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FOXO transcription factors at the interface between longevity and tumor suppression

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A wide range of human diseases, including cancer, has a striking age-dependent onset. However, the molecular mechanisms that connect aging and cancer are just beginning to be unraveled. FOXO transcription factors are promising candidates to serve as molecular links between longevity and tumor suppression. These factors are major substrates of the protein kinase Akt. In the presence of insulin and growth factors, FOXO proteins are relocalized from the nucleus to the cytoplasm and degraded via the ubiquitin–proteasome pathway. In the absence of growth factors, FOXO proteins translocate to the nucleus and upregulate a series of target genes, thereby promoting cell cycle arrest, stress resistance, or apoptosis. Stress stimuli also trigger the relocalization of FOXO factors into the nucleus, thus allowing an adaptive response to stress stimuli. Consistent with the notion that stress resistance is highly coupled with lifespan extension, activation of FOXO transcription factors in worms and flies increases longevity. Emerging evidence also suggests that FOXO factors play a tumor suppressor role in a variety of cancers. Thus, FOXO proteins translate environmental stimuli into changes in gene expression programs that may coordinate organismal longevity and tumor suppression.
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The FOXO family of Forkhead transcription factors

FOXO transcription factors belong to the large Forkhead family of proteins, a family of transcriptional regulators characterized by a conserved DNA-binding domain termed the 'forkhead box' (Kaestner *et al*., 2000). The Forkhead family is present in all eukaryotes. In humans, the Forkhead family is comprised of 39 distinct members, which have been divided into 19 subgroups (FOX for 'Forkhead Box' A to S). FOX transcriptional regulators play a wide range of roles during development, from organogenesis (FOXC) to language acquisition (FOXP) (Lehmann *et al*., 2003).

Among the Forkhead family, the FOXO subgroup contains four members (FOXO1, FOXO3, FOXO4, and FOXO6). The FOXO family was initially identified in humans because three members of this family, FOXO1/FKHR, FOXO3/FKHRL1, and FOXO4/AFX, were found at chromosomal translocations in human tumors (Galili *et al*., 1993; Davis *et al*., 1994; Parry *et al*., 1994; Borkhardt *et al*., 1997; Hillion *et al*., 1997; Anderson *et al*., 1998). These initial findings suggest that FOXO transcription factors might play an important role in tumor development.

FOXO1, FOXO3, and FOXO4 mRNAs are expressed to varying degrees in all tissues in mammals (Anderson *et al*., 1998; Furuyama *et al*., 2000; Biggs and Cavenee, 2001). FOXO1 mRNA is particularly abundant in adipose tissues, FOXO3 mRNA is highly expressed in the brain, and FOXO4 mRNA is abundant in the heart. FOXO6 mRNA is predominantly expressed in the developing brain, indicating that FOXO6 may play an important role in the nervous system (Jacobs *et al*., 2003) (Table 1).

Regulation of FOXO transcription factors in response to insulin and growth factors

Direct phosphorylation of FOXO transcription factors by the protein kinase Akt

The FOXO family of transcription factors is one of the major direct substrates of the protein kinase Akt in response to cellular stimulation by growth factors or insulin (Figure 1). Binding of growth factors or insulin to their tyrosine kinase receptors triggers the recruitment and activation of the phosphoinositide kinase (PI3K), which in turn activates several serine/threonine kinases, including the Akt family of protein kinases and the related SGK (serum and glucocorticoid inducible kinase) family of protein kinases (Cantley, 2002). The importance of the PI3K–Akt/SGK pathway in multicellular organisms is underlined by the conservation of this pathway from worms to mammals.

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Table 1 FOXO family members in mammals

Gene name	*Alternate names*	*Mouse name*	*Human chromosomal location*	*Chromosomal translocation*	*Cancer type associated with translocation*	*Expression pattern*	*Knockout phenotype*
FOXO1a	FKHR	fkhr1, Foxo1a	13q14.1	t(2:13)(q35;q14) PAX3:FOXO1 and t(1:13)(p36;q14) PAX7:FOXO1	Alveolar rhabdomyosarcomas	Ubiquitous. Highest in He, Sp, Ad, Ki, Br	E10.5 lethality angiogenesis defects
FOXO1b	FKHR pseudogene 1 (FKHRP1)		5q35.2–35.3				
FOXO3a	FKHRL1, AF6q21, FOXO2	fkhr2, Foxo3a	6q21	t(6;11)(q21;q23) MLL:FOXO3	Secondary acute myeloblastic leukemia	Ubiquitous. Highest in He, Br, Sp, Lu, Ki, Ad, Ov	Female sterility, anemia, glucose uptake defects, overproliferation of helper T cells, increased neutrophil apoptosis
FOXO3b	FKHRL1 pseudogene 1 (FKHRL1P1)		17p11				
FOXO4	AFX, AFX1, MLLT7	afx, Afxh, Foxo4, Mllt7	Xq13.1	t(X;11)(q13;q23) MLL:FOXO4	Acute leukemias	Ubiquitous. Highest in He, Br, Sp, Lu	Viable, no defects reported yet
FOXO5	zFKHR		Fish ortholog of FOXO3a				
FOXO6	FOXO6	Foxo6	1p34.1	None identified		Br, Th, Ki	Not done

