 min. Incubation with the HA polyclonal antibody and the flag M2 monoclonal antibody was for 1h, followed by 1h of incubation with anti-rabbit-CY3 and anti-mouse-FITC second antibody. Coverslips were washed and mounted on glass slides using Immuno-Mount (Shandon, Pittsburgh, Pa.).

Yeast-two-hybrid
The pMD4 β-catenin baits were independently transformed into the HF7C reporter yeast strain using a standard small-scale transformation protocol (Clontech). The resulting bait strains were subsequently transformed with 75 μg of a Matchmaker human fetal brain cDNA library according to the manufacturer's protocol (Clontech). Positive interacting clones were isolated and identified as previously described (Molenaar et al., 1996).
For two-hybrid analysis of interactions between β-catenin and FOXO, the HF7C yeast strain was co-transformed with bait and prey recombinant vectors in the presence of 20μg herring testis carrier DNA. Positive interactions were determined as previously described (Molenaar et al., 1996).

Dauer, lifespan and oxidative stress assays
Strains were cultured at 20°C using standard conditions as described (Brenner, 1974; Lewis and Fleming, 1995). Alleles used in this study were *daf-16(mu86)* (Lin et al., 1997), *pop-1(hu9)* (Korswagen et al., 2002), *pry-1(mu38)* (Korswagen et al., 2002; Maloof et al., 1999) and *daf-2(m41)* (Gems, 1998). Transgenes used were: *muIs32* (Ch'ng et al., 2003), *huIs29*, which contains pDE204 (*bar-1(+)* genomic sequence) (Eisenmann et al., 1998), pMH86 (*dpy-20(+)* genomic sequence) and pPD117.01 (a *mec-7::gfp* fusion construct, a gift from A. Fire), each injected at 50 ng/μl and *huIs33*, which contains pHCK74 (a *sod-3::gfp* fusion, see below) injected at 50 ng/μl and pRF4 (*rol-6(su1006*) injected at 150 ng/μl. Both *huIs29* and *huIs33* were selected as stable integrants after irradiation with 40 Gy of gamma-radiation.
To assay dauer development, strains were synchronized by hypochlorite treatment. The isolated eggs were distributed at approximately 100 eggs per NGM agar plate. Animals were grown at the indicated temperature and 4 days later the percentage of dauer larvae was scored. To test the effect of *daf-16* RNAi on dauer development, eggs were placed on NGM agar plates containing 1 mM IPTG and seeded with either non-transformed E. coli (OP50) or a bacterial strain expressing *daf-16* dsRNA (a gift from M. Vidal) as described (Kamath and Ahringer, 2003). To assay lifespan, strains were first treated with *glp-1* RNAi to produce sterile adults (Austin and Kimble, 1987). This was necessary, because *bar-1(ga80)* mutants have defects in vulva morphogenesis and are frequently egg-laying defective (Eisenmann et al., 1998). As a consequence, *bar-1(ga80)* hermaphrodites show premature death as a result of internal hatching of progeny. Thus, strains were synchronized by hypochlorite treatment and eggs were placed on 1 mM IPTG containing NGM agar plates seeded with a *glp-1* dsRNA expressing bacterial strain (Kamath et al., 2003). Sterile young adult hermaphrodites were placed on NGM agar plates containing 20 μg/ml 5-fluoro-2'-deoxyuridine (FUDR, Sigma) to arrest

the development of progeny produced during the experiment. Lifespan assays were as described (Tissenbaum and Guarente, 2001). Statistical tests (Kaplan-Meier survival analysis) were performed using Graphpad Prism 4 software.

To assay *sod-3* expression, a *sod-3::gfp* reporter was generated by amplifying a fragment containing the promoter and part of the coding sequence of *sod-3* from cosmid C08A9 (using primers sod-3F2SphI TCTGCAGTGATTCAGAGAGG and sod-3RbamHI TTCCAAAGGATCCTGGTTTG), which was cloned into vector pPD95.75 (a gift from A. Fire) (pHCK74). Strains containing *hulS33* were synchronized to L1 by hypochlorite treatment and overnight hatching in M9 buffer and were placed at a density of approximately 200 animals/plate on 0.25 mM paraquat (Sigma) containing NGM agar plates. *sod-3::gfp* expression levels were measured 48 hours later in L3 stage larvae. To quantify *sod-3::gfp* expression, GFP levels were measured in intestinal cells of the mid-body using digital imaging (image acquisition with a Zeiss AxioCam HRc camera using linear settings and image analysis using NIH image software).

Sensitivity to oxidative stress was tested essentially as described (Fujii et al., 2004). Strains were synchronized by hypochloride treatment and eggs were distributed at approximately 100 eggs per plate on NGM agar plates containing 20 μg/ml FUDR and the indicated concentration of paraquat. FUDR was used to arrest the development of progeny produced during the assay. The animals were grown at 20°C and 5 days later the percentage of L4 larvae and adult animals was determined. The minor differences in growth between the different strains was corrected by normalizing the data using growth values in the absence of Paraquat.

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Chapter 3

Interaction of FOXO with β-catenin inhibits β-catenin/ TCF activity

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To be Submitted

Wingless (Wnt) signalling regulates many aspects of development, and aberrant Wnt signalling can lead to cancer. Upon a Wnt signal β-catenin degradation is halted and consequently the level of β-catenin in the cytoplasm increases. This allows entry of β-catenin, into the nucleus, where it can regulate gene transcription by direct binding to members of the LEF/TCF family of transcription factors. Recently, we identified Forkhead box-O (FOXO) transcription factors as novel interaction partners of β-catenin. Here we show that the interaction between β-catenin and FOXO, results in inhibition of β-catenin/TCF transcriptional activity. This inhibition of transcription is probably due to competition between FOXO and TCF for binding to β-catenin, since activation of FOXO reduced TCF/β-catenin complex formation. The ability of FOXOs to inhibit TCF/β-catenin mediated transcription correlated also with the ability of FOXOs to shuttle β-catenin out of the nucleus. Mutants of FOXO that are predominantly localized within the nucleus are impaired in their ability to inhibit TCF/β-catenin transcriptional activity and do not increase cytosolic levels of β-catenin, whereas mutants of FOXO that efficiently inhibit TCF/β-catenin activity are predominantly localized within the cytosol and also increase the level of cytosolic β-catenin. Finally, cellular oxidative stress increases binding between β-catenin and FOXO and in keeping the ability of FOXOs to inhibit TCF/β-catenin signalling is further enhanced under conditions of oxidative stress. Taken together these results start to outline a cross-talk mechanism between FOXO and TCF signalling in which β-catenin plays a central regulatory role.

Introduction

Wnt proteins are closely related secreted glycoproteins that play critical roles in cell proliferation and cell fate determination at many stages of development (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998). In addition, deregulation of the Wnt signalling pathway resulting in stabilization and nuclear accumulation of β-catenin can lead to tumor formation (Polakis, 2000). Genetic and biochemical experiments in *Drosophila*, *Xenopus*, and mammalian cells have established a framework for the Wnt signalling pathway (summarized in figure 1). In the absence of a Wnt signal, cytoplasmic β-catenin is bound to a multiprotein β-catenin destruction complex that contains several proteins including Axin, adenomatous polyposis coli (APC), casein kinase Iα (CKIα) and ε (CKI ε), and glycogen synthase kinase 3 (GSK3). In this complex, CKIα and/or CKIε phosphorylate β-catenin at Ser45 (Liu et al., 2002). This in turn enables GSK3 to phosphorylate serine/threonine residues 41, 37, and 33 near the N-terminus of the β-catenin protein (Behrens et al., 1998; Ikeda et al., 1998; Kishida et al., 1998). Phosphorylation of these residues triggers ubiquitination of β-catenin by βTrCP, a component of the $SCF^{\beta TrCP}$ ubiquitin-protein ligase complex, and degradation of β-catenin by the ubiquitin-proteasome pathway (Aberle et al., 1997; Hart et al., 1999; Kitagawa et al., 1999). In the presence of Wnt, Dishevelled (Dsh) blocks β-catenin degradation by inducing the disassembly of the β-catenin destruction complex, thereby allowing accumulation of β-catenin within the cytosol

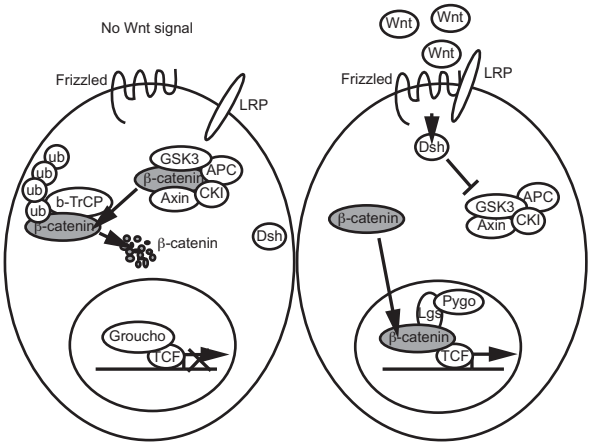

Figure 1. A simplified scheme of the Wnt signalling cascade
Left: in the absence of Wnt ligand, β-catenin levels in a cell are efficiently regulated by the β-catenin destruction complex, containing APC, Axin, CKI and GSK3. Transcription of β-catenin/TCF target genes is repressed by the presence of Groucho co-repressors. Right: Wnt ligand binding to the Frizzled receptor destabilizes the β-catenin degradation complex, allowing transportation to the nucleus. Once entered the nucleus, β-catenin recruits Legless (Lgs) and Pygopus (Pygo) and activates target genes. Ub = ubiquitin

and entry into the nucleus (Yanagawa et al., 1995). Within the nucleus β-catenin can bind to lymphoid enhancer factor (LEF)/T cell factor (TCF) family of transcription factors, and induce transcription of Wnt target genes (Behrens et al., 1996; Brunner et al., 1997; Molenaar et al., 1996; Riese et al., 1997; van de Wetering et al., 1997). Transcriptional activation of the β-catenin/TCF complex is mediated by the interaction of β-catenin with the histone acetyl transferase CBP, the chromatin remodeling SWI/SNF complex and Legless (Lgs) bound to Pygopus (Pyg) (Barker et al., 2001; Belenkaya et al., 2002; Hecht et al., 2000; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). In the absence of β-catenin, TCFs repress transcription by interaction with the corepressors CtBP and groucho bound to histone deacetylase (HDAC) (Brannon et al., 1999; Cavallo et al., 1998; Levanon et al., 1998; Roose et al., 1998).

The FOXO subfamily of transcription factors is critically involved in the regulation of apoptosis, proliferation and the control of oxidative stress (reviewed in Burgering and Medema, 2003). FOXOs are negatively regulated by the phosphoinositide-3 kinase (PI3K)/protein kinase B (PKB) pathway. Activation of PI3K/PKB will induce phosphorylation and nuclear exclusion of FOXO, thereby inhibiting FOXO transcriptional activity. In keeping with this regulation of FOXO by PI3K and Ras signalling, ligand independent activation of FOXO causes a cell cycle arrest in G_1, both in cells transformed with oncogenic Ras and cells with a deletion/mutation of PTEN (Medema et al., 2000). Interestingly, also colon carcinoma cells transformed by activated β-catenin/TCF signalling due to an APC truncation are arrested in G_1 by

activation of FOXO (Kops et al., 2002a), suggesting a link between β-catenin/TCF and FOXO signalling.
Recently, we identified a functional interaction between β-catenin and members of the FOXO subfamily of transcription factors (Essers *et al*, submitted, Chapter 2). β-catenin directly binds to FOXO and this binding leads to enhanced FOXO transcriptional activity. The binding of β-catenin to FOXO is increased under conditions of oxidative stress, and genetic analysis in *C. elegans* demonstrated that the interaction between FOXO and β-catenin is evolutionary conserved. Thus, these data reveal a novel and evolutionary conserved function for β-catenin, independent of TCF.
Here, we investigated the consequences of the binding between β-catenin and FOXO for Wnt signalling via the β-catenin/TCF complex. We show that activation of FOXO leads to inhibition of the transcriptional activity of the β-catenin/TCF complex. Binding of FOXO to β-catenin competes with binding of β-catenin to TCF. Competition only results in efficient inhibition of TCF signalling following nuclear exit of FOXO. Within the cytosol FOXO expression results in increased β-catenin levels and apparently binding to FOXO is thus sufficient to protect β-catenin from entering the destruction complex. Cellular oxidative stress increases binding between FOXO and β-catenin (Essers *et al*, submitted, Chapter 2), consequently the inhibitory effect of FOXO on β-catenin/TCF activity is further enhanced under conditions of oxidative stress. Taken together, the data start to outline a novel mechanism of cross talk between Wnt and PI3K signalling whereby β-catenin acts as a pivot between essential downstream elements of these signalling pathways, TCF and FOXO respectively.

Material and Methods

Cell culture, transfection, and infection
DL23 cells, DLD1 cells expressing a conditionally active FOXO3a.A3-ER fusion, were created as described (Kops et al., 2002a). DLD1 human colon carcinoma cells, DL23 cells, and LS174T human colon carcinoma cells were maintained in RPMI-1640 supplemented with L-glutamine, penicillin/streptomycin and 10% FCS. DL23 cells were treated with 500 nM of 4OHT for 8, 16 or 24h to activate the fusion protein. A14 cells (mouse NIH3T3 cells overexpressing the human insulin receptor) (Burgering and Coffer, 1995), HEK293T cells, and phoenix cells expressing the amphotrophe receptor were maintained in Dulbecco's Modified Eagle Medium supplemented with L-glutamine, penicillin/streptomycin and 10% FCS. A14 cells were transiently transfected using the calcium phosphate method. HEK293T, DLD1, and DL23 cells were transiently transfected using FuGENE6 reagent according to the manufacturer (Roche). Total amounts of transfected DNA were equalized using pBluescript KSII+.
LS174T cells were infected with pBabe-puro or pBabe-FOXO3a.A3 virus. For virus production Phoenix cells were transfected using superfect transfection reagent (3 mg/ml) with pBabe-puro or pBabe-FOXO3a.A3. 2 days after transfection medium of the transfected Phoenix cells was harvested. LS174T cells were infected by adding the medium of the phoenix cells together with 6 µg/ml hexadimethrine bromide. Infection was repeated 6h after the first round. The day after infection cells were seeded in selection medium containing 2 µg/ml puromycin.

Plasmids
The following constructs have been described before: pMT2-HA-FOXO4 (Kops et al., 1999); pMT2-HA-FOXO4-T447/451/454A (T1) (De Ruiter et al., 2001); pMT2-HA-FOXO4ΔDB; pMT2-HA-FOXO4-DB; pMT2-HA-FOXO4-ΔNLS (Δ198-216) (Brownawell et al., 2001); pCMV.p16^{INK4A}; pCMV.p21^{WAF1}; 6xDBE luciferase (Furuyama et al., 2000); and pSODLUC-3340 (Kim et al., 1999); pFOPGLOW-luc; pTOPGLOW (Vermeulen et al., 1996). pRL-Tk (Tk renilla luciferase) was purchased from Promega. pMT2-HA-FOXO4-ΔNES (Δ294-320) was constructed using PCR-based mutagenesis. Flag-β-catenin and flag-β-catenin.Δ45 were a gift from M. van de Wetering. GST-FOXO4-DB was purified from

bacteria using a standard GST-fusion-protein-purification protocol.

Antibodies
Monoclonal 12CA5 and 9E10 antibodies were produced using hybridoma cell lines. Monoclonal antibody β-catenin was obtained from Transduction Laboratories. Monoclonal flag antibody was obtained from Sigma. Polyclonal HA antibody was obtained from Santa Cruz.

Immunoprecipitation and western blots
Non-confluent cells were lysed in RIPA buffer (50 mM Tris pH 7.5, 1% Triton X-100, 0.5% deoxycholate, 10 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 mM sodium vanadate, 1 µg/ml leupeptin and 0.1 µg/ml aprotinin), and lysates were cleared for 10 minutes at 14000rpm at 4°C. Lysates were incubated for 2 hours at 4°C with either 1 µl 12CA5 or 9E10 antibody, or 7.5 µl β-catenin antibody and 50 µl pre-washed protein-A beads. The immunoprecipitations were washed four times with RIPA buffer, cleared for all liquid, and 25 µl of 1x laemmli sample buffer was added. Samples were subjected to SDS-PAGE and transferred to PVDF membrane (PerkinElmer). Western blot analysis was performed under standard conditions and using the indicated antibodies.

Luciferase reporter assays
DLD1 and DL23 cells were transiently transfected with either a reporter construct bearing multiple copies of an optimal TCF-binding site (pTOPglow) or a reporter construct bearing multiple copies of a mutant form of the optimal TCF binding site (pFOPglow) (Vermeulen et al., 1996). DLD1 cells were cotransfected with HA-FOXO4, HA-FOXO4 mutants or a control plasmid. DL23 and DLD1 cells were treated with 500 nM 4OHT for indicated times. Luciferase counts were normalized using Tk-renilla-luciferase. Cells were washed twice with PBS, lysed in passive lysis buffer (PLB) and luciferase activity was analyzed using a luminometer and a dual-luciferase assay kit according to the manufacturer (Promega). The fold induction of luciferase activity on the TOPglow construct was divided by the fold induction on the Fopglow construct, and this TOP/FOP ratio was plotted.

S100/P100 fractionation
Cell fractionation for analysis of the cytosolic pool of β-catenin was essentially done as previously described (Ding et al., 2000). Cells were harvested in Tris buffer (20 mM Tris pH 7.5, 50 mM NaF, and 1 mM EDTA), put on ice for 20 minutes, and homogenized through a 23 G 1 1/4 Microlance syringe. Nuclear and membrane components were separated by a sequential two times centrifugation step at 14000 rpm for 10 minutes at 4°C. The supernatant was collected as the soluble fraction (S100) and 5x sample buffer was added. The pellet fraction was taken up in 1x sample buffer (P100).

Electrophoretic mobility shift assay (EMSA)
Bandshift analysis of GST-FOXO4-DB was done as described (Kops et al., 1999). In summary, 5 µg of GST-FOXO4-DB was incubated with 1-2 ng of ^{32}P-labelled oligonucleotide probe in the presence of 2 µg of sonicated salmon sperm DNA and 200 ng of a comparable unlabeled double-stranded oligonucleotide in a total volume of 20 µl at 30 °C for 15 min. The reactions were electrophoresed on 4% polyacrylamide gels in 0.33 x Tris-buffered EDTA at 4 °C. The gels were then dried and exposed to x-ray films.

Immunofluorescence
DLD1 and A14 cells were cultured on coverslips, transfected with 1 mg of pMT2-HA-FOXO4 and/or 1 mg of flag-β-catenin, and fixed in 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS), and non-specific binding was blocked with 0.5% bovine serum albumin (BSA) in PBS for 45 minutes. Incubation with the HA polyclonal antibody was for 1h, followed by 1h of incubation with anti-rabbit-FITC and/or anti-mouse-TRITC second antibody. Coverslips were washed and mounted on glass slides using Immuno-Mount (Shandon, Pittsburgh, Pa).

Real time PCR analysis
The expression of endogenous TCF target genes in DLD1, DL23, and LS174T cells was determined by reverse transcription of total RNA followed by real time PCR analysis. Total RNA was isolated from DLD1, DL23, and LS174T cells using the RNAZol procedure (TELTEST, Inc). Using the AMV reverse transcriptase (Promega) single-stranded cDNA was synthesised from the total RNA. Real time PCR was performed on an ABI cycler using Sybr green (ABI) with the following primer sets;

Pitx2 (forward):	ACGCGAAGAAATCGCTGTG
Pitx2 (reverse):	CGACGATTCTTGAACCAAACC
Ephrin B2 (forward):	TGTCCAGACAAGAGCCATG
Ephrin B2 (reverse):	TTTATTCCTGGTTGATCCAGCAG
Cyclin D1 (forward):	GCCGAGAAGCTGTGCATCTAC
Cyclin D1 (reverse):	TCCACTTGAGCTTGTTCACCAG

p130 (forward): CTCAGGAATGCACCAAGTGAG
p130 (reverse): GCAATAGCCTGGGTTGGATC
Primers for Tspan5 were a gift from N. Barker.

Results

To investigate how the binding between β-catenin and FOXO would affect the function of the β-catenin/TCF complex as transcriptional activator, we first analyzed the effect of FOXO on β-catenin/TCF-dependent transcription. Therefore we used DL23 cells, which are DLD1 colon carcinoma cells expressing a conditionally active HA-FOXO3a.A3-ER fusion (Kops et al., 2002b). Due to a mutation in APC these cells contain high levels of stabilized β-catenin and thus display high β-catenin/TCF transcriptional activity. Activation of FOXO3a in these cells, induced by 4OHT treatment, resulted in inhibition of TCF-dependent transcription as measured by the TOP/FOP reporter assay (Vermeulen et al., 1996) (figure 2A). Activation of the TOPglow reporter, which contains multiple copies of an optimal TCF-binding site, was greatly reduced by activated FOXO, whereas FOXO had no effect on the FOPglow reporter, which contains multiple copies of a mutant form of the TCF binding site (Vermeulen et al., 1996). Also, expression of FOXO4 in DLD1 cells strongly inhibited TCF transcriptional activity (figure 3A). Thus, activation of FOXO inhibits the activity of the TCF transcription factor.

To investigate whether this inhibition is actually the consequence of the binding of FOXO to β-catenin, or whether FOXO could for example compete with TCF for binding to the TCF DNA binding sites present in the TOPglow reporter, we analysed in vitro binding of FOXO to known TCF DNA binding elements. Whereas *in vitro* the DNA binding domain of FOXO4 fused to GST (GST-FOXO4-DB) did bind to the IRE (T(G/A)TTT motif-containing insulin-response element) present within the IGFBP-1 promoter, it failed to bind to the TCF binding sites present within the Ultrabithorax (Ubx) homeotic gene promoter (Riese et al., 1997) (figure 2B), indicating that competition for DNA binding is unlikely. Furthermore, *in vivo* a FOXO4 mutant lacking the DNA binding domain was still able to inhibit β-catenin/TCF transcriptional activity (figure 3A). Thus, inhibition of TCF signalling by FOXOs is not due to direct competition between FOXO and TCF for DNA binding on the TCF consensus binding sites.

Activation of FOXO in DLD1 cells causes a cell cycle arrest in G_1 (Kops et al., 2002a). To exclude that the inhibition of TCF signalling by FOXOs in these cells was not secondary to the ability of FOXOs to induce a cell cycle arrest, we performed the TOP/FOP reporter assay in the presence of the cell cycle inhibitors $p16^{INK4}$ and $p21^{WAF1}$. Expression of these cell cycle inhibitors in DL23 and DLD1 cells induced a G1 arrest (data not shown), and this did result in a partial reduction of TCF dependent transcription (figure 2D), suggesting that indeed a cell cycle arrest in G_1 in these cells may contribute to the reduced β-catenin/TCF activity. More importantly, TCF activity was still further reduced upon activation of FOXO3a in the DL23 cells (figure 2D), indicating that although a cell cycle arrest may contribute to the inhibition of TCF signalling by FOXO this inhibition cannot account fully for the effect of FOXO and that FOXO-mediated inhibition is not solely secondary to the ability of FOXOs to

induce a cell cycle arrest in these cells.
To establish more directly whether binding between FOXO and β-catenin could compete with binding between TCF and β-catenin, we next analyzed binding between TCF and β-catenin in DL23 cells. Activation of FOXO3a in DL23 cells indeed reduced the binding between β-catenin and TCF4 (figure 2C). 4OHT treatment of the control DLD1 cells did not affect this binding. Thus we conclude that the inhibition of TCF signalling by FOXO reflects the ability of FOXO to compete with TCF for the binding to β-catenin.
Next, we tested whether expression of endogenous β-catenin/TCF target genes can be inhibited by activation of FOXOs. To this end we compared DL23 and DLD1 cells induced with 4OHT (figure 2E) or LS174T colon carcinoma cells infected with either a control virus or a constitutively active FOXO construct, FOXO3a.A3 (figure 2F). Q-PCRs for several target genes were performed on the isolated RNA from these cells. As described, FOXO3a activation in DL23 cells induced the expression of the known FOXO target gene p130 (figure 2E) (Kops et al., 2002b). On the other hand expression of cyclin D1 was inhibited (figure 2E). Cyclin D1 has been described as a target gene for the β-catenin/TCF complex (Tetsu and McCormick, 1999), and inhibition of cyclin D1 expression by FOXO has been described previously (Ramaswamy et al., 2002; Schmidt et al., 2002). Importantly, inhibition of cyclin D1 expression by FOXO occurs at the transcriptional level (Schmidt et al., 2002; Ramaswamy et al., 2002), although the cyclin D1 promoter does not contain a bona fide FOXO DNA binding element (Schmidt et al., 2002), and inhibition was shown to be independent of binding of FOXO to the cyclin D1 promoter region (Ramaswamy et al., 2002). This indicates the involvement of cofactors and thus would be consistent with FOXO inhibition of TCF signalling. However, gene expression regulation often summarizes the action of multiple signalling pathways and thus cyclin D1 regulation not necessarily reflects the status of only TCF and FOXO signalling, or the interaction between TCF and FOXO. Therefore, to exclude as much as possible interference of other signalling pathways we also analyzed other TCF target genes. Activation of FOXO3a by 4OHT treatment in DL23 cells also repressed the mRNA expression of the TCF target genes Pitx2 (Kioussi et al., 2002) and Ephrin B2 (Battle et al., 2002) (figure 2E). This result demonstrated that activation of FOXO3a inhibits the expression of multiple TCF target genes. To exclude that the repression of TCF target genes is specific to the DL23 cells and/or the 4OHT induction system, we also analyzed the effect of expression of active FOXO3a in LS174T colon carcinoma cells. Both DLD1 and LS174T cells are colon carcinoma cells with elevated levels of β-catenin, although the differentiation status of both cell lines is different. LS174T cells harbor mutated β-catenin with wild type APC and p53, whereas DLD1 cells have wild type β-catenin but mutant APC and p53. Again, we observed repression of Ephrin B2 mRNA levels by FOXO3a (figure 2F). Also the mRNA expression level of Tspan 5 (personal communication N. Barker), a β-catenin/TCF-dependent target gene specific to LS174T as compared to DLD-1 cells was repressed by FOXO3a (figure 2F). Thus FOXO3a can repress the transcriptional activation of multiple β-catenin/TCF-dependent target genes in cell types that differ with respect to the mechanism by which β-catenin is stabilized and therefore the effect of FOXO3a on endogenous gene regulation is again likely

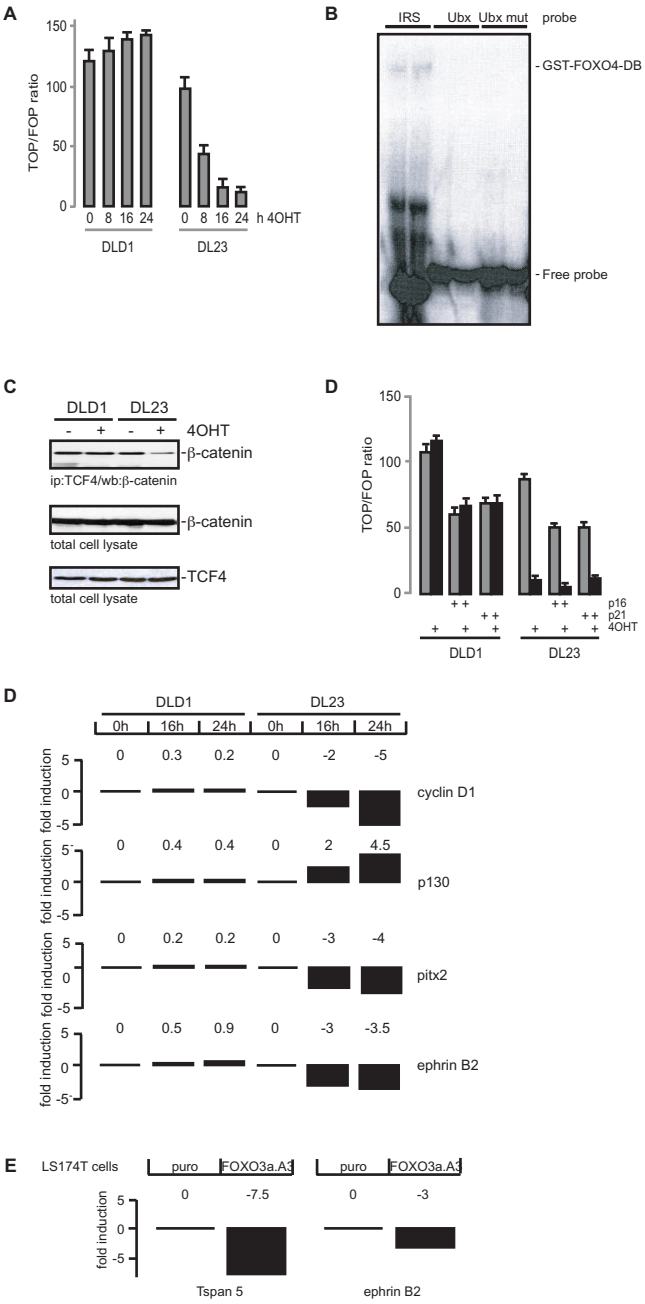
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Figure 2. The interaction of FOXO with β-catenin results in inhibition of β-catenin/TCF dependent transcription
A. Constitutively active FOXO3a inhibits transcription of the β-catenin/TCF complex. DL23 cells, DLD1 human colon carcinoma cells expressing a conditionally active HA-FOXO3a.A3-ER construct, and control DLD1 cells were transfected with pTOPglow, containing multiple copies of an optimal TCF binding site, or pFOPglow, containing multiple copies of a mutant form of a TCF binding site, together with Tk-renilla as an internal control. Cells were treated with 500 nM 4OHT for indicated times to activate the FOXO3a fusion construct and luciferase activity was measured. The ratio of TOP luciferase activity over FOP luciferase activity was plotted. Data presented are the average (+/- SED) of three independent experiments, performed in triplo. **B.** GST-FOXO4-DB was analyzed for the capacity to bind to TCF binding sites in the promoter of Ultrabithorax (Ubx). As a control the binding of GST-FOXO-DB to the insulin response elements (IRE) in the IGFBP-1 promoter was analyzed (left lanes). **C.** DLD1 or DL23 cells were treated with 500 nM 4OHT for 24h. TCF4 was immunoprecipitated and binding of β-catenin to TCF4 was analyzed on western blot. **D.** DLD1 and DL23 cells were transfected with pFOPglow or pTOPglow together with Tk-renilla. Cells were cotransfected with a control vector, $p16^{INK4}$ or $p21^{WAF1}$. 24h after 4OHT treatment luciferase activity was measured. Data presented are the average (+/- SED) of three independent experiments, performed in triplo. **E.** DLD1 and DL23 cells were treated with 500 nM for 24h. RNA was isolated and Q-PCRs were performed for p130, cyclinD1, ephrin B2 and pitx2. The fold induction is indicated. **F.** LS174T colon carcinoma cells were infected with a control virus or FOXO3a.A3. RNA was isolated and a Q-PCR for ephrin B2 and Tspan5 was performed. The fold induction is indicated.

to be at the level of β-catenin. Taken together, these data provide evidence that the interaction between β-catenin and FOXO can result in inhibition of β-catenin/TCF dependent transcription.

To further unravel the mechanism by which FOXO inhibits the β-catenin/TCF complex, we tested several FOXO mutants for their ability to inhibit TCF dependent transcription in DLD1 cells (figure 3A). Two mutants, FOXO4ΔNES and FOXO4-Thr447/451/454A (FOXO4-T1) displayed a loss of TCF repression, suggesting that these mutants no longer affect β-catenin/TCF activity. As mentioned, the ability of FOXO to inhibit TCF activity correlates with the ability of FOXO to bind β-catenin (figure 2), thus we hypothesised that these mutants are no longer capable of binding β-catenin. To test this we performed co-immunoprecipitation experiments with these and other FOXO mutants (figure 3B). Surprisingly, both FOXO4ΔNES and especially FOXO4-T1 still did bind to β-catenin and this binding was also enhanced by H_2O_2 treatment of cells, as reported previously (figure 3B) (Essers *et al*, submitted, Chapter 2). Thus, additional requirements are needed for FOXO to inhibit TCF activity besides the ability of FOXO to bind β-catenin.

Recently, it has been suggested that nuclear/cytoplasmic shuttling of APC is essential for the ability of APC to inhibit β-catenin/TCF signalling (Rosin-Arbesfeld et al., 2003; Rosin-Arbesfeld et al., 2000). Unlike TCF but similar to APC, FOXOs, are constantly shuttling between nucleus and cytoplasm. This suggests the possibility that the shuttling of FOXO from nucleus to cytosol combined with the binding of FOXO to β-catenin is needed for efficient inhibition of β-catenin/TCF signalling. To test this hypothesis we first determined the cellular localization of FOXOΔNES and FOXO4-T1, the two mutants that no longer inhibit β-catenin/TCF activity, but still bind β-catenin. Immunofluorescence staining in DLD1 cells shows that the steady state localization of both FOXO4ΔNES and FOXO4-T1 is exclusively nuclear (figure 3C). To further investigate the role of FOXO shuttling on β-catenin function we analyzed the effect of FOXO4 mutants on cytosolic levels of β-catenin by cellular fractionation (figure 3D). Indeed, FOXO4 can increase β-catenin levels in the S100 (soluble) fraction, indicating that FOXO can bind β-catenin and shuttle β-catenin out of the

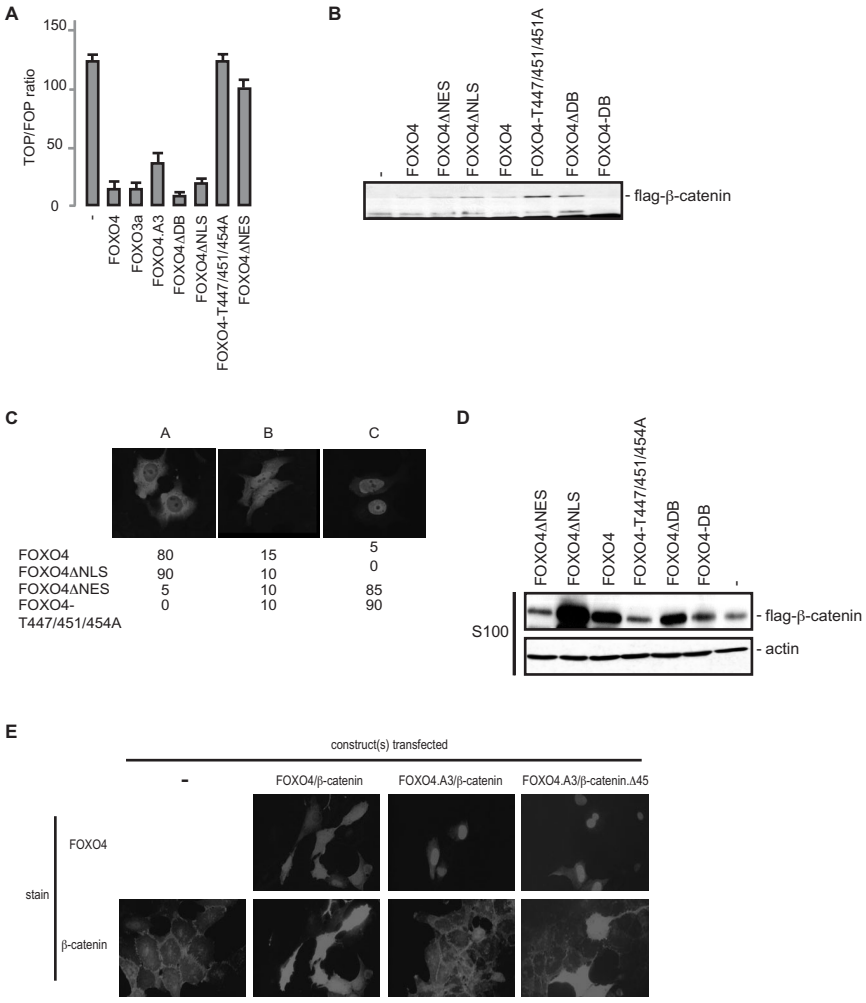

Figure 3. FOXO induced inhibition of β-catenin/TCF activity depends on the ability of FOXO to shuttle out of nucleus

A. DLD1 cells were transfected with pTOPglow or pFOPglow, together with Tk-renilla as an internal control. Cells were cotransfected with different FOXO4 mutant constructs and luciferase activity was measured. The ratio of TOP over FOP is plotted. Data presented are the average (+/- SED) of three independent experiments, performed in triplo. **B.** 293T cells were transfected with flag-β-catenin together with different HA-FOXO4 mutants. FOXO4 was immunoprecipitated using 12CA5 monoclonal antibody, and binding of β-catenin to FOXO4 was analyzed on blot. **C.** DLD1 cells were transfected with HA-FOXO4. Cells were fixed and stained for FOXO4. Photos A, B, and C are examples of the different FOXO staining observed in the cells. A = cytoplasmic staining, B = cytoplasmic and nuclear staining, C = nuclear staining. The % of cells with the different FOXO staining seen in the samples are indicated. **D.** A14 cells were transfected with flag-β-catenin and different HA-FOXO4 constructs. Cells were lysed and separated in a soluble (S100) and membrane (P100) fraction. β-catenin expression in the P100 fraction was equal in all lanes (data not shown). β-catenin expression in the S100 fraction is shown here. Expression of actin served as a loading control. **E.** A14 cells were transfected with either a control vector, HA-FOXO4, HA-FOXO4.A3, flag-β-catenin or flag-β-cateninΔ45, a stabilized mutant of β-catenin. Cells were fixed and stained for FOXO4 with rabbit-FITC and for β-catenin with mouse-TRITC.

nucleus. More importantly, the FOXO4ΔNES and FOXO4-T1 mutants did not increase β-catenin levels in the S100 fraction. Thus, these data suggest that the inability of these mutants to inhibit β-catenin/TCF activation relates to their apparent inability to shuttle β-catenin out of the nucleus. Finally, we analyzed β-catenin stability using immunofluorescence (figure 3E). FOXO4 co-transfection induces β-catenin stability, as indicated by strong expression of β-catenin, only in cells expressing FOXO4. This indicates that the effect of FOXO on β-catenin is cell autonomous and unlikely due to secreted factors. Furthermore, as in the cellular fractionation studies cells expressing a mutant of FOXO4 (FOXO4.A3) that is predominantly nuclear, showed a clearly reduced level of β-catenin expression. Furthermore, FOXO4.A3 expression did not affect the stability of a β-catenin mutant that is insensitive to degradation (β-catenin.Δ45), indicating again that the inability of FOXO4.A3 to stabilize β-catenin results from the inability to shuttle to the cytosol, rather than to interfer with β-catenin expression in general. Taken together, these data demonstrate that FOXO can inhibit β-catenin/TCF signalling through the ability of FOXO to bind β-catenin, shuttle it out

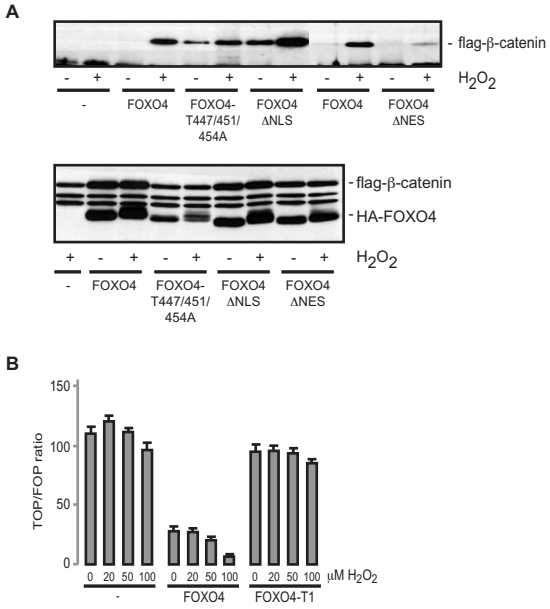

Figure 4. The inhibitory effect of FOXO on β-catenin function is enhanced under conditions of oxidative stress
A. 293T cells were transfected with flag-β-catenin together with different HA-FOXO4 constructs. Cells were treated with 100 μM H_2O_2 for 1h, where after FOXO4 was immunoprecipitated. Binding of flag-β-catenin to the FOXO4 mutants was analyzed on blot. **B.** DLD1 cells were transfected with pTOPglow, or pFOPglow, together with Tk-renilla as an internal control. Cells were cotransfected with FOXO4, or FOXO4-T1 and treated with indicated concentrations of H_2O_2 for 16h. Cells were lysed and luciferase activity was measured. Data presented are the average (+/- SED) of three independent experiments, performed in triplo.

of the nucleus, and keep it cytoplasmic.
We have previously shown that the interaction between β-catenin and FOXO is influenced by oxidative stress (Essers *et al*, submitted, Chapter 2). Treating cells with H_2O_2 to induce oxidative stress resulted in an increased binding between β-catenin and FOXO (figure 4A). To test whether this increased binding also resulted in an increase in the effect of FOXO on β-catenin/TCF activity we again performed a TOP/FOP luciferase assay wit DLD1 cells. Treatment of these cells with H_2O_2 did not affect basal TCF transcriptional activity. However, in the presence of FOXO4, inhibition of TCF activity by FOXO was further enhanced by H_2O_2 treatment (figure 4B). H_2O_2 treatment did not affect TCF activity in cells expressing FOXO4-T1 (figure 4B), indicating that the increased binding of this mutant to β-catenin after oxidative stress does not influence the function of the β-catenin/TCF complex, probably because of their inability to shuttle β-catenin out of the nucleus.

Discussion

Taken together, the data presented here suggest a model in which β-catenin forms a bridge between the Wnt signalling pathway and the insulin/PI3K/PKB/FOXO pathway. Here we show that FOXO inhibits TCF-dependent transcription by binding to β-catenin and shuttling β-catenin out of the nucleus. Cellular oxidative stress enhances β-catenin binding to FOXO and also enhances FOXO transcriptional activity. On the other hand cellular oxidative stress potently inhibits in a FOXO dependent manner TCF-dependent transcription. This suggests that especially under conditions of cellular oxidative stress it is essential for cells to enhance FOXO-dependent transcription and to repress and/or prevent TCF-dependent transcription.
The mechanism whereby nuclear-cytoplasmic shuttling of β-catenin occurs is still poorly understood. The primary sequence of β-catenin does not reveal the presence of either a NLS or NES sequence. Furthermore, shuttling of β-catenin occurs independent of importins and the Ran GTPase (Fagotto et al., 1998; Yokoya et al., 1999), although treatment of cells with the Crm1 inhibitor LMB induces nuclear accumulation of β-catenin (Henderson, 2000; Cong and Varmus, 2004). Other studies suggested that β-catenin can freely shuttle from the cytoplasm to the nucleus as β-catenin is structurally related to importins, and can therefore interact directly with the nuclear pore complex (Henderson and Fagotto, 2002). Consistent with this possibility that β-catenin acts as an importin, we observe that the FOXO4-T1 mutant displays highest binding to β-catenin and also displays considerable faster kinetics of nuclear entry compared to wild type FOXO4 (De Ruiter, 2002). Furthermore, oxidative stress increases binding between FOXO and β-catenin and, others and we have shown that oxidative stress enhances FOXO nuclear entry (Brunet et al., 2004, Essers *et al*, submitted, Chapter 4). Thus, this suggests the possibility that under conditions of cellular oxidative stress β-catenin may act as importin for FOXO. Components of the β-catenin destruction complex have also been shown to shuttle between cytoplasm and nucleus. By exporting β-catenin from the nucleus APC increases the cytoplasmic localization of β-catenin (Rosin-Arbesfeld et al., 2003; Rosin-Arbesfeld

et al., 2000). And it was shown that nuclear import and export of axin is also required for the axin induced shift of β-catenin to the cytoplasm (Cong and Varmus, 2004). Nuclear localization of β-catenin has also been shown to depend on the presence of the newly discovered proteins, Legless (Lgs) and Pygopus (Pygo) (Belenkaya et al., 2002; Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). Pygo is a protein, which is constitutively nuclear, whereas Lgs is nuclear only when Pygo is present (Townsley et al., 2004). Both proteins must be present for β-catenin to localize to the nucleus (Townsley et al., 2004). In the nucleus Pygo, Lgs, and β-catenin form a complex, which will bind TCF and induce transcriptional activity of this transcription factor (Townsley et al., 2004). How APC, axin, Lgs, Pygo and FOXO may act together to regulate the nuclear-cytoplasmic localization of β-catenin needs to be investigated further.