He, heart; Sp, spleen; Ad, adipose tissue; Ki, kidney; Br, brain; Lu, lung; Ov, ovaries; Th, thymus

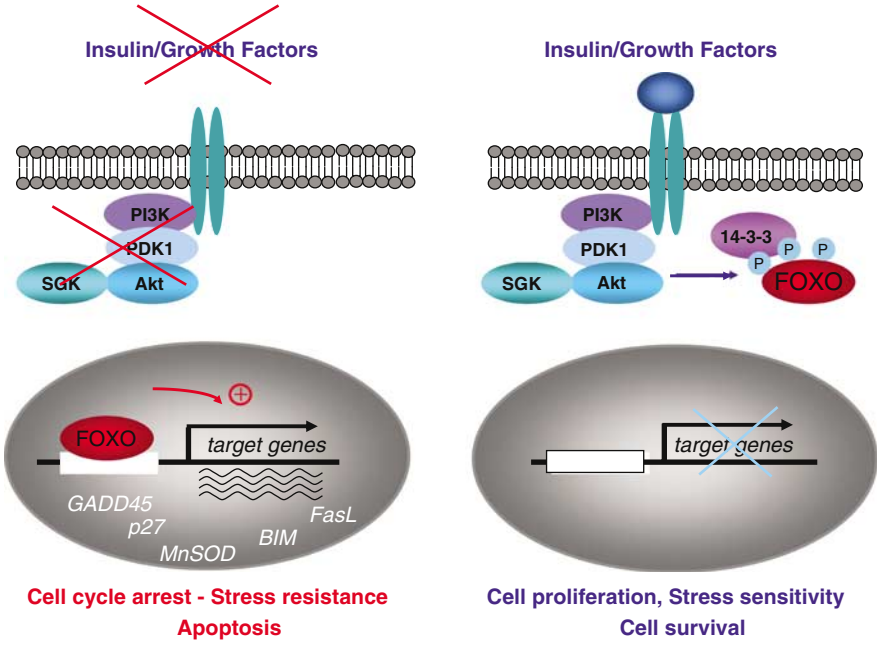

Figure 1 Regulation of FOXO transcription factors by insulin and growth factors. In the absence of insulin or growth factors, FOXO transcription factors are localized in the nucleus, where they cause cell cycle arrest, stress resistance, and cell death, by upregulating a series of key target genes. In the presence of insulin or growth factors, the PI3K–Akt/SGK pathway is activated. Akt and SGK translocate to the nucleus where they directly phosphorylate FOXO transcription factors on three conserved residues. Phosphorylated FOXO factors bind to 14-3-3 proteins, which result in the export of FOXO factors from the nucleus into the cytoplasm. The sequestration of FOXO into the cytoplasm inhibits FOXO-dependent transcription and allows cell proliferation, stress sensitivity, and cell survival. p27, cyclin-dependent kinase inhibitor (p27KIP1); MnSOD, manganese superoxide dismutase; FasL, Fas ligand; GADD45, growth arrest and DNA damage-inducible protein 45

Initial genetic studies in worms indicated that the FOXO family is a key downstream target of the PI3K–Akt pathway in development and longevity (Lin *et al*., 1997; Ogg *et al*., 1997). Biochemical studies in mammalian cells have shown that Akt directly phosphorylates FOXO transcription factors (Biggs *et al*.,