Our data establish a potential point of interaction between insulin/IGF1 signalling and Wnt signalling. FOXO can inhibit β-catenin dependent TCF signalling by shuttling β-catenin out of the nucleus. Insulin/IGF1 signalling causes relocalization of FOXO from the nucleus to the cytoplasm and would therefore be able to inhibit Wnt/TCF signalling. Indeed, it has been shown that insulin/IGF1 can inhibit Wnt signalling at the level of β-catenin (Desbois-Mouthon et al., 2001; Playford et al., 2000). Several other studies have explored the possibility that insulin/IGF1 would influence β-catenin by activation of PKB/Akt and inhibition of GSK3 (Fukumoto et al., 2001; Harwood, 2001). However, although these studies did indicate that insulin/IGF1 to some extend may regulate TCF activity, the involvement of PKB regulated GSK-3 activity appeared unlikely and this has led to the suggestion that different pools of GSK-3 may exist and that these pools are restricted to either Wnt or insulin signalling (Ding et al., 2000; Harwood, 2001). Here we now show that insulin/IGF1 may influence TCF activity via the regulation of FOXO. Our findings also indicate that through FOXO interaction β-catenin levels increase in the cytoplasmic S100 fraction, whereas actual TCF transcriptional activity is repressed. This indicates that stabilization of β-catenin per se, as measured by an increase in β-catenin present in the S100 (cytosolic) fraction cannot be taken as an indication of TCF activity in a cell.

Taken together, the data presented here provide a new level of interplay between insulin/IGF1 signalling and Wnt/TCF signalling, two important pathways in cell proliferation and carcinogenesis.

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Chapter 4

FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK

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EMBO J*, in press*

Forkhead transcription factors of the FOXO class are negatively regulated by PKB/c-Akt in response to insulin/IGF signalling, and involved in regulating cell cycle progression and cell death. Here we show that, in contrast to insulin signalling, low levels of oxidative stress generated by treatment with H_2O_2 induce the activation of FOXO4. Upon treatment of cells with H_2O_2, the small GTPase Ral is activated and this results in a JNK dependent phosphorylation of FOXO4 on Threonine 447 and Threonine 451. This Ral-mediated, JNK-dependent phosphorylation is involved in the nuclear translocation and transcriptional activation of FOXO4 after H_2O_2 treatment. In addition, we show that this signalling pathway is also employed by TNFα to activate FOXO4 transcriptional activity. FOXO members have been implicated in cellular protection against oxidative stress via the transcriptional regulation of MnSOD and catalase gene expression (Burgering and Medema, 2003). The results reported here, therefore outline a homeostasis mechanism for sustaining cellular ROS that is controlled by signalling pathways that can convey both negative (PI-3K/PKB) and positive (Ras/Ral) inputs.

Introduction

Reactive oxygen species (ROS) are oxygen free radicals that are highly reactive towards cellular constituents including protein, lipid and DNA. Formation of ROS can be caused by exogenous sources such as UV or ionising radiation or by endogenous sources such as normal aerobic metabolism or by pathological conditions such as ischemia. Also in normal cell signalling, ROS are generated by growth factor-induced activation of enzyme complexes such as NADH oxidase (reviewed in Finkel, 2000). Furthermore, cellular levels of ROS fluctuate throughout the cell cycle and in fact ROS are required for cell proliferation (Clopton and Saltman, 1995; Shackelford et al., 2001). Nevertheless, cells have developed numerous antioxidant systems to prevent excess generation of ROS as the highly reactive nature of ROS will easily result in ROS-induced adverse modifications of protein, lipid or DNA. Normally, ROS-induced modifications or damage is either repaired, as in the case of chromosomal DNA damage, or removed by degradation and subsequent resynthesis. In the case of excessive damage or ineffective repair, cell death (apoptosis) is triggered. Thus, cells require both a stringent homeostasis mechanism for ROS and efficient repair to tolerate levels of ROS required for normal cell function that will otherwise result in certain cell death.

Consistent with its role in normal growth factor-induced signalling, ROS generation by exogenous sources such as H_2O_2 treatment has been shown to trigger most of the signalling pathways downstream of growth factor receptors (reviewed in Finkel, 2000). For example, ROS has been shown to activate members of the JNK/p38 stress kinase family, MAPkinases, PI-3K signalling, NF-κB and many more. Activation by ROS of some of these signalling cascades has been implicated primarily in the induction of apoptosis (JNK/p38), whereas others have been implicated in cell survival (PI-3K).

Protein kinase B (PKB/c-Akt) mediates many of the anti-apoptotic effects of PI-3K signalling. A large number of PKB substrates have been implicated in the regulation of cellular survival (reviewed in Lawlor and Alessi, 2001). However, little is known as to how PI-3K/PKB signalling may regulate the cellular level of ROS. Recently, others and we have shown that the PKB-regulated Forkhead transcription factor FOXO3a can reduce the level of cellular oxidative stress by directly increasing mRNA and protein levels of manganese superoxide dismutase (MnSOD) and catalase (reviewed in Burgering and Medema, 2003). PKB-mediated phosphorylation of FOXO results in translocation of FOXO from the nucleus to the cytosol. Consequently, PKB activation decreases MnSOD and catalase levels and this is likely to contribute to an increase in cellular ROS. As cell cycle progression requires increased ROS level, this is in agreement with the role of PI-3K signalling in stimulating cell proliferation.
Because of the inverse relationship between PI-3K/PKB signalling and FOXO activity, we were interested whether an increase in ROS could regulate FOXO activity and oppose the effect of PI-3K/PKB signalling. Here, we show that oxidative stress induced by treatment of cells with H_2O_2 results in the activation of the small GTPase Ral. Activation of Ral results in the phosphorylation and activation of JNK and JNK-mediated phosphorylation of FOXO4 on Thr447 and Thr451. Phosphorylation of these residues is critical to FOXO4 transcriptional activity. Thus, H_2O_2 can induce FOXO4 transcriptional activity and this is further confirmed by the observation that H_2O_2 treatment results in translocation of FOXO4 from the cytosol to the nucleus. In addition, we show that TNFα, a ligand known to increase cellular H_2O_2 levels, also activates FOXO4 transcriptional activity and that this involves cellular ROS, Ral and JNK. These results indicate that FOXOs can function in a negative feedback loop to control the cellular level of oxidative stress in a cell and therefore these results start to outline a novel homeostasis mechanism of ROS control.

Materials and methods

Cell culture, transfection, and treatment
A14 cells (mouse NIH3T3 cells overexpressing the human insulin receptor (Burgering and Coffer, 1995), wt MEFs (wild type mouse embryo fibroblasts), JNK9 MEFs (JNK1-/-, JNK2-/- MEFs) (Sabapathy et al., 1999), HEK293T cells, and mouse C2C12 myoblast cells were maintained in Dulbecco's Modified Eagle Medium supplemented with L-glutamine, penicillin/streptomycin and 10% FCS. DLD1 human colon carcinoma cells were maintained in RPMI 1640 medium supplemented with L-glutamine, penicillin/streptomycin and 10% FCS. For luciferase assays, cells were cultured in 6-wells plates. For the GST-RalBD pull down assay cells were cultured in 90 mm dishes. For all other experiments, cells were cultured in 60 mm dishes. HEK293T cells, wt MEFs, JNK9 MEFs, and DLD1 cells were transiently transfected using FuGENE6 reagent according to the manufacturer (Roche). A14 cells were transfected using the calcium phosphate method. Total amounts of transfected DNA were equalized using pBluescript KSII+. Anisomycin, EGF, insulin, TNFα and H_2O_2 were added at 10 µg/ml, 20 ng/ml, 1 µg/ml, 20 ng/ml and 20-500 µM respectively as indicated. For luciferase assays, H_2O_2 was added at 5-20 µM overnight.

Plasmids and recombinant proteins
The following plasmids have been described before: pMT2-HA-FOXO4 (Kops et al., 1999), pMT2-HA-FOXO4-T447A, pMT2-HA-FOXO4-T451A, pMT2-HA-FOXO4-T447/451/454A, pMT2-HA-FOXO4-T447D, pMT2-HA-FOXO4-T451D, (De Ruiter et al., 2001), pMT2-HA-RalN28, pMT2-HA-Rlf-CAAX, pMT2-HA-Rlf-CAAXΔGEF, pSVE-RASV12 (Wolthuis et al., 1997), pMT2-HA-RalGEF2 (de Bruyn et al., 2000), pMT2-HA-JNK1, pMT2-HA-JNK3 (de Groot et al., 1997), 6xDBE luciferase (Furuyama et

al., 2000), and pSODLUC-3340 (Kim et al., 1999). pRL-Tk (Tk renilla luciferase) was purchased from Promega.
pMT2-HA-FOXO4-T451E and pMT2-HA-FOXO4-T447E were generated using mutagenesis PCR. 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.
GST-FOXO4(C) and GST-FOXO4-T447/451A(C) (Kops et al., 1999), GST-Jun (de Ruiter et al., 2000), and GST-RalBD (Wolthuis et al., 1998) have been described before.

Antibodies
Monoclonal 12CA5 and 9E10 antibodies were produced using hybridoma cell lines. Monoclonal antibody against Ral was obtained from Transduction laboratories. Phosphospecific polyclonal antibodies recognizing PKB-Ser473, FOXO4-Ser193, and Jun-Ser73 were obtained from Cell Signalling. Phosphospecific polyclonal antibody against FOXO3a-Thr32 was obtained from Upstate Technology. Polyclonal HA antibody and polyclonal 14-3-3β (K19) antibody used for immunofluorescence was obtained from Santa Cruz.
The phospho-specific antibody against the phosphorylated Thr447 was made by using the peptide KALGTpPVLTPPTEAC to immunize rabbits (Sigma). The phospho-specific antibody against the phosphorylated Thr451 was generously provided by Cell Signalling.

Immunoprecipitations and Western blots
Cells were lysed in RIPA buffer (50 mM Tris-HCl [pH 7.5], 0.1% NP-40, 0.5% deoxycholate, 10 mM EDTA, 150 mM NaCl, 50 mM NaF, 1 μM leupeptin, and 0.1 μM aprotinin). Lysates were cleared for 10 minutes at 14000 rpm at 4°C, and incubated for 2 hours at 4°C with either 1 μl 12CA5 (HA) or 1 μl 9E10 (myc) and 50 μl pre-washed protein A beads. Immunoprecipitations were washed four times in lysis buffer, cleared of all supernatant, and 25 μl of 1x Laemmli sample buffer was added. Samples were subjected to SDS-PAGE and transferred to PVDF (Perkin Elmer). Western blot analysis was performed under standard conditions, using indicated antibodies. For phosphospecific antibodies, membranes were blocked in 1% BSA and washed in TBS-Tween.

In vitro kinase assay
GST-FOXO4 and GST-FOXO4-T447/451A were precoupled to glutathione beads and washed twice with reaction buffer (50 mM HEPES pH 7.4, 15 mM $MgCl_2$, 200 μM sodium vanadate). For kinase reactions, the beads were incubated in kinase buffer (containing 100 μM ATP or 5 μM ATP and 10 μCi of γ-^{32}P-ATP per reaction) at 30 °C for 30 minutes, resuspended in sample buffer, and analysed by SDS-PAGE followed by autoradiography.

[^{32}P] orthophosphate labeling
In vivo labeling of A14 cells transfected with HA-FOXO4 was performed as described previously (Burgering and Coffer, 1995).

Determination of Ral-GTP
Ral-GTP levels were determined using a Gst-RalBD pull down assay as described previously (Wolthuis et al., 1998).

Immunofluorescence
DLD1 cells were cultured on coverslips, transfected with 1 μg of pMT2-HA-FOXO4, and fixed in 4% paraformaldehyde. Cells were permeabilized with 0.1% Triton X-100 in PBS, and non-specific binding was blocked with 0.5% BSA in PBS for 45 min. One hour incubation with the HA polyclonal antibody was followed by 1h of incubation with anti-rabbit-CY3 secondary antibody. Coverslips were washed and mounted on glass slides using Immuno-Mount (Shandon, Pittsburgh, Pa.).

Luciferase assays
Cells were transfected with a reporter construct bearing six canonical FOXO binding sites (6xDBE-luciferase) or bearing a –3340 basepair promoter fragment of the human SOD2 gene for MnSOD (pSODLUC-3340). Cells were cotransfected with indicated constructs. Transfections were performed in triplicate. Luciferase counts were normalised using Tk-Renilla-luciferase. After overnight treatment with H_2O_2 or TNFα, or forty hours post transfection, cells were lysed in passive lysis buffer (PLB) and luciferase activity was analysed using a luminometer and dual-luciferase assay kit according to the manufacturer (Promega).

Measurement of mitochondrial membrane integrity
Cells were glucose deprived for 48 h by adding DMEM medium lacking glucose and pyruvate, but supplemented with 8% FCS. Note that owing to the small amount of glucose in FCS, glucose amounts are estimated to be about 10–20-fold less in this medium than under normal culture conditions. Cells

were digested with trypsin and incubated with 10 µg/ml rhodamine-1,2,3 (Scaduto and Grotyohann, 1999) at 37 °C for 30 min. After two washes with PBS mitochondrial membrane staining was measured by standard flow cytometry.

Results

To investigate whether FOXO could function in a feedback mechanism to control cellular redox, we first analysed the possibility that, similar to insulin signalling, cellular oxidative stress generated by H_2O_2 treatment of cells could induce FOXO phosphorylation. Cells transiently expressing HA-FOXO4 were labelled with ^{32}P-orthophosphate and treated with various concentrations of H_2O_2. At the lowest concentration tested (20µM), H_2O_2 treatment induced phosphorylation of FOXO4 (figure 1A). Phosphorylation by H_2O_2 did not correlate with H_2O_2-induced PKB activation, as increased PKB phosphorylation in these cells was only observed at the highest concentration of H_2O_2 (200 µM; figure 1A). By mutational analysis, we have previously defined two residues within FOXO4, Threonine 447 (Thr447) and Threonine 451 (Thr451), which can be phosphorylated independently of PKB activation (De Ruiter et al., 2001) and recently, we confirmed phosphorylation of these residues on FOXO4 by Mass spectrometry (data not shown). To study regulation of Thr447/451 phosphorylation, we obtained phospho-specific antibodies against both phosphorylated Thr451 (Thr451P) and Thr447 (Thr447P). H_2O_2 treatment induced both Thr451 and Thr447 phosphorylation (figure 1B). The Thr451P antibody did not recognize HA-FOXO4-Thr451A and the Thr447P antibody did not recognize HA-FOXO4-Thr447A isolated both from untreated and H_2O_2 treated cells, indicating

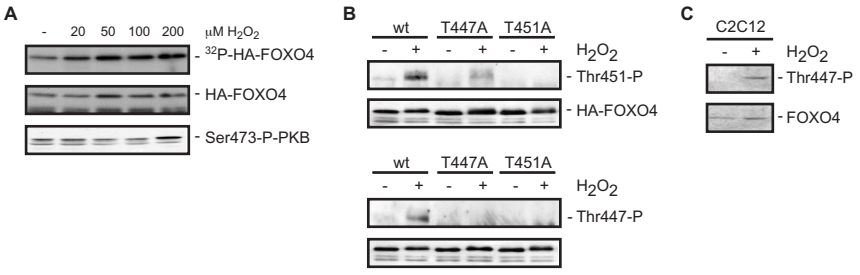

Figure 1. H_2O_2 induced phosphorylation of FOXO4 on Thr447 and Thr451
A. A14 cells, transfected with HA-FOXO4, were labelled with ^{32}P-orthophosphate for 3 hr and left untreated or treated for 60 minutes with indicated H_2O_2 concentrations. Cells were lysed and HA-FOXO4 was immunoprecipitated. Following exposure to film, the blot was probed with 12CA5 monoclonal antibody to ensure equal expression of HA-FOXO4 in each lane. H_2O_2 treatment induced a 2.5 fold increase in phosphorylation of FOXO4. In parallel, samples were analysed on western blot for Ser473 phosphorylation of PKB (lower panel). **B**. 293T cells, transfected with HA-FOXO4, HA-FOXO4-Thr447A, or HA-FOXO4-Thr451A, were left untreated or treated with 100 µM H_2O_2 for 60 minutes. HA-FOXO4s were immunoprecipitated and analysed on western blot for Thr447 or Thr451 phosphorylation. Same results were obtained with 200 and 400 µM H_2O_2. **C**. Mouse C2C12 cells were left untreated or treated with 100 µM of H_2O_2 for 60 minutes. Endogenous FOXO4 was analysed on blot for Thr447 phosphorylation. Same results were obtained using 200 or 400 µM H_2O_2.

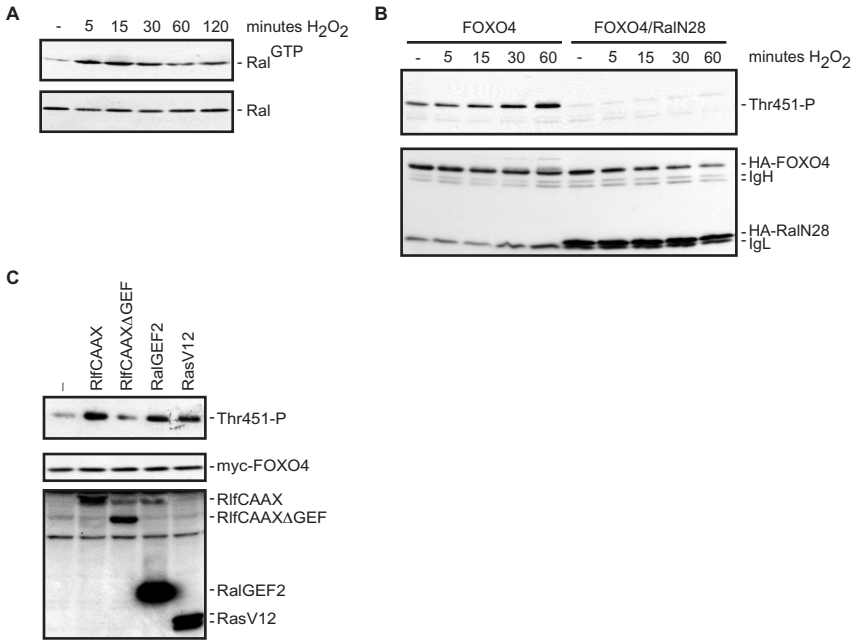

Figure 2. H_2O_2 induces Ral activation and Ral activation is necessary and sufficient for H_2O_2-induced Thr451 phosphorylation
A. A14 cells were treated with 100 μM of H_2O_2 for indicated time, and Ral GTP levels were analysed on western blot using a Ral pull down assay (upper panel). Lower panel shows endogenous Ral protein levels. Same results were obtained using 200 and 400 μM H_2O_2. **B**. 293T cells, transfected with HA-FOXO4 and HA-RalN28 or a control construct, were treated with 100 μM H_2O_2 for indicated time and Thr451 phosphorylation was analysed on western blot. Same results were obtained using 200 or 400 μM H_2O_2. **C**. 293T cells were transfected with myc-FOXO4 together with the indicated constructs. Thr451 phosphorylation was analysed.

their specificity. The Thr447P antibody also did not recognize HA-FOXO4-Thr451A, suggesting that Thr451 is an essential part of the epitope for the Thr447P antibody. Moreover, we analysed phosphorylation of endogenous FOXO4. Mouse C2C12 cells expressing endogenous FOXO4 were treated with H_2O_2 and displayed increased Thr447 phosphorylation (figure 1C). As Thr451 is not conserved between human and mouse FOXO4, we could not test endogenous Thr451 phosphorylation in these cells. These results show that *in vivo* FOXO4 becomes phosphorylated at Thr447 and Thr451 following treatment of cells with H_2O_2. The Thr451P antibody is of better quality compared to the Thr447P antibody. Therefore, the results with the Thr451P antibody are shown in the following figures, but similar results were obtained using the Thr447P antibody.
In insulin signalling, phosphorylation of Thr447 and Thr451 occurs in a Ral-dependent manner (De Ruiter et al., 2001). Thus, we analysed whether H_2O_2-induced phosphorylation of Thr447 and Thr451 was dependent on activation of the small GTPase Ral. Therefore, we first analysed whether H_2O_2 could induce the activation

of Ral. Cells treated for various periods of time with H_2O_2 were lysed and the level of active Ral (Ral-GTP) was determined by a pull down assay (Wolthuis et al., 1998). H_2O_2 treatment induced a rapid and time dependent increase in RalGTP levels (figure 2A). To determine whether this H_2O_2-induced Ral activation mediated the phosphorylation of Thr451, we expressed HA-FOXO4 in the presence or absence of dominant-negative Ral (RalN28). Expression of dominant-negative Ral completely blocked phosphorylation of Thr451 (figure 2B), indicating the involvement of Ral in H_2O_2-induced phosphorylation. Subsequently, we examined whether activation of endogenous Ral could mediate Thr451 phosphorylation. Activation of endogenous Ral, both through the expression of active Ras (RasV12) and through the expression of active Ral guanine nucleotide exchange factors (RlfCAAX and RalGEF2), but not the expression of a control in which the catalytic domain was mutated (RlfCAAXΔGEF), resulted in increased Thr451 phosphorylation (figure 2C). Taken together, these data demonstrate that H_2O_2 treatment of cells results in the activation of the small GTPase Ral, which is necessary and sufficient to induce phosphorylation of Thr451 on FOXO4.