1999; Brunet *et al*., 1999; Kops and Burgering, 1999; Nakae *et al*., 1999; Rena *et al*., 1999; Tang *et al*., 1999) and have elucidated the mechanisms by which Akt regulates FOXO transcription factors (Biggs *et al*., 1999; Brunet *et al*., 1999). Phosphorylation of FOXO factors by Akt triggers the rapid relocalization of FOXO proteins from the nucleus to the cytoplasm. Akt phosphorylates FOXO1, FOXO3, and FOXO4 family members at three key regulatory sites (Thr32, Ser253, and Ser315 in the FOXO3 sequence) that are conserved from *Caenorhabditis elegans* to mammals and are part of a perfect consensus sequence for Akt phosphorylation (RXRXX(S/T)) (Alessi *et al*., 1997) (Figure 2). The related protein kinase SGK also phosphorylates FOXO factors (Brunet *et al*., 2001). Surprisingly, Akt and SGK preferentially phosphorylate a different combination of sites in FOXO factors (Brunet *et al*., 2001). Akt preferentially phosphorylates Ser253 and SGK favors the phosphorylation of Ser315. Thr32 is phosphorylated by both kinases. The three FOXO regulatory sites are phosphorylated in response to a number of growth factors, including insulin-like growth factor I (IGF-I) (Brunet *et al*., 1999; Rena *et al*., 1999), insulin (Kops and Burgering, 1999; Nakae *et al*., 1999), interleukin 3 (Dijkers *et al*., 2000a), erythropoietin (Kashii *et al*., 2000), epidermal growth factor (Jackson *et al*., 2000), and nerve growth factor (Zheng *et al*., 2002). Thus, FOXO transcription factors integrate a broad range of external stimuli via phosphorylation of three conserved residues by Akt and SGK. The preferential phosphorylation of FOXO residues by different protein kinases may allow FOXO factors to selectively respond to closely related but different stimuli, such as insulin and IGF-I (Nakae *et al*., 2000).

Insulin and growth factors also trigger the phosphorylation of several other sites of FOXO factors. For example, FOXO1 is phosphorylated at Ser322 and Ser325 in response to growth factor stimulation (Rena *et al*., 2002). Phosphorylation of Ser322 and Ser325 in FOXO1 appears to be mediated by casein kinase 1 (CK1) and is 'primed' by the phosphorylation of Ser329 (Rena *et al*., 2002) (Figure 2). Ser329 can be phosphorylated by the dual tyrosine (Y) phosphorylated regulated kinase 1 (DYRK1) (Woods *et al*., 2001), a member of the MAP kinase family. Interestingly, all these phosphorylation events participate in the regulation of FOXO subcellular localization (see below).

Regulation of FOXO factors by changes in subcellular localization

The major consequence of the phosphorylation of FOXO transcription factors by Akt and SGK is a change in the subcellular localization of these transcription factors (Biggs *et al*., 1999; Brunet *et al*., 1999; Takaishi *et al*., 1999). In the absence of growth factors, when Akt and SGK are inactive, FOXO factors are localized within the nucleus. When cells are exposed to growth factors, the PI3K–Akt/SGK cascade is activated and triggers the export of FOXO factors to the cytoplasm. Mutation analyses have revealed that one or two leucine-rich domains in the conserved C-terminal