To investigate which kinase could mediate Ral-induced phosphorylation of Thr451, we treated cells with a variety of kinase inhibitors prior to H_2O_2 treatment. The MEK inhibitor PD98059, the PI3K inhibitor LY294002 or the p38 inhibitor SB203580 could not inhibit H_2O_2-induced phosphorylation of Thr451 and Thr447 on FOXO4 (data not shown). These results indicate that Thr451 phosphorylation is not mediated by PI3K, MAPK or p38. To study a potential involvement of JNK, we used immortalized MEFs derived from JNK1,2-/- mice, since there is no specific JNK inhibitor available. As these MEFs also do not express JNK3, they lack any JNK activity (Sabapathy et al., 1999). H_2O_2 treatment of JNK1,2-/- MEFs did not induce phosphorylation of Thr451, whereas H_2O_2 treatment of control MEFs (wt MEFs) did, strongly indicating that *in vivo* JNK mediates Thr451 phosphorylation (figure 3A). To further confirm this, we rescued JNK expression in JNK1,2-/- MEF cells by co-expression of either JNK1 or JNK3. This restored H_2O_2-induced JNK activity and the induction of T451 phosphorylation (figure 3B). JNK is often observed bound to its potential substrates. We therefore analysed the binding between JNK and FOXO4. Treatment of cells with increasing concentration of H_2O_2 induced the binding of JNK1 (data not shown) and JNK3 to FOXO4 (figure 3C). Consistent with the *in vivo* data, active JNK1, but not p38α could efficiently phosphorylate Thr451/447 of FOXO4 *in vitro* (figure 3D). Thus, we conclude that JNK phosphorylates FOXO4 *in vitro* and *in vivo* at Thr451 and that this phosphorylation can be induced by H_2O_2 treatment.

Our results thus far suggest a role for Ral in mediating H_2O_2-induced JNK activation *in vivo*. To test this directly, we expressed HA-JNK1 (not shown) or HA-JNK3 either in the absence or the presence of dominant-negative RalN28 and stimulated JNK activity by H_2O_2 treatment. Dominant-negative Ral inhibited, especially at later time points, the induction of JNK phosphorylation and activation by H_2O_2 (figure 3E). Again, we tested whether activation of endogenous Ral would be sufficient to increase JNK activity. Indeed, as was shown for Thr451 phosphorylation, co-expression of active RalGEFs but not that of the inactive GEF increased JNK activity (figure 3F). To analyse the specificity of the involvement of Ral in H_2O_2 induced JNK activation,

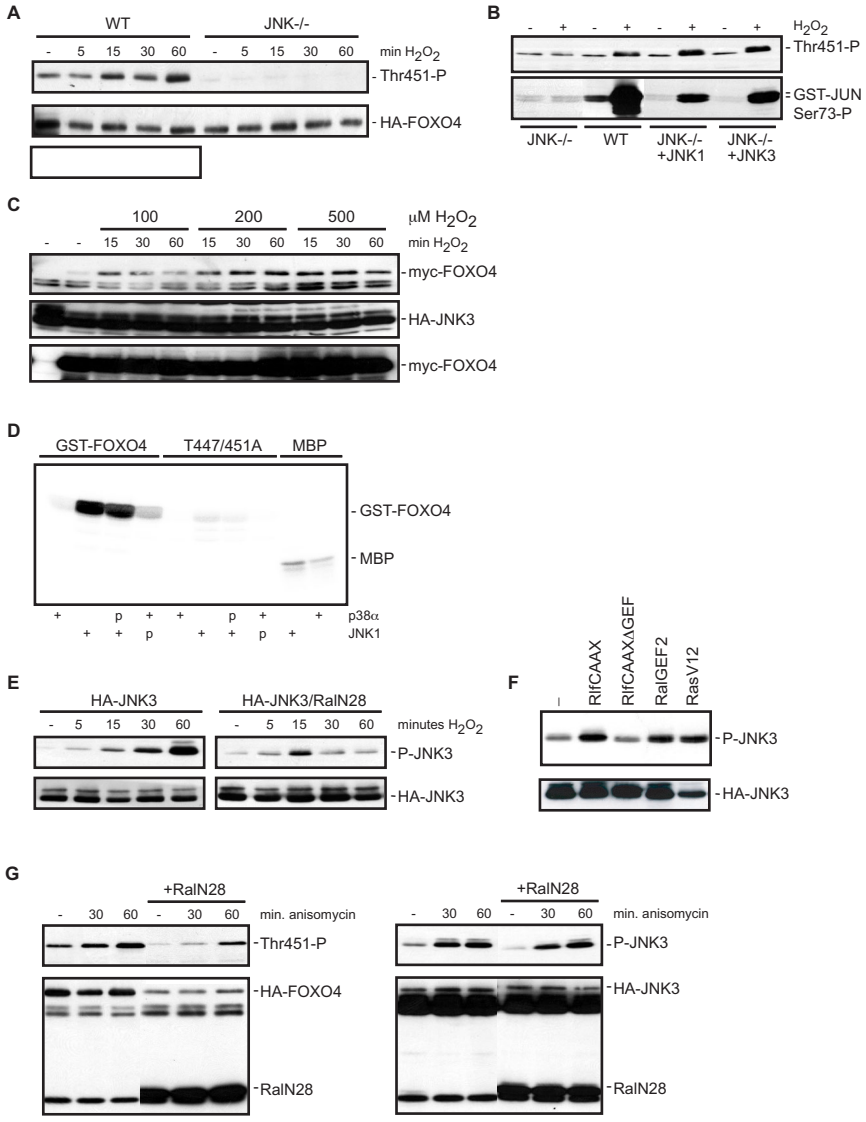

Figure 3. JNK is involved in the H_2O_2 induced Ral mediated phosphorylation of Thr451 and Thr447 on FOXO4
A. JNK 1,2-/- MEFs, transfected with HA-FOXO4 together with JNK1, JNK3 or an empty vector, were treated with 100 μM H_2O_2 for indicated time, and Thr451 phosphorylation was analysed on western blot. Wt MEFs were included as control. Similar results were obtained using 200 or 400 μM H_2O_2. **B**. JNK1,2-/- MEFs, wt MEFs, and JNK-/- co-transfected with either JNK1 or JNK3, and transfected with HA-FOXO4, were left untreated or treated with 100 μM of H_2O_2 for 60 minutes. Thr451 phosphorylation was analysed. In parallel, a GST-Jun pull down was performed to measure JNK activity (lower panel). Same results were obtained using 200 or 400 μM H_2O_2. **C**. 293T, transfected with myc-FOXO4 and HA-JNK3 were treated with different concentrations of H_2O_2 for indicated times. HA-JNK3 was immunoprecipitated and binding of

we also tested whether Ral is involved in anisomycin-induced JNK activation. Consistent with a role for JNK in mediating Thr447/451 phosphorylation of FOXO4, anisomycin treatment also induced Thr451 phoshorylation. However, RalN28 did not block the anisomycin-induced phosphorylation of both JNK and Thr451 (figure 3G). Therefore, we conclude that FOXO4 is phosphorylated by JNK at Thr447 and Thr451 and that JNK is differentially regulated following cellular stress: JNK activation following oxidative stress as generated by H_2O_2 treatment is mediated by the small GTPase Ral, whereas JNK activation following ER stress as generated by anisomycin treatment occurs independently of Ral.

Both others and we have previously shown that insulin signalling results in the translocation of FOXO from the nucleus to the cytosol (Biggs et al., 1999); (Brownawell et al., 2001). Thus, we analysed the effect of increased oxidative stress on FOXO4 localization and transcriptional activity. As was shown by others (Brunet et al., 2004), treatment of cells with H_2O_2 cultured in the presence of serum, when FOXO4 is predominantly localized in the cytosol, induced a relocalisation of FOXO4 from cytosol to nucleus (figure 4A). Translocation induced by H_2O_2 appeared to be stochastic. For reasons that are not clear, cells appear to respond in an all-or-none fashion. An example of that is shown in figure 4A, middle panel. To analyse the effect on transcriptional activity, we performed reporter assays using FOXO responsive promoters. Interestingly, we only observed a small but reproducible increase of FOXO transcriptional activity at low H_2O_2 concentrations, as measured by an increase in activity on the MnSOD promoter construct (figure 4B) (Kim et al., 1999). Similar results were obtained using the 6xDBE or p27 promoter constructs, both of which are FOXO responsive promoters (Furuyama et al., 2000); (Medema et al., 2000); and data not shown). The decrease in FOXO transcriptional activity following overnight H_2O_2 treatment at higher concentration is often accompanied by a decrease in HA-FOXO4 expression (figure 4B, loading control). Whether this decrease in FOXO transcriptional activity observed at higher H_2O_2 concentration is also due to PKB/c-Akt signalling, which is switched on at high concentrations of H_2O_2, or that other H_2O_2-induced modifications inhibit FOXO activity and/or expression in a dominant fashion, is at present unknown.

To further demonstrate that phosphorylation of either Thr447 or Thr451 results in activation of FOXO4, we tested a series of Thr447 and Thr451 mutants that can no

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myc-FOXO4 to HA-JNK3 was analysed on western blot (upper panel). Lower panels show expression of the constructs. **D**. Purified bacterially expressed GST-FOXO4(C) and GST-FOXO4-Thr447/451A(C) were incubated in the presence (+) or absence of active JNK or active p38α. P indicates a pretreatment with either active JNK or p38α in the presence of unlabeled rATP. Pre-phosphorylation by by JNK or p38 α did not enhance the ability of p38α or JNK to subsequently phosphorylate GST-FOXO4. MBP substrate was included as control for the activity of active JNK and p38α. **E**. 293T cells transfected with HA-JNK3 with or without HA-RalN28 were treated with 100 μM of H_2O_2 for increasing periods of time. JNK phosphorylation was analysed on western blot. Similar results were obtained using 200 or 400 μM of H_2O_2. **F**. 293T cells were transfected with HA-JNK3 together with indicated constructs. JNK phosphorylation was analysed on western blot. **G.** 293T cells transfected with HA-FOXO4 or HA-JNK3 with or without HA-RalN28 were untreated or treated with 10 μg/ml anisomycin for 30 or 60 minutes. FOXO4-Thr451 and JNK phosphorylation were analysed on western blot. 60 minutes treatment of anisomycin induced a 3 fold increase in FOXO4-Thr451 phosphorylation, both in the presence and absence of RalN28.

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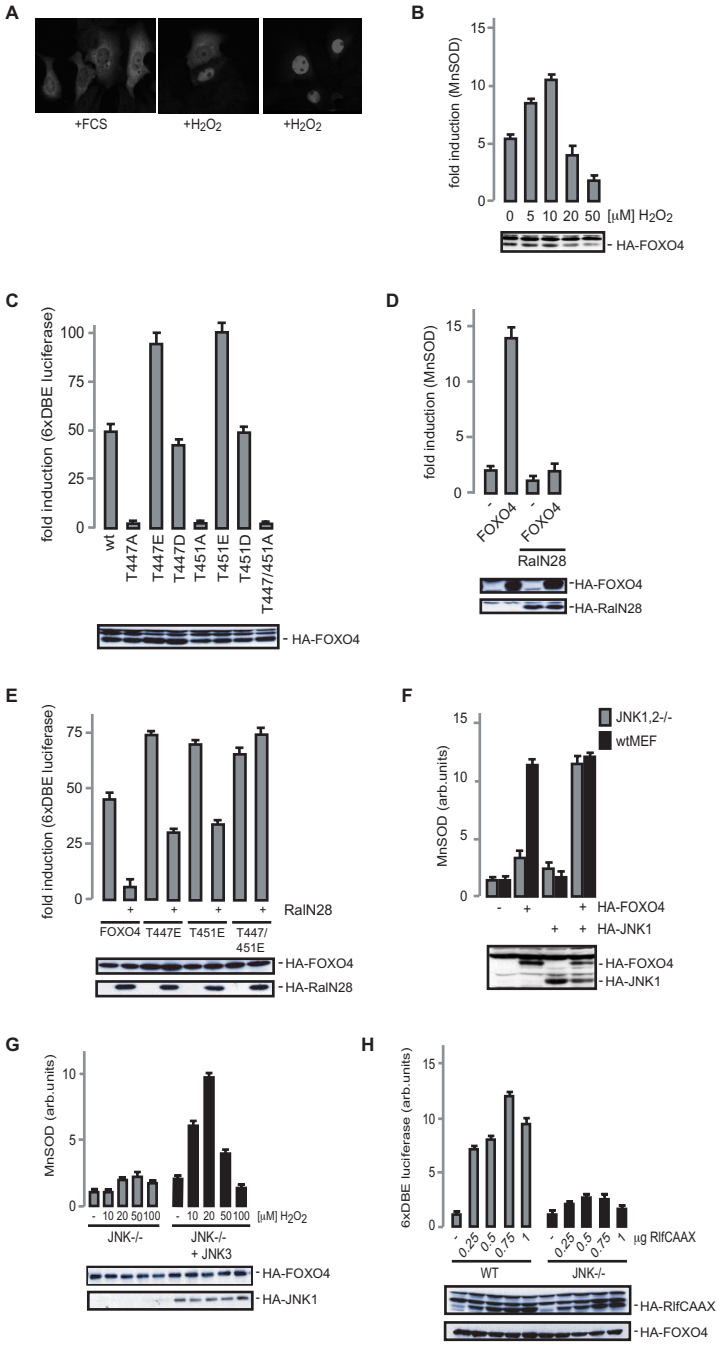
longer be phosphorylated at these sites or could mimic phosphorylation (figure 4C). Mutating either Thr447 or Thr451 to alanine was already sufficient to almost completely block transcriptional activity. Importantly, both the phospho-mimicking Thr447E and the Thr451E mutant displayed enhanced transcriptional activity compared to wild type FOXO4. Thus, these data suggest that phosphorylation of either Thr447 or Thr451 is sufficient to activate FOXO4 transcriptional activity. In agreement with a role for Ral, introduction of the dominant negative RalN28 completely blocks FOXO4 activity (figure 4D), whereas the phospho-mimicking mutants were either partially (Thr447E and Thr451E) or not inhibited by RalN28 co-expression (Thr447/451E, figure 4E). These data indicate that the effect of Ral on FOXO4 transcriptional activity is entirely through the regulation of Thr447 and Thr451 phosphorylation. To confirm the role of JNK in transcriptional activation via Thr447/451 phosphorylation, we analysed FOXO transcriptional activity in JNK1,2-/- cells. In these cells, FOXO activity was lower compared to wild type MEFs and re-introducing JNK, clearly enhanced FOXO4 transcriptional activity (figure 4F). H_2O_2-induced activation of FOXO activity was also reduced in JNK,2-/- MEFs. However, reintroduction of JNK3 in these cells restored stress-induced FOXO4 activity (figure 4G). Previously, we reported that the effect of RlfCAAX on FOXO4 mediated transcription was sensitive to the amount of RlfCAAX used (De Ruiter et al., 2001). Here, we show that the effect of RlfCAAX on FOXO activity is also dependent on JNK, since RlfCAAX induced FOXO activity is lowered in JNK-/- cells (figure 4H). The dose-dependent effect of RlfCAAX shows similarities with the dose-dependent effect of H_2O_2 treatment.

Next, we tested whether the loss of transcriptional activity as a result of the Thr447/451A mutation in FOXO4 as measured by the reporter assays resulted in a change in FOXO function. Previously, we have shown that FOXOs can protect cells from glucose deprivation-induced mitochondrial membrane instability (Kops 2002) and thus we tested whether the FOXO4 mutants were compromised in this respect.

Figure 4. H_2O_2 induces nuclear translocation and activation of FOXO4
A. DLD1 cells, transfected with HA-FOXO4, were maintained in the presence of serum and left untreated (left panel) or treated with 100 μM of H_2O_2 for 60 minutes (middle and right panel). Cells were fixed and HA-FOXO4 was stained. Middle panel shows differential response to oxidative stress with respect to localisation. Same results were obtained using 200 or 400 μM of H_2O_2. **B**. DLD1 cells, transfected with pSODLUC-3340 in the absence or presence of HA-FOXO4, were treated with indicated concentrations of H_2O_2 for 16 hours, and subjected to luciferase assays. Equal expression was tested by western blot. Data represent the average of three independent experiments. **C**. DLD1 cells were transfected with 6xDBE-luciferase together with the indicated constructs, and subjected to luciferase assays as described for panel B. **D**. DLD-1 cells were transfected with pSODLUC-3340 in the absence or presence of HA-FOXO4 and absence or presence of dominant-negative Ral (RalN28), and subjected to luciferase assays as described for panel B. **E**. A14 cells were transfected with 6xDBE-luciferase in the presence of HA-FOXO4 or the indicated mutant constructs and either in the presence or absence of dominant-negative Ral (RalN28), and subjected to luciferase assays as described for panel B. **F**. JNK1,2-/- MEFs and wt MEFs were transfected with pSODLUC-3340 in the absence or presence of HA-FOXO4, HA-JNK1 or both, and subjected to luciferase assays as described for panel B. **G**. JNK1,2-/- MEFs, transfected with pSODLUC-3340 with or without JNK3, were treated with indicated concentrations of H_2O_2 for 16 hours, and subjected to luciferase assays as described for panel B. **H**. Wt MEFs and JNK1,2-/- were transfected with 6xDBE-luciferase together with indicated amounts of HA-RlfCAAX, and subjected to luciferase assays as described for panel B. **I**. A14 cells were transfected with the indicated constructs and a puromycin selection vector. 36 hr after transfection, cells were put on 2 μg/ml puromycin and either cultured in medium containing FCS with glucose or medium with FCS lacking glucose. After 48 hr of glucose deprivation, cells were harvested, stained with Rhodamine 1,2,3 and analysed for mitochondrial membrane stability.


A
+FCS
+H2O2
+H2O2
B
fold induction (MnSOD)
0 5 10 20 50 [μM] H2O2
HA-FOXO4
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wt
T447A
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T451A
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T447/451A
HA-FOXO4
D
fold induction (MnSOD)
FOXO4
FOXO4
RalN28
HA-FOXO4
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T447E
T451E
T447/ 451E
HA-FOXO4
HA-RalN28
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MnSOD (arb.units)
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6xDBE luciferase (arb.units)
μg RlfCAAX
WT
JNK-/-
HA-RlfCAAX
HA-FOXO4

Inhibition of Ral signalling and expression of the Thr447/451A mutant reduced the ability of FOXO4 to protect cells from glucose deprivation and consistent with this the phospho-mimicking Thr447/451D mutant displayed slightly enhanced protection (figure 4I).

Thus, from these data we conclude that H_2O_2 induces a translocation of FOXO4 from the cytosol to the nucleus and that this translocation is part of the mechanism whereby H_2O_2 induces transcriptional activation of FOXO4. Mutant analysis shows that this transcriptional activation involves Thr447 and Thr451 phosphorylation and is dependent on the presence of JNK.

Finally, we tested whether stimuli other than H_2O_2, which are known to influence intracellular ROS levels, could activate FOXO transcriptional activity. Tumor necrosis factor α (TNFα) is a cytokine that has been shown to increase intracellular H_2O_2 levels and likely concomitantly to increase cellular oxidative stress (Goossens et al., 1995). This increase in cellular ROS may mediate the cytotoxic action of TNFα, although the exact mechanism is largely unknown. For example, overexpression of antioxidants including catalase to reduce cellular H_2O_2, has been shown to enhance (Bai and Cederbaum, 2000), or reduce TNFα cytotoxicity (Wong et al., 1989). There are also reports that demonstrate unaltered TNFα cytotoxicity after overexpression of antioxidants (O'Donnell et al., 1995). Interestingly, TNFα has also been shown to increase the expression of MnSOD (Wong et al., 1989) and FOXOs can induce MnSOD expression (Kops et al., 2002). A14 cells are sensitive to TNFα, as measured by activation of NF-κB, but show no obvious signs of TNFα-induced cell death (data not shown). Treatment of A14 cells with increasing concentrations of TNFα-induced a dose-dependent increase in FOXO4 transcriptional activity (figure 5A). Similar to the H_2O_2-induced FOXO4 transcriptional activity, the TNFα-induced increase is mediated by Ral, as it is inhibited by expression of the dominant-negative RalN28. Moreover, it is mediated by JNK, as it is absent in JNK1,2-/- cells and can be restored by reintroducing JNK in JNK1,2-/- cells (figure 5B). Importantly, TNFα-induced JNK activation involved an increase in cellular oxidative stress, as pretreatment with N-acetyl-L-cysteine (NAC), which enhances the scavenging of oxygen radicals, reduced TNFα-induced JNK activation (figure 5C).

The ability to increase cellular oxidative stress is not unique to TNFα, and for several growth factors it has been suggested that a change in intracellular redox contributes to activation of downstream signalling pathways (Sundaresan et al., 1995); (Bae et al., 1997). However, in general these growth factors repress FOXO transcriptional activity through PKB-mediated nuclear exclusion. Thus, we compared the levels of PKB activation with the activation of JNK by the stimuli employed in this study. As already indicated by our initial experiments (figure 1), H_2O_2 at relatively low concentrations induces Thr447/451 phosphorylation, and consistently H_2O_2 induces considerable JNK activity, whereas little or no induction of PKB activity could be demonstrated (figure 5C). Similar to H_2O_2, and consistent with the ability to induce FOXO transcriptional activity, TNFα induced considerable JNK activity, whereas only a small and transient increase in PKB activity was observed. In contrast, insulin induces a robust increase in PKB activity and represses FOXO transcriptional activity (Kops et al., 1999); data not shown), despite the induction of JNK activity comparable

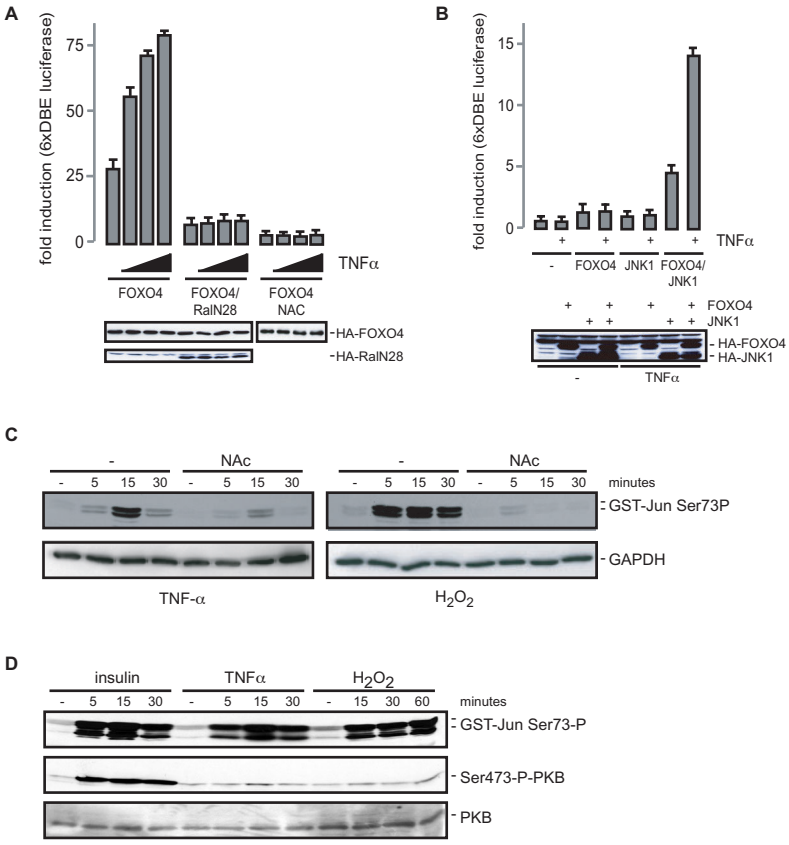

Figure 5. TNFα induces FOXO4 transcriptional activity
A. A14 cells were transfected with the HA-FOXO4 with or without HA-RalN28, together with 6xDBE-luciferase and Tk-renilla. Cells were treated with increasing concentrations of TNFα (5,10,20 ng/ml). Pretreatment with NAC was performed by adding 10mM of NAC 16 hr before treatment with TNFα. 16hr after TNFα treatment, luciferase activity was measured. Data represent the average of three independent experiments. **B**. JNK1,2-/- MEFs were transfected with HA-FOXO4 with or without HA-JNK1 together with 6xDBE-luciferase and Tk-renilla, and treated for 16 hr with TNFα (20 ng/ml). Luciferase activity was measured as descrived for panel B. **C.** A14 cells were treated with 10mM NAC and the next day treated with TNFα (20 ng/ml) or H_2O_2 (100 μM) for the indicated time points. JNK activity was measured by a GST-Jun pull down. **D.** A14 cells were treated with TNFα (20ng/ml), H_2O_2 (100 μM) or insulin (1 μg/ml) for the indicated time points. JNK activity was measured by means of a GST-Jun pull down and PKB activity was monitored using the Ser473 phospho-specific antibody.

to TNFα and H_2O_2. This comparison therefore indicates that PKB activation can act dominantly over JNK activation and explains how TNFα and H_2O_2 result in FOXO activity.

A possible mechanism whereby the opposing effects of JNK- and PKB-mediated phosphorylation of FOXO4 can be integrated is through the regulation of binding of co-factors such as 14-3-3. Binding of 14-3-3 is suggested to function as cytoplasmic

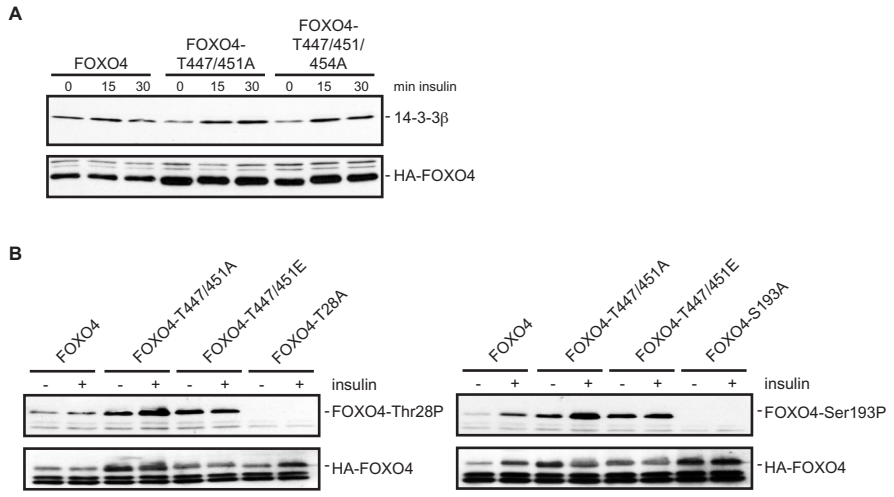

Figure 6. JNK phosphorylation does not affect 14-3-3 binding to FOXO
A. A14 cells were transfected with the indicated constructs and stimulated with insulin (1 μg/ml) for the indicated time points. Binding of endogenous 14-3-3 to HA-FOXO4 and HA-FOXO4 mutants was assayed by immunoprecipitation of HA-FOXO4 followed by western blotting using an antibody directed against 14-3-3β. **B**. A14 cells were transfected with the indicated constructs and treated with insulin (1 μg/ml) for 30 minutes. Phosphorylation of Thr28 and Ser193 was assayed using phosphospecific antibodies. The specificity of these antibodies was demonstrated by including the FOXO4-Thr28A and FOXO4-Ser193A mutants respectively.

anchor for FOXO and thereby to inhibit FOXO transcriptional activity. Therefore, we tested whether phosphorylation of Thr447/451 would affect the ability of PKB-mediated 14-3-3 binding to FOXO4 (figure 6A). We could not observe reproducible changes in insulin-induced 14-3-3 binding to FOXO4 and the FOXO4 mutants Thr447/451A and Thr447/451D. Also insulin-induced PKB-mediated phosphorylation of Thr28 and Ser193 did not appear significantly changed (figure 6B). Thus JNK-mediated phosphorylation of Thr447/451 appears to increase transcriptional activity, rather than relieve the inhibition by PKB imposed via 14-3-3 binding.

Discussion.

Activation of PI3K/PKB signalling decreases FOXO activity and thus the levels of FOXO target genes like MnSOD and catalase (Kops et al., 2002). MnSOD and catalase belong to a large and diverse family of anti-oxidant enzymes. Their regulation via PI3K/PKB/FOXO signalling therefore implies that insulin, through this signalling cascade, may modulate the cellular ROS level. Consistent with this hypothesis, we have previously shown that FOXO-mediated upregulation of MnSOD expression results in considerable lowering of cellular ROS (Kops et al., 2002). Here, we demonstrate that an increase in ROS will enhance FOXO transcriptional activity,

and thus functions as a feedback mechanism. An increase in ROS levels induces activation of the small GTPase Ral, which will in turn lead to the phosphorylation and activation of the stress kinase JNK. Active JNK induces the phosphorylation of Thr447 and Thr451 on FOXO4. Phosphorylation of these residues is essential for FOXO4 transcriptional activity as shown by mutational analysis. Consistent with this, H_2O_2 treatment increases FOXO transcriptional activity and translocation of FOXO4 from the cytoplasm to the nucleus and activation of the transcription factor. Activation of FOXO4 through Thr447/451 phosphorylation can now induce transcription of MnSOD and catalase, leading to a decrease in ROS levels. Thus, activation of FOXO4 by oxidative stress is part of a negative feedback loop to reduce the levels of oxidative stress in a cell, preventing damage to DNA, lipids and proteins (figure 7).
The homeostasis mechanism for controlling ROS levels presented here is controlled by signalling pathways that can provide both negative (PI3K/PKB) and positive (Ras/Ral/JNK) inputs on FOXO. Growth factors, including insulin, have the ability to regulate both pathways simultaneously. The question remains what would then determine the effective outcome of growth factor signalling of cells? H_2O_2 induces nuclear translocation in the presence of serum growth factors (figure 4A), and we performed our transcriptional assays in the presence of serum growth factors. In the presence of serum, cells maintain a basal level of PKB activity and apparently H_2O_2 treatment even at relatively low concentration can sufficiently activate Ral/JNK signalling in order to activate FOXO. Thus, when PKB activity is relatively low, for example as compared to insulin-induced PKB activity, the Ral/JNK signalling pathway acts opposite and dominant over PKB. At higher concentrations of H_2O_2, or for example following treatment of cells with insulin, PKB activity increases to a level

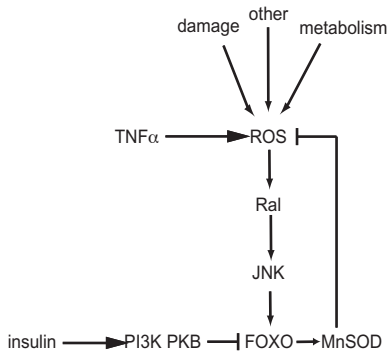

Figure 7. A model for FOXO-mediated control of cellular ROS levels
During periods of increased oxidative stress, FOXO4 is activated by Ral and JNK mediated phosphorylation on Thr447 and Thr451. Increased activity of FOXO4 will lead to an induction of the transcription of the anti-oxidant enzymes MnSOD and catalase, leading to a decrease of ROS levels. Thus, activation of FOXO4 by oxidative stress is part of a negative feedback loop to reduce the levels of ROS in a cell. Growth factors can balance cellular ROS through the activation of PKB or JNK, depending on the magnitude and kinetics of PKB and JNK activation. Cellular ROS can be increased through a variety of events including cellular metabolism and various forms of damage (DNA, protein).

at which it can act dominant over JNK activity. The importance of this differential activation of intracellular signalling pathways is further illustrated by our finding that TNFα, unlike insulin, increases FOXO transcriptional activity in A14 cells and this occurs with a concomitant strong activation of JNK and weak activation of PKB. If PKB activity outweighs the JNK activity this will result in relocalization of FOXO to the cytosol. In addition, it has been suggested that PKB-mediated phosphorylation of FOXO also results in ubiquitination and consequent degradation of the FOXO proteins (Matsuzaki et al., 2003). This would be consistent with the reduced FOXO expression we frequently observe following long-term treatment with higher concentrations of H_2O_2.

Recent studies have also revealed another mechanism via which the activity of FOXOs can be regulated. In response to H_2O_2 treatment, FOXO3a and FOXO4 are a substrate for acetylation by the acetylase transferases p300/CBP and subsequent deacetylation by Sir2^{SIRT1} and other deacetylases (Brunet et al., 2004); (Motta et al., 2004); (van der Horst et al., 2004). Results on the effect of acetylation and deacetylation on FOXO transcriptional activity are contradictory. Both negative (Brunet et al., 2004); (van der Horst et al., 2004) and positive (Motta et al., 2004) regulation of FOXO transcriptional activity by acetylation have been reported. Taken together and consistent with our own data on the role of acetylation in regulating FOXO4 (van der Horst et al., 2004), the data presented here suggest that during periods of low oxidative stress, FOXOs are initially activated by JNK-mediated phosphorylation, but thereafter, or at higher concentrations or longer periods of time inactivated by acetylation and/or ubiquitination. Deacetylation of FOXO by Sir2^{SIRT1} can thus prolong the FOXO activity induced by H_2O_2 treatment in order to ensure full activation of the anti-oxidant targets of FOXO. To test this hypothesis, we are currently investigating the kinetics of H_2O_2-induced phosphorylation and acetylation of FOXO and the effect on FOXO transcriptional activity.

The human FOXO family of transcription factors consists of three different isoforms. Here we show the regulation of FOXO4 via Thr447/451 phosphorylation. Clustal W alignment of FOXO4 with the other isoforms, FOXO1 and FOXO3a, did not reveal conserved T447/451 phosphorylation sites in the C-terminal parts of the protein. However, for example for FOXO3a, it has been shown that several sites within the C-terminal part of the protein can be phosphorylated upon H_2O_2 treatment (Brunet et al., 2004). These phosphorylation sites resemble potential JNK phosphorylation sites. We are currently investigating whether JNK can induce a phosphorylation dependent activation of FOXO1 and FOXO3a upon treatment with H_2O_2, which of the phosphorylation sites are involved, and thus are functionally equivalent to Thr447/451.

The model that derives from the data presented here bears striking similarities to the proposed role and regulation of DAF-16, the *C.elegans* homologue of mammalian FOXO. In *C.elegans,* a variety of stresses including oxidative stress have been shown to induce translocation of DAF-16/GFP from the cytosol to the nucleus (Henderson and Johnson, 2001). Oxidative stress strongly induces the expression of SOD-3, the *C.elegans* homologue of MnSOD, in a DAF-16 dependent manner (Honda and Honda, 1999). Thus, both in *C.elegans* and in mammalian cells oxidative stress

activates DAF-16/FOXO to act in a negative feedback control of ROS. In *C.elegans,* little is known as to what mediates stress-induced activation of DAF-16. Both Ral and JNK isoforms are present in *C.elegans* and it will be of interest to investigate whether these are involved in DAF-16 control by certain forms of oxidative stress. Interestingly, it has recently been demonstrated that in *Drosophila* increased JNK signalling also increases oxidative stress resistance (Wang et al., 2003). Similarly, dFOXO has also been shown to increase oxidative stress resistance in *Drosophila* (Junger et al., 2003). In the regulation of cell shape in *Drosophila,* there is genetic evidence for a connection between dRal and dJNK (Sawamoto et al., 1999). However, here dRal appears to regulate cell shape changes through inhibition of the JNK pathway, which is in contrast to what we observe here. The genetic interaction between FOXO, Ral and JNK in regulating oxidative stress resistance has not been examined in *Drosophila* yet. However, the JNK-mediated FOXO activation as described here may likely be a mechanism whereby increased oxidative stress resistance is also achieved in *Drosophila*.

Studies in both *C. elegans* and in *Drosophila* support a link between stress-resistance and prolonged longevity. For example, in both organisms, removing oxidants by overexpressing superoxide dismutase and catalase resulted in animals with a prolonged lifespan (Taub et al., 1999); (Honda and Honda, 1999). In *C. elegans,* prolonged lifespan, protection against oxidative stress, and regulation of superoxide dismutase and catalase are all dependent on the status of DAF-16 (Murphy et al., 2003). In mammals, the pathways involved in the basic controls for aging will likely be more complex. Recently, FOXO3a has been linked to the important mammalian gerontogene p66shc. In cells lacking p66shc, the activity of FOXO3a is increased. H_2O_2 treatment of these p66shc-/- cells reduced the activation of PKB and consequent PKB mediated FOXO3a inactivation, indicating that the redox-dependent FOXO inactivation is reduced (Nemoto and Finkel, 2002). Yet, a mechanism as to how p66Shc ablation could result in FOXO activation, rather than solely preventing its inhibition in an indirect manner, remains to be determined. Our results may provide such a possible mechanism. It has been shown that p66Shc inhibits the p46/p52Shc isoforms. The latter isoforms are involved in growth factor-induced Ras and MAPK activation. We have shown that insulin-induced Ras activation is mediated almost exclusively by p46/p52 Shc (Pronk et al., 1994) and insulin-induced Ral activation is mediated by Ras (Wolthuis et al., 1998). Thus, loss of p66Shc would be expected to specifically enhance the Ras/Ral signalling branch following insulin stimulation. A major consequence would be enhanced FOXO activity due to a shift from negative regulatory signalling (PI-3K/PKB) to positive regulatory signalling (Ras/Ral). This possibility is currently under investigation.

In summary, our data describe a mechanism by which FOXO activity is regulated by oxidative stress, resulting in prevention of cellular damage. They bring us one step closer to unravelling the mechanisms that control aging in mammalian cells.

Acknowledgements

We would like to thank Arjan Brenkman, Fried Zwartkruis, Armando van der Horst, Pieter van den Heuvel, Leo Price and Hans van Dam for discussions and critical reading of the manuscript. We would like to thank Cell Signalling for providing samples of Thr451P antibody. Erwin Wagner for providing JNK-/- cells. This research was supported by grants from the Dutch Cancer Foundation (KWF) and the Netherlands Organization for Scientific Research (NWO).

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Addendum Chapter 4

Role for FOXO in oxidative stress and UV induced signalling

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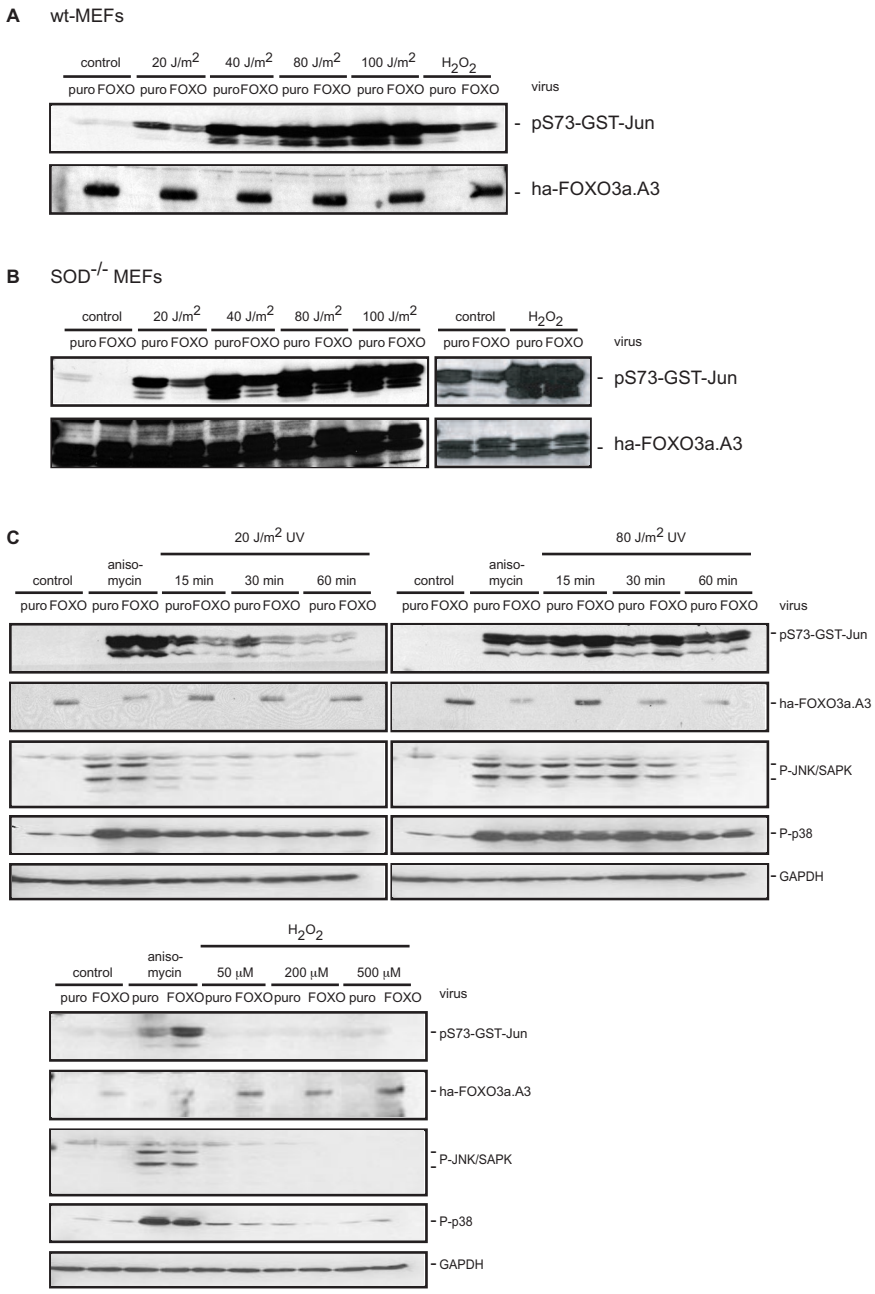
Recently, others and we have shown the regulation of anti-oxidant enzymes like MnSOD and catalase by FOXO (Kops et al., 2002; Nemoto and Finkel, 2002). Moreover, we have shown that FOXO-mediated upregulation of MnSOD expression will result in a decrease of cellular ROS levels in cells (Kops et al., 2002). In chapter 4, we now show that in turn an increase in ROS levels in a cell will signal back to FOXO to increase the FOXO transcriptional activity. Thus, FOXO is part of a feedback mechanism to control levels of ROS in a cell, thereby preventing cells from damage by oxidative stress.

JNK is a stress kinase, which is activated by treatment of cells with cytokines and by exposure of cells to many forms of environmental stress, including osmotic stress, oxidative stress and radiation (reviewed in (Ip and Davis, 1998)). Consistent with the activation of JNK by cellular stress and the ability of FOXOs to lower cellular oxidative stress, we have shown previously that the activation of JNK by glucose-deprivation is impaired following FOXO3a activation (Kops et al., 2002). To investigate whether the ability of FOXOs to protect cells from oxidative stress, by increasing MnSOD and catalase expression, is specific to certain types of stress or represents a general defense mechanism against all sorts of stress, we employed the activation of JNK by different stressors and the effect of FOXO on this activation as a read-out. Wt MEFs were infected with either a control virus (pBabe-puro) or a virus containing a constitutively active FOXO3a (HA-FOXO3a.A3). Cells were ionized using increasing doses of UV, or treated with H_2O_2. As shown in figure 1A, UV treatment induces an increase in JNK activity, as analyzed by phosphorylation of GST-jun on Ser73, following a GST-jun pull down experiment. Infection of wt MEFs with FOXO3a reduced the levels of phosphorylated GST-jun, following H_2O_2 treatment and at low dose of

Figure 1. FOXOs use diverse mechanisms to protect cells from oxidative stress or stress induced by UV irradiation

A/B. Wt MEFs (Figure 1A) or SOD-/- MEFs (Figure 1B), infected with pBabe-puro or pBabe-FOXO3a.A3, were treated with increasing doses of UV or 100 μM H_2O_2 for 1h. A GST-Jun kinase assay was performed to analyze the JNK activity in the cells. Phosphorylated GST-Jun was analyzed on blot. Lower panel shows expression levels of HA-FOXO3.A3. **C.** TKO MEFs, infected with pBabe-puro or pBabe-FOXO3a.A3, were treated with increasing doses of UV or 100 μM H_2O_2 for 1h. A GST-Jun kinase assay was performed to analyze the JNK activity in the cells. Phosphorylated GST-Jun was analyzed on blot. In parallel, total lysates were analyzed for phosphorylated JNK or p38 kinases. Lower panel shows expression levels of HA-FOXO3.A3.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wt-MEFs
control
20 J/m2
40 J/m2
80 J/m2
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P-JNK/SAPK
P-p38
GAPDH

UV irradiation, indicating that FOXO3a may also decrease JNK activation induced by UV treatment. To analyze whether the ability of FOXO to impair JNK activation in response to UV damage or oxidative stress by H_2O_2 depends on the presence of MnSOD, we performed the same experiment as described above, but now in SOD-/- MEFs which lack MnSOD. FOXO3a could not prevent increased activation of JNK after H_2O_2 treatment in SOD-/- MEFs, whereas FOXO3a still induced a reduction of phosphorylated GST-jun after low doses of UV irradiation (figure 1B). Again at high doses of UV no difference between control and FOXO3a infected cells could be observed. Taken together, we conclude that the ability of FOXO3a to protect cells from oxidative stress as generated by H_2O_2 and measured by JNK activation is dependent on the presence of MnSOD. FOXO3a apparently also impairs UV-induced JNK activation but this does not require MnSOD.

Oxidative stress induced by H_2O_2 treatment induces a G_1 and possibly G_2/M cell cycle arrest in cells (Barnouin et al., 2002; Clopton and Saltman, 1995). Activation of FOXO will also lead to an arrest of cells in the G_1 and possibly G_2/M phase of the cell cycle (Alvarez et al., 2001; Medema et al., 2000; Nakamura et al., 2000). The ability of cytostatic drugs and irradiation to induce apoptosis is influenced by the cell cycle status of the responding cells and this may account for JNK activation as well. Thus we asked whether the FOXO-induced cell cycle arrest contributes to the observed repression by FOXO of UV-induced JNK activation. Therefore, we used TKO MEFs, which are mouse embryo fibroblasts lacking the Rb family (pRb, p107, and p130) (Sage et al., 2000), and these cells are therefore incapable of going into a cell cycle arrest. Again, cells were infected with constitutively active FOXO3a and treated with UV or H_2O_2. To our surprise TKO MEFs were not sensitive to oxidative stress induced by H_2O_2 treatment (Figure 1C). On the other hand, TKO MEFs did still respond to anisomycin and UV irradiation did induce phosphorylation of GST-jun, which importantly was also decreased when cells were infected with FOXO3a. This indicates that FOXO-induced inhibition of JNK in response to UV occurs independent of its ability to induce a cell cycle arrest in G_1.

Our results indicate that FOXOs can repress UV-induced JNK activation independent of MnSOD and a G_1 cell cycle arrest. This is in contrast to JNK activation by glucose-deprivation and H_2O_2 treatment, which is repressed by FOXO in a MnSOD-dependent manner (Kops et al., 2002; Essers *et al*, Chapter 4). This difference between UV and H_2O_2/glucose deprivation could be that the type of oxidative stress generated by UV differs from that generated by H_2O_2/glucose deprivation. Indeed H_2O_2 treatment will predominantly generate OH^- radicals, whereas radiation in general and also UV will generate predominantly singlet oxygens (Davies, 2004). Cysteine derivatives such as taurine and hypotaurine have been shown to act as efficient scavengers for these singlet oxygens (Aruoma et al., 1988), and thus might be candidates for targets of FOXO in response to UV. Whether FOXO induced protection of cells against UV indeed signals via these taurines or other scavengers of singlet oxygen will be subject for further investigation.

Material and Methods

Cell culture, and infection

Wt MEFs (wildtype mouse embryo fibroblasts), SOD-/- MEFs (mouse embryo fibroblasts knocked out for SOD2-/- gene) (Huang, 1997), TKO MEFs (mouse embryo fibroblasts derived from pRB-/- p107-/- p130-/- triple knockout (TKO) mice) (Sage et al., 2000), and phoenix cells were maintained in Dulbecco's Modified Eagle Medium supplemented with L-glutamine, penicillin/strecptomycin and 10% FCS.

Wt MEFs, SOD-/- MEFs and TKO MEFs were infected with pBabe-puro or pBabe-HA-FOXO3a.A3 using phoenix cells expressing the ecotrophic receptor. Phoenix cells were transfected using superfect transfection reagent (3 mg/ml). 2 days after transfection, MEFs were infected by adding the medium of the phoenix cells together with 6 μg/ml hexadimethrine bromide. Infection was repeated 6h after the first round. The day after infection cells were seeded in selection medium containing 2 μg/ml puromycin.

Plasmids

pBabe-puro and pBabe-HA-FOXO3a.A3 have been described (Kops et al., 2002). GST-cJun1-79 was purified from bacteria using a standard GST-fusion-protein-purification protocol.

Antibodies

Antibody specific for JNK phosphorylated at Thr 183/Tyr185 (anti-phospho-Thr183/Tyr185-JNK/SAPK), for p38 phosphorylated on Thr180/Tyr182 (anti-phospho-Thr180/Tyr182-p38 MAPK) and the anti-phospho-Ser73-Jun antibody were from Cell signalling. pBabe-FOXO3a.A3 was detected using polyclonal HA antibody (Santa Cruz). GAPDH antibody was from Chemicon.

Stress treatments

Anisomycin and H_2O_2 were added to the cells at 10 μg/ml and 100 μM respectively. Before irradiation cells were washed with PBS, PBS was removed, and UV treatment of 20-100 J/m^2 was given using a UV Statalinker 2400 (Stratagene). After irradiation conditioned medium was added back for another 30 min before harvesting the cells.

JNK activity

MEFs were lysed in lysis buffer (10% glycerol, 1% NP-40, 40 mM Tris pH 7.5, 200 mM NaCl, 2 mM MgCl2, 1 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, 50 μM NaF and 1 mM sodium orthovanadate), and endogenous JNK was immunoprecipitated using 10 mg of GST-Jun1-79 per ml precoupled to glutathione beads. The beads were washed twice with kinase lysis buffer and twice with kinase reaction buffer (25 mM Tris pH 7.5, 20 mM MgCl2, 2 mM DTT). For kinase reactions, the beads were incubated in 25 μl kinase buffer, with 100 μM ATP, at 37 °C for 30 min, taken up in sample buffer, and analyzed by SDS-PAGE followed by Western blotting with phosphospecific c-Jun antibody. (anti-phospho-Ser73-Jun).

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Chapter 5

General discussion

An increase in reactive oxygen species (ROS) in a cell by exogenous sources or as a consequence of endogenous enzyme activity can lead to increased damage to proteins, lipids and DNA. When the oxidative stress is severe, survival of the cell is dependent on the ability of the cell to adapt to, or resist the stress, and to repair, or replace damaged molecules. To protect themselves against increases in ROS levels, cells harbor a number of antioxidant defense systems against ROS, which includes the enzymatic scavengers superoxide dismutase (SOD), catalase, glutathione peroxidase, and other proteins and peptides whose function is to reduce the cumulative load of ROS within a cell. In recent years, others and we have identified a role for FOXO transcription factors in the defense against oxidative stress (Kops et al., 2002; Nemoto and Finkel, 2002). FOXOs regulate the expression of the antioxidant enzymes MnSOD and catalase, thereby reducing ROS levels in the cell. In this thesis, we have described the role of FOXO in oxidative stress signalling in more detail. We show that FOXO is part of a feedback mechanism, in which an increase in ROS levels in a cell will signal towards FOXO, leading to phosphorylation and activation of FOXO. This results in activation of defense mechanisms against oxidative stress, and thus protection of the cell (Chapter 4). We also identified a new binding partner for FOXOs, β-catenin (Chapter 2 and 3). Interaction between the two proteins is increased under oxidative stress conditions and affects both insulin and Wnt signalling in a cell, indicating a bridge between two important pathways in proliferation, development and carcinogenesis.

FOXO and β-catenin, partners in crime

Interaction between insulin/IGF1 signalling and Wnt signalling has been reported, although in most cases cross talk of these two pathways was suggested to be at the level of glycogen synthase kinase-3 (GSK3) (Fukumoto et al., 2001; Harwood, 2001). GSK3 is a downstream target of insulin stimulation, negatively regulated by activated PKB/Akt (Doble and Woodgett, 2003). On the other hand, GSK3 is also a central element in Wnt signalling, where it is part of the β-catenin destruction complex, together with axin, APC, and CKI (Doble and Woodgett, 2003). Wnt stimulation inhibits the activity of GSK3 within this complex, allowing unphosphorylated β-catenin to accumulate in the cytoplasm and nucleus (Doble and Woodgett, 2003). PKB phosphorylation of GSK3 in the destruction complex has been observed after chronic and prolonged Wnt stimulation, and alanine substitution of the PKB phosphorylation site within GSK3 increases GSK3 activity within the Wnt pathway (Haq et al., 2003). This indicates that sequestering GSK3 within the destruction complex is not sufficient to prevent cross talk with components of the insulin pathway. On the other hand, PKB phosphorylation does not elicit the Wnt response in cells, and no direct link between insulin and TCF activity has been elucidated. This indicates that, although both pathways signal to GSK3, the effects of insulin and Wnt are different.

In **Chapter 2** and **3**, we now show evidence for cross talk between the insulin and Wnt pathway at the level of TCF: the interaction between FOXO and β-catenin. Activation of FOXO leads to inhibition of β-catenin/TCF signalling, thereby inhibiting Wnt signalling (**Chapter 3**). The mechanism of FOXO induced inhibition of TCF activity

includes stabilization of β-catenin in the cytoplasm, and probably increased nuclear-cytoplasmic shuttling of β-catenin, thus preventing binding of β-catenin to TCF in the nucleus (**Chapter 3**). Inhibition of TCF activity by FOXO is further enhanced under conditions of oxidative stress, indicating a role for the Wnt signalling pathway in oxidative stress signalling. Further evidence for the involvement of the Wnt pathway in stress signalling comes from a report, showing that Wnt-1 mediated activation of TCF-dependent transcription can inhibit apoptosis by suppressing the mitochondrial release of cytochrome C that is induced by chemotherapeutic agents (Chen et al., 2001). Furthermore, recently Shin et al also showed that H_2O_2 treatment of cells leads to inhibition of TCF activity (Shin et al., 2004). However, the mechanism, signalling from increased oxidative stress to inhibition of TCF activation was unclear. Our data indicate a role for FOXO in this mechanism.

Binding of β-catenin and FOXO does not only influence β-catenin/TCF signalling, it also leads to an increase in FOXO activity (**Chapter 2**). This increase is probably due to increased shuttling of FOXO from cytoplasm to the nucleus. Nuclear-cytoplasmic localization is an important element of regulating FOXO activity in a cell. Nuclear import and export of FOXO is an active process, depending on the small GTPase Ran (Biggs et al., 1999; Brownawell et al., 2001; Brunet et al., 2002). Nuclear-cytoplasmic shuttling of FOXO is thought to be dependent on the binding of FOXO to 14-3-3 via two 14-3-3 binding motifs (Obsil et al., 2003). However it is unclear how 14-3-3 is actually involved. Initially it was thought that following PKB mediated phosphorylation of FOXO within the nucleus, 14-3-3 would bind and that this results in recruitment of crm-1 and subsequent nuclear export (Brunet et al., 2002; van Hemert et al., 2004). However, we have previously shown that crm-1 binding to FOXO4 does not require 14-3-3 (Brownawell et al., 2001) and furthermore recent results with another exportin, i.e. exportin 7 (Mingot et al., 2004) suggest that at least 14-3-3sigma shuttles via exportin7 and not crm-1. Finally our analysis of various FOXO4 mutants indicates an inverse relation between 14-3-3 binding and β-catenin binding. Increased 14-3-3 binding correlates with constitutive cytosolic localization, whereas increased β-catenin binding correlates with constitutive nuclear localization (**Chapter 3).** The importin for active shuttling of FOXO from cytosol to the nucleus is not identified yet and β-catenin is a good candidate for this importin, since the armadillo repeats are structurally related to importin-β HEAT repeats that bind to the nuclear pore complex (Henderson and Fagotto, 2002). This would indicate that, upon binding in the cytoplasm, β-catenin shuttles FOXO into the nucleus, where it can function as a transcription factor (figure 1). It is of course to be sorted out, whether β-catenin under all conditions will be able to act as importin for FOXO or whether this may only occur after increased cellular oxidative stress. Phosphorylation of FOXO in the nucleus will result in binding to 14-3-3 and sequestering out of the nucleus (figure 1). Whether or not 14-3-3 is actually involved in nuclear export or solely serves as a cytosolic retention signal remains to be determined.

Why would both β-catenin and FOXO activity be affected under conditions of oxidative stress? Following its role in wnt-signalling one may conclude that β-catenin is likely to function as a proliferative signal. However, under conditions of oxidative stress sufficient to cause damage to cells, cells will try to repair damage and for this

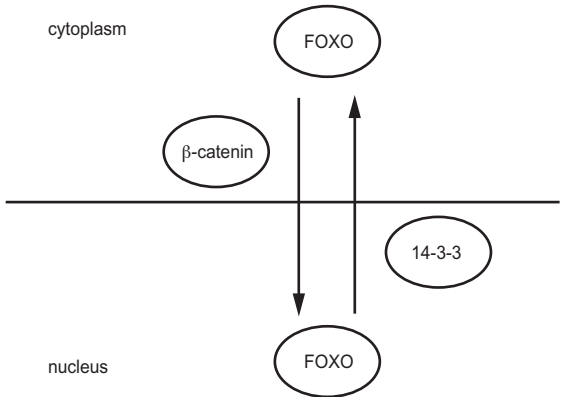

Figure 1. A model for nuclear import and export of FOXO
Upon binding in the cytoplasm, β-catenin shuttles FOXO into the nucleus, where it can bind promoters of target genes and induce transcription. Phosphorylation of FOXO in the nucleus will result in binding to 14-3-3 and sequestering out of the nucleus.

a proliferative arrest is required. Thus, by decreasing β-catenin/TCF activity, this proliferation signal will actively be repressed and in addition, by inducing activation of FOXOs, cells will actively induce a FOXO-dependent cell cycle arrest. Thus cellular oxidative stress will hereby prevent proliferation and induce separately also arrest. This will then be accompanied by FOXO-dependent increase in antioxidant defense systems to prevent and/or to repair damage induced by oxidative stress. Thus, by inhibiting β-catenin and inducing FOXO activity in response to oxidative stress, a cell can activate several lines of defense against an increase in ROS.
Overexpression of β-catenin/TCF signalling is thought to be a major cause for the development of cancer. In most colon carcinomas, mutations in the Wnt pathway, leading to stabilization of β-catenin, are accompanied by mutations in the Ras pathway (Bos et al., 1987). These mutations in Ras are gain of function mutations, resulting in increased PI3K/PKB signalling. Thus, in colon carcinoma cells FOXO activity will often be repressed. Given our observation that FOXOs can inhibit several aspects of oncogenic transformation of tumor cells of varying genetic background (i.e. mutant Ras, deletion of PTEN, mutation/deletion of APC) suggests that inactivation of FOXO may be a general requirement for obtaining a tumorigenic phenotype and as such FOXO may be an excellent target for drug development.

Regulation of FOXO under conditions of oxidative stress
An increase in cellular ROS by treatment of cells with H_2O_2 results in the activation of FOXO, and thus an increased protection against oxidative stress. In **Chapter 4**, we show that oxidative stress induces phosphorylation of Thr447/Thr451 in FOXO4. Phosphorylation of these sites seems to be specific for FOXO4, since no conserved Thr447/Thr451 phosphorylation sites are found in the C-terminal

parts of FOXO1 and FOXO3a. However, Brunet et al showed that oxidative stress also induces phosphorylation of FOXO3a (Brunet et al., 2004). Several of these phosphorylation sites are located in the C-terminus (figure 2) (Brunet et al., 2004), and resemble potential JNK phosphorylation sites. Mutation of Thr447/Thr451 in FOXO4 completely blocks transcriptional activity of the protein. This suggests that mutating the phosphorylation sites in FOXO3a involved in oxidative stress induced, JNK dependent activation of FOXO to alanine will also result in an inactive protein. We are currently using this approach to scan through the C-terminus of FOXO3a in search of phosphorylation sites homologous to Thr447/Thr451 in FOXO4.
As mentioned, FOXO3a is phosphorylated upon H_2O_2 treatment on several phosphorylation sites. Using Mass spectrometry eight serine and threonine residues were identified (figure 2) (Brunet et al., 2004). Preliminary data from our lab also show additional phosphorylation events upon induction of oxidative stress (figure 2) (A. Brenkman, personal communication). These sites include the PKB phosphorylation sites Thr28 and Ser258, and the DYRK1a phosphorylation site Ser268. This indicates that JNK dependent phosphorylation of Thr447/Thr451 is not the only pathway controlling FOXO activity under conditions of oxidative stress. Other stress kinases can be involved as well. Future experiments will elucidate other signalling pathways involved in stress signalling to FOXOs.
Recently, another important modification of FOXO was identified upon increases

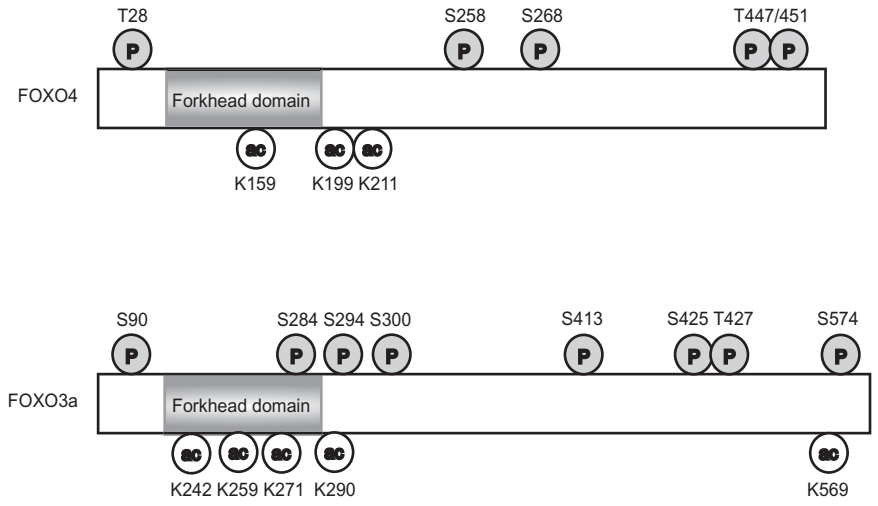

Figure 2. FOXO phosphorylation and acetylation induced by oxidative stress
Mass spectrometry data have revealed several sites in FOXO4 and FOXO3a, which are phosphorylated or acetylated upon induction of oxidative stress by treatment with H_2O_2. P = phosphorylated serine or threonine, ac = acetylated lysine.

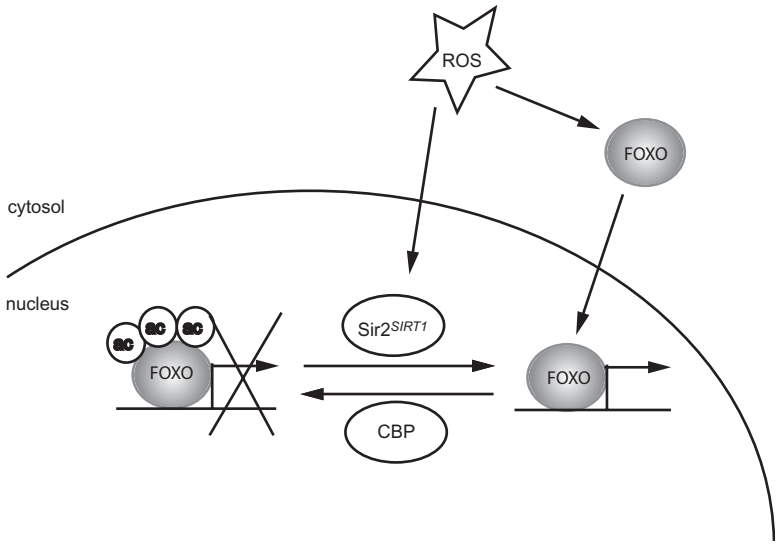

Figure 3. Model for oxidative stress induced phosphorylation and acetylation of FOXO.
During periods of low oxidative stress, FOXO is initially activated by JNK-mediated phosphorylation. Thereafter, at higher concentrations or longer periods of time of oxidative stress FOXOs will be inactivated by acetylation by the acetyl transferases p300/CBP. Subsequently, FOXOs will be deacetylated by Sir2SIRT1 and other deacetylases.

in oxidative stress. Others and we showed that H_2O_2 treatment induced acetylation of FOXO by the acetyl transferases p300/CBP and subsequent deacetylation by Sir2*SIRT1* and other deacetylases (Brunet et al., 2004; Motta et al., 2004; van der Horst et al., 2004). Using Mass spectrometry several different acetylated lysine residues were identified on FOXO3a upon H_2O_2 treatment (Brunet et al., 2004), or after co-transfection of FOXO4 with CBP (A.Brenkman, personal communication). Results on the effect of acetylation and deacetylation on FOXO activity were contradictory. Both negative and positive regulation of FOXO transcriptional activity was reported. Data from our lab on acetylation of FOXO4 showed a decrease in FOXO activity after H_2O_2 treatment (van der Horst et al., 2004). Coupling this to the phosphorylation data, we propose a model in which during periods of low oxidative stress, FOXO is initially activated by JNK-mediated phopshorylation, but thereafter at higher concentrations or longer periods of time inactivated by acetylation (figure 3). This hypothesis will be further tested by investigating the kinetics of H_2O_2 induced phosphorylation and acetylation of FOXO and their effect on FOXO activity.
Also other modifications like ubiquitination and degradation could play a role in the regulation of FOXO during oxidative stress. We frequently observed reduced expression of FOXO4 following long term treatment with higher concentrations of H_2O_2 (**Chapter 4**). This could indicate degradation of the protein, although the mechanism via which FOXO would be degraded is currently unknown.

Phosphorylation of FOXO by JNK

The c-Jun NH2-terminal kinase (JNK) is activated in response to treatment of cells with inflammatory cytokines and by exposure of cells to environmental stress (reviewed by Ip and Davis, 1998). In **Chapter 4**, we show that oxidative stress can induce activation of JNK via the small GTPase Ral. This activation of JNK will induce activation of FOXO by directly phosphorylating the transcription factor on Thr447/ Thr451. JNK belongs to the family of mitogen-activated protein kinases (MAPK), which represent an evolutionary conserved signalling mechanism that is used by cells to respond to changes in their environment. Activation of MAPK is mediated by dual phosphorylation of MAPK on Thr and Tyr by members of a group of MAPK kinases. Two different MAPK kinases (MAPKK), MKK4 and MKK7, are implicated in the activation of JNK (Davis, 2000). Although the two MAPKK can phosphorylate both Thr and Tyr, MKK4 has a preference for phosphorylating Tyr, whereas MKK7 preferentially phosphorylates Thr (Lawler et al., 1998). Targeted gene-disruption studies were performed to unravel the role of MKK4 and MKK7 in vivo. Disruption of both MKK4 and MKK7 is required for a complete block in JNK activation caused by exposure of cells to environmental stress (Tournier et al., 2001). In contrast, disruption of MKK7 alone is sufficient to prevent JNK activation caused by proinflammatory cytokines (Tournier et al., 2001). Since we investigate the role of oxidative stress on this pathway, it is most likely that both MKK4 and MKK7 are necessary for a full activation of JNK after treatment with H_2O_2 (figure 4). Experiments with MKK4-/- MKK7-/- double knockout MEFs or by using RNAi against both proteins will reveal the role of MKK4 and MKK7 in the Ral-mediated activation of JNK induced by

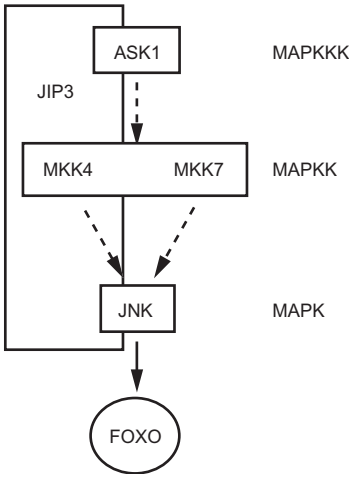

Figure 4. A model for activation of FOXO by stress-activated MAPK signalling
In Chapter 4 we show direct phosphorylation of FOXO by JNK. The regulation of JNK in this pathway is still unknown. However, the kinases outlined in this figure are likely to be involved.

oxidative stress.
Several MAPKKK have been reported to activate the JNK signalling pathway by phosphorylating MAPKK. These include members of the MEKK group (MEKK1 to 4) (Blank et al., 1996; Gerwins et al., 1997; Lange-Carter and Johnson, 1994; Minden et al., 1994; Takekawa et al., 1997; Yan et al., 1994), the mixed-lineage protein kinase group (MLK1, MLK2, MLK3, DLK, and LZK) (Dorow et al., 1993; Fan et al., 1996; Hirai et al., 1997; Sakuma et al., 1997; Teramoto et al., 1996), the ASK group (ASK1 and ASK2) (Ichijo et al., 1997; Wang et al., 1996; Wang et al., 1998), TAK1 (Yamaguchi et al., 1995), and TLP2 (Salmeron et al., 1996). In most cases, the evidence is based on *in vitro* protein kinase assays. However, it is unclear whether all these MAPKKK are physiological regulators of the JNK pathway *in vivo*, and which MAPKKK are relevant to specific physiological stimuli. A good candidate for the involvement in oxidative stress induced phoshorylation of JNK is the apoptosis signal-regulating kinase (ASK) 1 (figure 4), since an increase in oxidative stress will induce activation of ASK1 (Chang et al., 1998; Ichijo et al., 1997), by inhibiting the binding of ASK1 to 14-3-3 (Goldman et al., 2004). Moreover, mice lacking functional ASK1 show a loss of oxidative stress-induced sustained activation of both JNK and another stress kinase, p38, and a loss of oxidative stress-induced apoptosis (Tobiume et al., 2001). These observations indicate that ASK1 plays an essential role in oxidative stress signalling and it will be interesting to investigate whether ASK1 is involved in the oxidative stress-induced, JNK-dependent activation of FOXO.
Normal function of the JNK signalling pathway depends on protein-protein interactions. Indeed, signalling specificity of JNK may be mediated through the formation of protein complexes. Recent studies have identified JIP1, JIP2 and JIP3 as scaffold proteins that interact with multiple components of the JNK pathway and facilitate JNK activation *in vivo* (Kelkar et al., 2000; Whitmarsh et al., 2001; Yasuda et al., 1999). From these three scaffold proteins, JIP3 is most likely to be involved in the oxidative stress induced activation of JNK, since this scaffold protein was shown to bind JNK, MMK4, MKK7 and ASK1 (figure 4) (Matsuura et al., 2002). Moreover, exogenous JIP3 enhanced AKS1 and H_2O_2-induced activation of JNK3 (Matsuura et al., 2002). We are currently investigating the role of the JIP3 scaffold protein in H_2O_2-induced activation of JNK in our system in an attempt to further elucidate the pathway leading from an increase in oxidative stress to the activation of FOXO via Ral and JNK. Since phosphorylation of Thr447/Thr451 is not the only phosphorylation event occurring upon H_2O_2 treatment, it will also be interesting to investigate the role of other MAPK in the regulation of FOXO upon oxidative stress.

Role of FOXO in oxidative stress, metabolism and aging
At the beginning of the last century is was noted that animals with higher metabolic rates often have shorter life spans, indicating a link between metabolism and life expectancy. However, the mechanism behind this hypothesis was unknown. In the middle of the last century, Denham Harman postulated a ‘free radical theory of aging’, in which he hypothesised that endogenous oxygen radicals were generated in cells, resulting in a pattern of cumulative damage (Figure 5) (Harman, 1956). This would indicate that factors that increase resistance to stress in a cell should

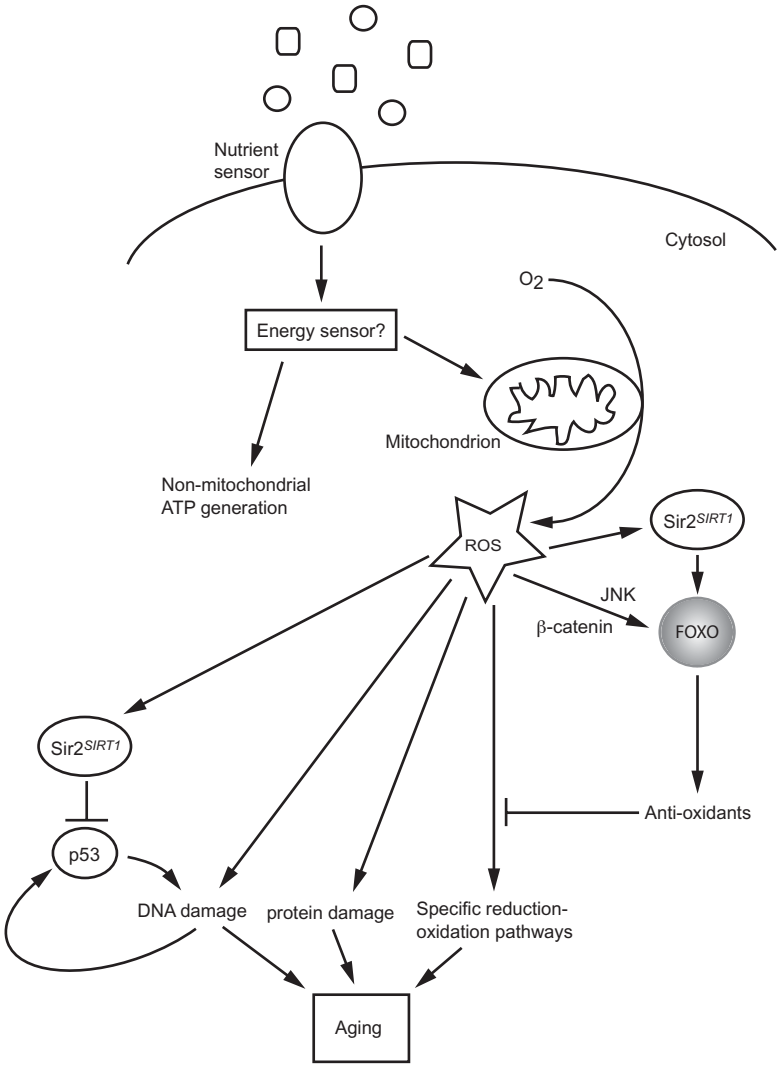

Figure 4. A current version of the free-radical theory of aging
Nutrients, detected by sensors such as the insulin receptor at the cell membrane, and oxygen fuel metabolism in the mitochondria, generate reactive oxygen species (ROS). The levels of ROS might determine the rate of aging by inducing random damage in proteins and DNA, and by activating specific pathways that depend on the reduction-oxidation status of proteins. DNA damage or recombination might be decreased by proteins that influence lifespan, including members of the Sir2^{SIRT1} family.

lead to enhanced life span of an animal. In support of this hypothesis, genetic links between stress responsiveness and longevity have been established in *C. elegans*, *Drosophila* and mice. Worms deficient in PI3K/PKB signalling have an increased

lifespan, increased SOD and catalase activities and thus increased resistance to oxidative stress (Honda and Honda, 1999). Both the increased lifespan and resistance to oxidative stress in these animals depend on the activation of DAF-16, the FOXO homologue in *C. elegans* (Honda and Honda, 1999). In *Drosophila*, elevated expression of SOD and catalase also result in enhanced stress tolerance and increased lifespan (Orr and Sohal, 1994; Parkes et al., 1998; Sun and Tower, 1999). Moreover, overexpression of dFOXO in the brain and fat body of the fly results in increased lifespan and increased resistance to paraquat (Giannakou et al., 2004; Hwangbo et al., 2004). These genetic data thus provide a link between longevity and oxidative stress, although it remains to be determined whether levels of oxidative stress are cause or consequence of aging.

One of the few genetic alterations demonstrated to increase lifespan in mammals is a homozygous deletion of the p66shc gene (Migliaccio et al., 1999). p66shc is one of the isoforms of the shc family of proteins, who are involved in insulin signalling. Insulin induces Ras activation primarily through Grb2/SOS binding to the p48/54shc isoforms, whereas Ras activation can be inhibited by p66shc (Bonfini et al., 1996). Mice lacking p66shc also display increased resistance to oxidative stress and it has been suggested that this may involve FOXO (Nemoto and Finkel, 2002). In p66shc-/- cells, oxidative stress-induced activation of PKB and consequently PKB-mediated phosphorylation and inactivation of FOXO3a is impaired (Nemoto and Finkel, 2002). However, a molecular mechanism as to how p66shc ablation could result in FOXO activation, rather than solely prevent its inhibition by PKB, remains to be determined. Since insulin induced Ras activation will lead to Ral activation (Wolthuis et al., 1998), this would suggest that p66shc can block Ral activation, thereby inhibiting Ral-dependent activation of FOXO under conditions of oxidative stress. This hypothesis is under current investigation.

Lifespan of mice lacking FOXO genes have not been investigated yet, but expression of FOXOs is increased in aging muscles or muscles from mice, which were caloric restricted. Together with the genetic data in other organisms and the potential link between p66shc and FOXO in mice, this indicates an important role for FOXOs in the protection against oxidative stress and aging.

In this thesis, we provide further evidence for a role of FOXO in the protection against oxidative stress. Together with the data on the effect of FOXO on proliferation, this indicates that also in mammals, FOXO is involved in the regulation of both processes, providing a link between aging and oxidative stress resistance in mammals. Both in *C. elegans* and *Drosophila*, FOXO signalling in specific tissues is responsible for the regulation of lifespan and protection against oxidative stress (Hwangbo et al., 2004; Libina et al., 2003) (Giannakou et al., 2004). Especially, signalling in the nervous system, intestine, and fat body seems to be important, the last two being important tissues involved in metabolism. Experiments with tissue-specific knockdowns of FOXO in mice might reveal whether a same pattern can be observed in mammals as well.

FOXO can protect cells from oxidative stress by increasing expression of MnSOD and catalase. Recently, several screens have been performed in *C. elegans* to identify genes involved in longevity in the worm (Lee et al., 2003) (Murphy et al.,

2003). The most frequent category of gene product that appeared to extend lifespan when knocked down in the worm comprised regulators of mitochondrial function. RNAi directed against several different elements of mitochondrial electron transport, a crucial step in metabolism, also increased lifespan. This indicates the importance of the mitochondria in the process of aging, by converting food into energy, thereby producing ROS. Thus, tight control of the function of mitochondria might be a critical step in the process of aging. This is further supported by the observation of premature aging in mice expressing a defective mitochondrial DNA polymerase, leading to accumulation of mutations in the mitochondrial DNA, and thus disfunction of the mitochondria (Trifunovic et al., 2004). Since FOXO seems to be an important regulator of aging, it will be interesting to investigate a possible link between FOXO signalling and the function of the mitochondria.

In conclusion, we have identified an interaction between two important pathways in proliferation and carcinogenesis. The interaction between FOXO and β-catenin signalling affects both signalling pathways and is further enhanced under conditions of oxidative stress. Furthermore, we unraveled the signalling pathway from increases in ROS levels to the activation of FOXO. These findings indicate an important role for FOXO in the defense against oxidative stress and provide further evidence for a link between protection against oxidative stress and longevity via the regulation of one single subfamily of transcription factors, FOXOs.

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Summary

FOXO Forkhead transcription factors are a subfamily of the large superfamily of Forkhead transcription factors, which consists of over 100 different members, expressed in species ranging from yeast to humans. Members of this family of transcription factors have been demonstrated to play important roles in cell proliferation and differentiation, both during development and in the adult organism. In addition to these roles in normal development, several Forkhead transcription factors have also been suggested to be involved in neoplasia. FOXO transcription factors, for example, have been shown to be part of chromosomal translocations associated with several forms of human cancer.

In recent years, a lot of research is performed to investigate the regulation and the role of the FOXO Forkhead transcription factors. Members of this subfamily have been identified in several organisms including in *Caenorhabditis elegans*, *Drosophila Melanogaster*, mice, and humans. FOXOs are mainly regulated by the phosphoinositide 3-kinase (PI-3K)/protein kinase B (PKB) pathway. Upon growth factor stimulation FOXOs are directly phosphorylated by PKB, resulting in nuclear export of FOXO and inhibition of the transcriptional activity. Several other kinases, which regulate phosphorylation of FOXO, have been identified as well. Also other processes like acetylation and ubiquitination may play a role in the regulation of FOXO activity. FOXO transcription factors have been implicated in to play a role in the regulation of a multitude of biological processes including cell cycle, apoptosis, DNA repair, differentiation, metabolism, and protection from oxidative stress. These data indicate a role for FOXOs as a meeting point within the cell, integrating several signalling pathways and regulating cell fate.

In *C. elegans*, the FOXO orthologue DAF-16 is involved in the regulation of longevity, dauer formation and stress resistance. Overexpression of DAF-16 in the worm will lead to an increased lifespan, correlated with increased resistance to oxidative stress. Recent data in mammalian systems show that FOXOs are involved in inducing a cell cycle arrest and quiescence. On the other side, FOXOs can protect cells from oxidative stress by regulating the expression of MnSOD and catalase, two anti-oxidant enzymes important in the defense to oxygen radicals. Thus, these data indicate, that, like in *C. elegans*, also in mammalian systems FOXOs can regulate both quiescence and stress resistance.

In this thesis we tried to further understand the role of mammalian FOXOs in the control of cellular oxidative stress. We focus on the possibility that FOXOs not only repress cellular oxidative stress by increasing anti-oxidants expression, but that cellular oxidative stress itself also signals to FOXOs, creating a feedback mechanism.

FOXO transcription factors can directly bind to regions within promoters of their target genes, thereby regulating the transcription of these genes. However, in recent years, a variety of cofactors for FOXO-induced effects on transcription have been described, for example p300 and several nuclear receptors. A yeast-two-hybrid screen for new interactors for β-catenin revealed FOXOs as potential binding partners. β-catenin is a multifunctional protein that regulates gene transcription within the Wnt signalling pathway by direct binding to members of the Lef-1/TCF family of transcription factors.

The results described in **Chapter 2** show that the binding of FOXO to β-catenin also occurs in cells, and that this binding enhances the transcriptional activity of FOXO. The binding between FOXO and β-catenin is induced under conditions of increased cellular oxidative stress, further increasing FOXO transcriptional activity under these conditions. In addition to the biochemical data, our genetic analysis in *C. elegans* demonstrate that this interaction is evolutionary conserved. The β-catenin homologue BAR-1 is required for DAF-16 dependent dauer formation, lifespan regulation, oxidative stress resistance and the expression of the DAF-16 target gene SOD-3 following oxidative stress. These data implicate β-catenin binding to FOXO as part of the mechanism by which cellular oxidative stress signals to FOXO.

The binding of β-catenin to FOXOs not only influences the function of FOXOs; this interaction also results in the inhibition of β-catenin/TCF transcriptional activity (**Chapter 3**). The β-catenin/TCF complex is activated upon Wnt signalling and it regulates transcription of several downstream target genes. β-catenin levels in a cell are tightly controlled by the APC/GSK3/Axin complex. In the absence of a Wnt signal, this complex targets β-catenin for degradation by inducing phosphorylation of β-catenin. The inhibition of β-catenin/TCF transcriptional activity is probably due to competition between TCF and FOXO for binding to β-catenin. The FOXO mediated inhibition also correlated with the ability of FOXOs to shuttle β-catenin out of the nucleus. Mutants of FOXO that are predominantly localized within the nucleus are impaired in their ability to inhibit β-catenin/TCF transcriptional activity and do not increase cytosolic levels of β-catenin, whereas mutants of FOXO that do inhibit β-catenin/TCF activity are predominantly localized in the cytosol and also increase cytosolic β-catenin levels. Finally, the increased binding of FOXO to β-catenin upon cellular oxidative stress also results in a further enhancement of the ability of FOXOs to inhibit β-catenin/TCF activity. These results further demonstrate a cross talk between FOXO and TCF signalling in which β-catenin is the central player.

Phosphorylation of FOXOs is regulated by several kinases, including PKB. Activation of the Ras/Ral pathway also results in phosphorylation of FOXO, as was described for FOXO4. Activation of Ral results in phosphorylation of FOXO4 on two residues in the C-terminal part of the protein, Thr447 and Thr451. In **Chapter 4** we show that phosphorylation of these sites by the Ral pathway is increased under conditions of cellular oxidative stress. Upon treatment of cells with H_2O_2, Ral is activated and this results in JNK dependent phosphorylation of Thr447 and Thr451. Oxidative stress induces nuclear localization of FOXO4 and an increase in the transcriptional activity of the protein. In addition, we show that this signalling pathway is also employed by TNFα to activate FOXO4 transcriptional activity. Taken together, these data provide evidence for a feedback mechanism from oxidative stress to FOXO transcription factors, involving the small GTPase Ral and the stress kinase JNK. In the **Addendum** of **Chapter 4** we compare different forms of stress and the ability of FOXOs to reduce JNK activation in cells in response to these stresses. Inhibition of JNK activation by FOXOs in response to oxidative stress is dependent on MnSOD, since FOXOs can no longer inhibit JNK activity after H_2O_2 treatment in SOD-/- MEFs. However, FOXOs can still reduce JNK activation in response to UV treatment in these cells. Experiments in TKO MEFs, which lack all members of the RB family and thus are

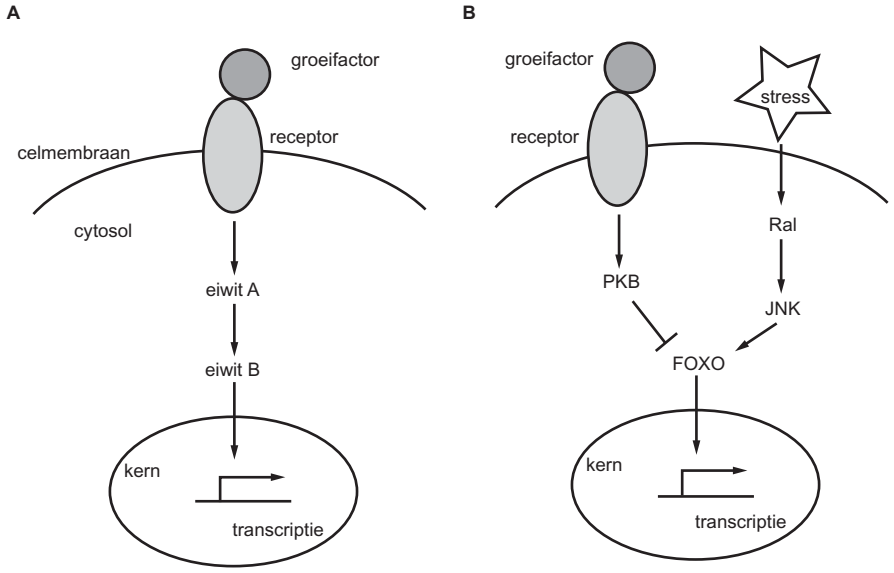
incapable of going into cell cycle arrest, indicate an involvement of a cell cycle arrest in stress induced by H_2O_2, whereas UV induced stress is likely to be independent of the ability to go into cell cycle arrest.
In conclusion, this thesis describes the role of FOXOs in oxidative stress signalling in more detail. We show that FOXOs are part of a feedback mechanism, in which an increase in ROS levels in a cell will signal towards FOXO, leading to Ral-mediated, JNK-dependent phosphorylation and activation of FOXO. This results in activation of antioxidant mechanisms in a cell, and thus protection of the cell. Furthermore, we have identified a new binding partner for FOXOs, β-catenin. Interaction between these two proteins is increased under conditions of oxidative stress and affects both FOXO and β-catenin/TCF signalling in a cell. These findings indicate an important role for FOXO in the defense against oxidative stress. Together with its role in cell cycle regulation this further indicates a link between the protection against oxidative stress and the regulation of the cell cycle and quiescence via the regulation of one subfamily of transcription factors, FOXOs.

Nederlandse samenvatting voor de leek

Het menselijk lichaam is opgebouwd uit weefsels, organen en systemen, zoals spieren, longen en de bloedsomloop. Al deze systemen samen zorgen ervoor dat ons lichaam optimaal kan functioneren. De weefsels in het lichaam zijn weer opgebouwd uit miljoenen cellen. Deze cellen zijn microscopisch kleine eenheden die de werkelijke bouwstenen vormen van ons lichaam. Een cel bestaat uit een kern met daaromheen het cytosol (figure 1). In de kern van een cel bevindt zich het DNA. DNA bevat de code waarin onze erfelijke informatie is vast gelegd. DNA is opgedeeld in kleine stukjes, genen genaamd, die coderen voor de aanmaak van eiwitten. Elk gen bevat de code voor een ander eiwit. Al die genen bij elkaar bevatten dus de code voor heel veel eiwitten. Die eiwitten zijn essentieel voor de cel. Met die eiwitten kan de cel namelijk zijn vorm en stevigheid bepalen, eiwitten maken uit of een cel een spiercel of een huidcel wordt, en sommige eiwitten zijn betrokken bij allerlei processen in een cel. Het zijn de eiwitten die reguleren of een cel moet groeien. Ook zorgen zij ervoor dat cellen kunnen verdubbelen als er andere cellen moeten worden vervangen. En eiwitten zorgen ook voor het contact van de cel met zijn naburige cellen. Kortom, het zijn belangrijke werkpaarden voor een cel en het is daarom belangrijk dat de cel, op het moment dat dat nodig is, op een goede manier van de code in het DNA een eiwit maakt. Hier mogen geen fouten plaats vinden anders kan het gebeuren dat er een eiwit gemaakt wordt, dat niet meer functioneert of juist te goed zijn best gaat doen. Een fout in het DNA waardoor het maken van het gecodeerde eiwit ontregeld raakt noemen we een mutatie. Het ontstaan van deze fouten kan allerlei oorzaken hebben: ze kunnen ontstaan door factoren van buiten de cel, zoals de rook van sigaretten, maar ook door fouten in het kopieren en verdubbelen van het DNA, als een cel gaat delen.
Het onderzoek dat ik beschreven heb in dit proefschrift valt onder de noemer signaal transductie, maar wat bedoelen we daar nu mee? Zo nu en dan zijn er in het lichaam cellen die vervangen moeten worden, bv omdat de cellen te oud zijn, niet meer goed functioneren of dood zijn gegaan. Dit houdt in dat de cel zich moet gaan delen waardoor uit die ene cel twee cellen zullen ontstaan. De regulatie van deze celdeling is heel strak gecontroleerd. Als er te weinig nieuwe cellen worden gemaakt, kunnen de versleten cellen niet worden vervangen en kan het weefsel minder goed functioneren. Als echter te veel nieuwe cellen worden gemaakt kan er een tumor ontstaan. Een goede controle van dit proces is noodzakelijk voor het goed blijven functioneren van je lichaam. Naburige cellen helpen bij het controleren van de deling van een cel door een groeifactor uit te scheiden. Een groeifactor is een klein eiwit dat kan binden aan specifieke eiwitten die op de buitenkant van een cel zitten, de receptoren (figure 1). Wanneer een groeifactor aan de receptor bindt wordt de receptor aangezet, geactiveerd. Deze actieve receptor kan op zijn beurt andere eiwitten in de cel activeren. Zo ontstaat er een cascade aan eiwitten die elkaar activeren of inactiveren. Deze eiwit cascade eindigt in de kern waar de laatste eiwitten in de cascade de celdeling regelen door het aan- of uitzetten van genen. Deze cascade van eiwitten, die ervoor zorgen dat het binden van de groeifactor aan de receptor leidt tot het regelen van de celdeling in de kern wordt een

signaaltransductie route genoemd. Als er nu onverhoopt een mutatie optreedt in het DNA kan het dus gebeuren dat een eiwit uit zo'n signaaltransductie route abnormaal gaat functioneren. Een eiwit kan niet meer functioneren waardoor het signaal niet goed door gegeven kan worden. Maar het kan ook gebeuren dat een eiwit te goed zijn best gaat doen en signalen blijft doorgeven, ongeacht de aanwezigheid van een groeifactor. Dit kan leiden tot een ontregeling van de celdeling en dus een ongecontroleerde groei van aantal cellen zonder dat dat nodig is. In het ergste geval kan dat leiden tot een tumor.

Wij proberen te achterhalen welke eiwitten in deze signaaltransductie routes zitten en welk effect het doorgeven van een bepaalde signaal heeft op het functioneren van de cel. Wij kijken hierbij naar een specifieke signaaltransductie route: de route waarbij het eiwit FOXO een centrale rol spelt. FOXO is een eiwit dat aan of uit gezet kan worden door een ander eiwit, PKB. Als er een groeifactor op de receptor van de cel gaat zitten, bv insuline, wordt het eiwit PKB geactiveerd. Dit actieve PKB zorgt ervoor dat ons eiwit FOXO geremd wordt en in het cytosol van de cel blijft zitten. Maar als er geen groeifactoren aanwezig zijn aan de buitenkant van de cel wordt PKB niet geactiveerd en dus FOXO niet geremd. FOXO gaat dan naar de kern van



Signaal transductie van buiten de cel naar de kern
A. Buiten de cel zijn er allerlei factoren, zoals groeifactoren, die invloed hebben op het delingsproces van een cel. Wanneer een groeifactor aan de receptor gaat binden, wordt de receptor actief. De actieve receptor kan eiwitten in de cel activeren en zo een signaal van de buitenkant van de cel doorgeven naar de kern. In de kern vindt dan de regulatie plaats van onder andere de aanmaak van nieuwe cellen en de celdeling. **B.** Groeifactoren activeren het eiwit PKB, dat ervoor zorgt dat het FOXO eiwit inactief blijft. Oxidatieve stress activeert de eiwitten Ral en JNK die ervoor zorgen dat het FOXO eiwit actief wordt en in de kern als transcriptie factor aan het werk kan.

de cel, waar het kan functioneren als een transcriptie factor. Transcriptie factoren zijn eiwitten die zich bezig houden met het al dan niet activeren van genen, en dus met het aanmaken van nieuwe eiwitten. Als FOXO in de kern komt zal FOXO ervoor zorgen dat er eiwitten gemaakt worden die ervoor zorgen dat de cel stopt met delen, of zelfs dood gaat. Ook kan het eiwitten laten maken die de cel zullen beschermen tegen radicalen, moleculen die voor fouten in eiwitten en DNA kunnen zorgen.

Wij hebben in meer detail gekeken naar de manier waarop FOXO de cel kan beschermen tegen die schadelijke radicalen, die onder andere ontstaan bij oxidatieve stress. We zijn op zoek gegaan naar eiwitten die samen met FOXO een gen kunnen activeren. **Hoofdstuk twee** beschrijft de samenwerking van FOXO met zo'n eiwit, β-catenin. β-catenin is een belangrijk eiwit in een ander signaaltransductie pad. Wij hebben gevonden dat FOXO en β-catenin in cellen aan elkaar kunnen binden. De binding wordt sterker als de cellen oxidatieve stress krijgen. De binding van β-catenin aan FOXO zorgt ervoor dat FOXO beter gaat functioneren en dus een betere bescherming kan geven tegen de stress. Wij hebben niet alleen naar deze eiwitten in cellen gekeken, maar ook naar het functioneren van deze eiwitten in een heel organisme, de worm C. elegans. Als je in deze worm zorgt dat FOXO altijd actief is zal de worm langer leven en beter tegen stress kunnen. Wij laten zien dat FOXO β-catenin nodig heeft om optimal te functioneren in de worm. Al deze proeven laten zien dat er cross talk plaats vindt tussen twee signaaltransductie routes, en dat deze cross talk belangrijk is als cellen onder oxidatieve stress staan.
De binding tussen FOXO en β-catenin blijkt niet alleen effect te hebben om de functie van het FOXO eiwit. Onder normale omstandigheden werkt het eiwit β-catenin samen met een ander eiwit, TCF. TCF is ook een transcriptie factor, net als FOXO, en kan dus in de kern genen aanzetten tot het maken van nieuwe eiwitten. Echter, als FOXO aanstaat, zal FOXO binden aan β-catenin. Daardoor kan b-catenin niet meer binden aan TCF en is TCF niet meer in staat om genen te activeren (**Hoofdstuk drie**). Bovendien zorgt FOXO ervoor dat β-catenin uit de kern gaat, weg van TCF en het DNA.
FOXO kan er dus voor zorgen dat eiwitten worden gemaakt die de cel beschermen tegen radicalen die ontstaan bij oxidatieve stres. Wij vroegen ons af of een toename aan radicalen in een cel effect heeft op FOXO. In **Hoofdstuk vier** laten we inderdaad zien dat bij een toename aan radicalen door het geven van oxidatieve stress FOXO aangezet wordt. We laten zien welke eiwitten ervoor zorgen dat FOXO geactiveerd wordt en dat dit ervoor zorgt dat FOXO naar de kern gaat. Deze proeven laten zien dat er een feedback mechanisme in de cel aanwezig is: een toename in radicalen zorgt ervoor dat FOXO actief wordt, FOXO gaat naar de kern en zorgt ervoor dat eiwitten aangemaakt worden die weer voor kunnen zorgen dat de radicalen opgeruimd worden. In het **Addendum** van **Hoofdstuk vier** laten we zien dat FOXO mogelijk ook belangrijk is bij de bescherming tegen een andere vorm van stress, UV straling. Hoe dit precies werkt hebben we nog niet ontrafeld.

Samenvattend hebben we dus aangetoond dat FOXO een belangrijke rol speelt in de bescherming van cellen tegen oxidatieve stress. Daarbij krijgt FOXO hulp van

een ander eiwit, b-catenin, dat ervoor zorgt dat FOXO nog beter gaat functioneren. Oxidatieve stress zorgt voor het ontstaan van radicalen in de cel. Die radicalen kunnen grote schade aanbrengen aan eiwitten en DNA, waardoor de kans bestaat dat de signaaltransductie in een cel, en dus de controle op de celdeling, verstoord wordt, met allerlei mogelijke gevolgen. Onze resultaten duiden erop dat het belangrijk is om normaal functionerend FOXO in je cellen te hebben om schade door radicalen te voorkomen.

Curriculum Vitae

Marieke A.G. Essers werd geboren op 29 juli 1976 te Zevenaar. Na het behalen van het VWO diploma aan het Elshof College te Nijmegen, begon zij in 1994 met de studie Medische Biologie aan de Universiteit Utrecht. In de laatste fase van de studie deed zij onderzoekservaring op bij de afdeling Hematologie van het Universitair Medisch Centrum Utrecht onder begeleiding van Drs. V.A.J. Smits en Dr. R.H. Medema, en bij de vakgroep Moleculaire Biologie van het Nederlands Kanker Instituut in Amsterdam onder begeleiding van Dr. S.R. Wicks en Prof. Dr. R. Plasterk. Het doctoraal-diploma werd behaald in december 1999. Van januari 2000 tot september 2004 was zij werkzaam als assistant in opleiding bij de afdeling Fysiologische Chemie van het Universitair Medisch Centrum Utrecht, onder leiding van Prof. Dr. Ir. B.M.T. Burgering en Prof. Dr. J.L. Bos. De resultaten van dit onderzoek zijn beschreven in dit proefschrift.

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Dankwoord

Nog een paar woorden en dan is het klaar, de afgelopen AIO jaren gebundeld in iets meer dan 100 pagina's. Gelukkig heb ik het niet alleen hoeven doen en zijn er heel veel collega's, vrienden en familie die me met raad en daad hebben bij gestaan. Mede daardoor zijn het fantastische jaren geworden. Bedankt!

Natuurlijk zijn er een aantal mensen die ik in het bijzonder wil bedanken. Beste Boudewijn, lieve Bom. Wat hebben wij veel geleerd de afgelopen jaren. Ik hoe je eerst de kleine goede proef moet doen voordat je mega experimenten inzet voor niks, hoe je positief moet kijken naar je proeven en bij een presentatie niet altijd de negatieve kant moet benadrukken. Jij hoe je een eigen agenda moet bijhouden, je moet bewegen in de politieke kringen van een universiteit en hoe je moet toezien dat je postdocs, AIOs en analisten de proeven doen en jij achter de computer zit. Bedankt voor de stroom aan nieuwe modellen, de positieve draai aan mislukte proeven, kritische noten en support. Wie wordt nu je assistente?
Beste Hans, ik heb bewondering voor de manier waarop jij je groep leidt en in de wetenschap staat. We hebben elkaar vanaf de zijlaan gevolgd, maar zonder jouw kritische kanttekeningen was ik zeker niet zo ver gekomen. En blijf vooral doorgaan met die meetings op Spetses, want een AIO kan zich geen betere kennismaking met de grote namen in de wetenschap voorstellen dan met een Mythos op het strand!

Naast twee promotoren waren er nog veel meer mensen op het lab die ik wil bedanken. Lieve Lydia, waar zou ik zijn zonder jouw geweldige proeven en heerlijk geklets over van alles en nog wat. Heel erg bedankt voor alles wat je op het lab voor me gedaan hebt en voor het er gewoon zijn. Je bent een top analist en vrouw! Ingrid, een eigenwijze AIO en nog eigenwijzere analist. Toch heeft dat mooie proeven op geleverd. Ik hoop dat je je plekje snel zult vinden, succes daarmee. Rik, het heeft wat bloed, zweet en tranen gekost maar het is ons gelukt, hoofdstuk 2 is een stuk om trots op te zijn! Zonder jouw mooie proeven en secure kijk op het manuscript was dat zeker niet gelukt.
Pieter, AIO van het eerste uur, heerlijk om iemand te hebben waar je al die jaren mee kan optrekken en mee kan delen. Heel veel succes in de States. Wanneer gaan we nou tequila's drinken op een zonig, mexicaans strand? Drs J, zonder jouw grapjes en goede gesprekken waren de afgelopen jaren een stuk saaier en moeilijker geweest. Nog effe doorzetten en dan ligt de wereld aan je voeten! Lieve Sanne, van nieuwe collega's al heel snel naar maatjes op en buiten het lab. Het worden spannende tijden voor ons allebei, maar waar ik ook zit, ik zal er voor je zijn. Fried, uitlaatklep, grapjes uithalen, Heidelberg onveilig maken, gesprekken over de wetenschap en het leven, bedankt voor heel veel. Ik hoop dat de toekomst brengt wat jij ervan verwacht en dat we nog vaak samen een biertje zullen pakken, waar dan ook ter wereld. Armando, wat moet ik nou zonder jouw gezang en gegil? Wat zal het leven saai zijn op een lab zonder jouw aanwezigheid. En wanneer mag dat sikje er nou af? Bea, samen met Pieter de

laatste maanden door schept zeker een band. Maar nu zijn we alle drie klaar, lekker he! Arjan, onze top postdoc, jouw kritische blik is absoluut een aanwinst voor de groep. Blijf je me op de hoogte houden van alle roddels! Margarita, vanaf het moment dat je met je zuidelijke temperament het lab opkwam ben je niet meer weg te denken uit mijn (lab)leven. Heel veel geluk met je nieuwe gezinnetje (wel na het hora est graag).Holger, thanks for the company during all the evening and weekend experiments. When will be the next campfire? Marta, thanks for being such a nice, kind and open person. You're the best. Marta B., why do we work so far apart? Always a pleasure to have you around in Utrecht. Wendy, het gaat goed komen met jou en je toekomstdromen, ik weet het zeker. Anna, don't be afraid for the big boys in the lab, you can handle them, I'm sure. Good luck with shc. Niels, doet de mass spec het alweer? Succes met alle banden. Paulien, vrouw naar mijn hart. Recht door zee, niet miepen maar aanpakken. Nog even en dan commandant van de brandweer? Joost, met gezellige, brabantse mensen op het lab wordt het altijd feest. Miranda, wat heerlijk om je een dag in de week terug te hebben op het lab, ook al betekende dat elke Vrijdag weer puzzelen over Lydia's blotjes. John, waar ben je? We missen je op het lab. Roland, Oss valt best mee, ik geef het toe. Matthijs, wat jammer dat we maar zo kort collega AIOs zijn geweest. Wanneer gaan we weer eens skien, ik wil revanche! Jerome, wormen zijn toch veel leuker dan cellen. Doe je wel rustig aan met alle vrouwen in Boston? Karen, good luck with the Dutch people and language. Leo, mooi voorbeeld van zelfstandige postdoc en echte engelsman. Jun, good luck with all your stainings. Fons, ik hoop dat het snel beter met je gaat. Piet, het onderwijs zit er voor ons beiden op. Geniet van je vrije tijd! Annelies, succes met de brug slaan tussen wetenschap en samenleving.

Natuurlijk wil ik ook alle studenten bedanken die de afgelopen jaren op het lab hebben rond gelopen. In het bijzonder Anja, jouw stageperiode was voor ons allebei een leerzame tijd. Succes met je toekomstplannen. En waar zou ik zijn geweest zonder Wim, Arnoud en Patrick! Mijn laptop en ik hebben dit proefschrift avontuur overleefd, met dank aan jullie. Niet te vergeten natuurlijk de meiden van het secretariaat. Felicia, Marit, Marianne, Saskia, en Beatrice, het waren niet alleen de hulp bij pakjes versturen en dingen regelen, maar zeker ook de gezellige praatjes en luisterende oren die jullie zo bijzonder maken.

Er kwamen mensen bij maar er gingen ook mensen weg. Geert, Johan, Nancy, Joy, Kris, en Kim, bedankt voor de leuke tijden samen. Dominique, you can come back now, I stopped doing wester blots. Jorrit, ja ik geef toe, ik heb je grappen en grollen de afgelopen maanden wel gemist. Marita, thanks for being a friend, although you're there and I'm here. Marion, samen met de trein naar Heidelberg, heerlijke taartjes eten, en Hefenweissen drinken. Nu maar eens met de mannen naar het Sauerland!

Er waren niet alleen Bossers en Burgerings. De laatste maanden ben ik op het lab geadopteerd door de Timmers. Bedankt voor jullie gastvrijheid en ik snap

nu een stuk beter waarom de deur af en toe dicht gegooid wordt als Armando weer gaat gillen! Ook de van der Vliets en Holsteges wil ik bedanken voor hulp en gezelligheid de afgelopen jaren. En niet te vergeten de mensen achter de schermen; Marcel, Kees, Ina, Marjan, Ton, Gerrit en Marjoleine, bedankt.

Naast alle mensen op het lab waren er ook heel mensen achter de schermen aanwezig. Lieve oude wijven, weten jullie wel hoe bijzonder ons clubje meiden is! En voor de mensen die zich afvragen waarom ik toch telkens naar het buitenland ging; deze meiden zijn het antwoord. Mas, huisgenoot zijn schept een bijzondere band. Ik ben dan ook vereerd dat je als paranimf vandaag weer dichtbij me staat. Ben benieuwd wat de toekomst ons allebei gaat brengen. Wordt het eerst Cuba of toch Suriname? Eel, het had zo leuk kunnen zijn; samen de laatste maanden door en tegelijk promoveren. Helaas, het mocht niet zo zijn. Maar ik weet zeker dat het weer helemaal goed komt met je en dat we volgend jaar weer een feestje vieren. Je kunt het! Ingrid, al zat er de laatste jaren wat water tussen, voor een nuchtere kijk op de zaak of een uitlaatklep heb ik je altijd weten te vinden. Wanneer gaan we je in mantelpakje zien? Viv, het waren roerige tijden maar het nieuwe avontuur is voor je begonnen. Bedankt voor je lieve zorgen en tot in Boston! Nienke, ondanks je drukke leven met Jasper blijf je altijd geinteresseerd en betrokken bij iedereen om je heen. Op nog vele kopjes thee in jullie tuin na een wandeling bij de plassen! Simone, een paar honderd kilometer snelweg weerhield je er niet van om er voor me te zijn in goede en slechte tijden. Heel veel geluk samen met Alwin. Sas, je was de eerste die ondanks een drukke agenda een vliegticket boekte om vandaag hier te zijn. Geweldig! Wendy, jouw creatieve geest zorgt altijd weer voor geweldige ideeen en leuke uitstapjes. Lieve meiden, op naar nog veel meer thee, chocola, wijn, weekendjes, knutselavondjes (ja, ik wil wel een stukje hoor) en gezelligheid! Mark, Jasper, Edu, Martijn en Alwin, ook jullie bedankt voor de leuke tijden!
Lieve Merijn, je komt elkaar tegen in de Alpen op een berg, je groeit naar elkaar toe, gaat dalen en bergen met elkaar delen en nu sta je naast me als paranimf! En natuurlijk, want het laatste stukje naar de top heb ik je zeker nodig. De toekomst wordt spannend voor ons allebei, op naar nieuwe toppen!
Juul en Suuz, jullie gaven mij de perfecte afleiding waardoor ik ook nog tijd met Rob heb door gebracht de laatste maanden. Brainstormen, organiseren en wijntjes in de tuin, het waren heerlijke avonden. Nu maar weer eens de Alpen in. Nadia en Sjoerd, vrienden vanaf het moment dat ik nog een klein, verlegen meisje was. Heel bijzonder om jullie nog steeds om me heen te hebben! Het bestuur van de regio midden Nederland en het hockeyteam van de provincie, bedankt voor de nodige ontspanning.

En dan de familie, zowel van mij als van Rob, bedankt voor jullie interesse en steun. Lieve Wim en Lia, vanaf het eerste moment stond jullie huis voor mij open. Ik mag dan wel eens een afwijkende mening hebben, ik bedoel het goed. Bedankt voor een tweede thuis. Rene, Lena, Lex en Mariella, na drukke tijden voor ons allemaal moeten we binnenkort maar weer eens lekker gaan stappen en

bijkletsen. Nu een keertje in Utrecht?

En last but not least, lieve pap en mam. Zonder jullie basis had ik hier nooit gestaan. Weten jullie wel hoe fijn het is om te weten dat er altijd twee mensen zijn die er voor je zijn, die je steunen, onvoorwaarderlijk achter je staan! Bedankt voor jullie liefde, kritische blik, levenslessen en heerlijke thuis. Jullie zijn super mensen!
Lieve zussen, natuurlijk vergeet ik jullie niet. Thekla, ik heb enorme bewondering voor de manier waarop jij het afgelopen jaar hebt aangepakt en ben erg benieuwd waar de toekomst je zal brengen. Heel veel succes met jouw ontdekkingstocht door de wereld! Eline, je blijft mijn kleine zusje, maar wel een die heel snel groot geworden is. Ik hoop dat je een leuke werkplek gaat vinden waar je door kunt groeien en mensen kunt helpen. Maar vergeet bovenal niet te genieten van je vrijheid en alle leuke dingen om je heen! Arnoud, het zal niet mee vallen om in een meidengezin binnen te komen, maar je houdt je goed staande. Succes met de toekomst. Oma, heel bijzonder dat u dit mag mee maken.

Lieve Rob, rots in de branding, de laatste woorden zijn voor jou. Het viel niet mee, de laatste maanden, maar we hebben het ook wel uitgezocht. Ik kan me geen twee werkvloeren bedenken die zover van elkaar liggen als die van jou en mij. Bedankt voor je geduld, steun, vertrouwen, liefde en nog veel meer. Nu breken spannende tijden aan, de wereld ligt aan onze voeten, wat gaan we ermee doen. De weide wereld in natuurlijk, jij en ik, op een nieuwe ontdekkingstocht. Ik heb er zin in!

Marieke