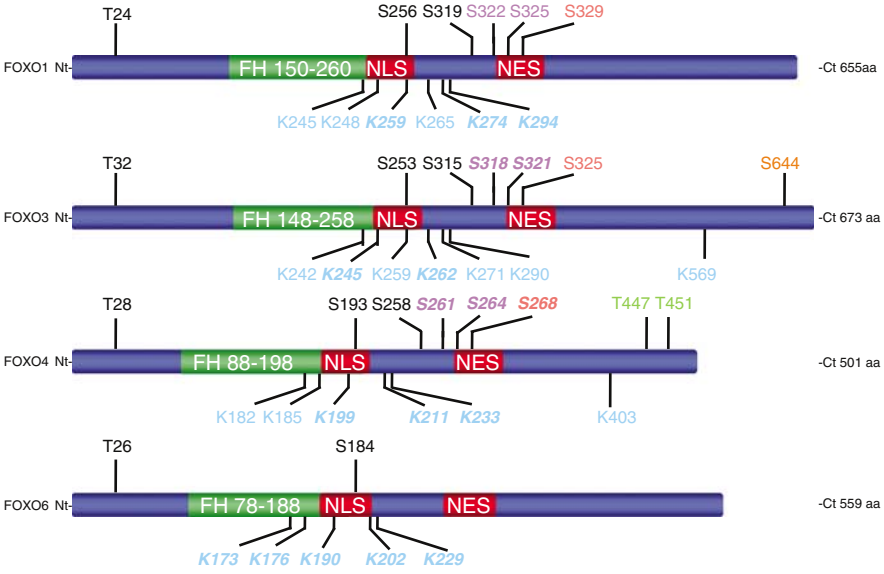

Figure 2 Phosphorylation and acetylation sites of FOXO family members. FOXO transcription factors are regulated by phosphorylation and acetylation in response to insulin, growth factors, and stress stimuli. FOXO post-translational modifications alter mostly FOXO subcellular localization, and also affect FOXO degradation, DNA-binding ability, transcriptional activity, or protein–protein interactions. Sites that are conserved in FOXO members but that have not yet been confirmed to be modified in a particular isoform are italicized. Stress-induced phosphorylation sites of FOXO3 identified by tandem mass spectrometry (Ser90, Ser284, Ser294, Ser300, Ser413, Ser425, Thr427, and Ser574) were not included because of space. Akt sites (black); SGK, serum and glucocorticoid inducible kinase (black); IKKβ, IκB kinase β (orange); JNK, Jun N-terminal kinase (green); DYRK, dual-specificity tyrosine (Y) phosphorylation-regulated kinase (red); CK1, casein kinase 1 (purple); acetylation sites (blue); FH, Forkhead domain; NLS, nuclear localization signal; NES, nuclear export sequence

region of FOXO proteins function as a nuclear export sequence (NES) (Biggs *et al*., 1999; Brunet *et al*., 2002). In addition, phosphorylated FOXO factors have been shown to specifically interact with 14-3-3 proteins, which serve as chaperone molecules to escort FOXO proteins out of the nucleus (Brunet *et al*., 1999, 2002). Several mechanisms have been proposed to explain how 14-3-3 binding to FOXO factors promotes the relocalization of FOXO factors from the nucleus to the cytoplasm. While 14-3-3 proteins are mostly present in the cytoplasm at equilibrium, these chaperone molecules have been found to bind to their substrates in the nucleus (Brunet *et al*., 2002). Consistently, 14-3-3 binds to FOXO3 in the nucleus (Brunet *et al*., 2002). 14-3-3 binding may decrease the ability of FOXO factors to bind DNA, releasing FOXO proteins from a nuclear DNA anchor (Cahill *et al*., 2000). 14-3-3 binding to FOXO factors may actively promote the nuclear export of FOXO factors, perhaps by inducing a conformational change in FOXO molecules that would expose the NES and allow interaction with Exportin/Crm1 (Brunet *et al*., 2002). 14-3-3 binding to FOXO factors may also prevent the nuclear reimport of these transcriptional regulators by masking FOXO nuclear localization signal (NLS) (Brownawell *et al*., 2001; Rena *et al*., 2001). Finally, the phosphorylation of FOXO factors at Ser322 and Ser325 appears to accelerate FOXO relocalization to the cytoplasm in response to growth factors by increasing the interaction between FOXO and the export machinery (Ran and Exportin/Crm1) (Rena *et al*., 2002) (Figure 5a). These various mechanisms for regulating the translocation of FOXO transcription factors from the nucleus to the cytoplasm may serve as a fail-safe mechanism to ensure a complete sequestration of FOXO factors away from their target genes.

Mutational analysis of the three regulatory Akt/SGK sites have revealed that the phosphorylation of each site contributes to the nuclear exclusion of FOXO factors (Brunet *et al*., 2001). One attractive possibility is that each site participates in different aspects of the mechanisms that ensure the relocalization of FOXO proteins into the cytoplasm. Thus, phosphorylation of FOXO factors may represent a way of modulating the extent of the relocalization of these transcription factors to the cytoplasm in different cell types or in response to different combinations of signals.

The most recently identified FOXO member, FOXO6, only contains two of the three Akt/SGK regulatory sites (Thr26 and Ser184 in mouse FOXO6) (Jacobs *et al*., 2003). Unlike the other FOXO isoforms, FOXO6 is mostly nuclear. However, FOXO6 phosphorylation at Thr26 and Ser184 appears to decrease the transcriptional activity of this FOXO isoform (van der Heide *et al*., 2005). These findings suggest that the regulations and functions of FOXO6 may differ from those of FOXO1, FOXO3, and FOXO4.

The protein phosphatases that dephosphorylate FOXO transcription factors at the sites that are targeted by Akt and SGK remain elusive. These phosphatases would have the capacity to counteract Akt/SGK actions and to rapidly activate FOXO proteins, by allowing these transcription factors to translocate to the nucleus. As FOXO factors appear to play an important role in cell cycle arrest (see below), identifying ways to activate FOXO factors may be critical to counteract tumor formation.

Regulation of FOXO factors by ubiquitin-dependent protein degradation

While FOXO transcription factors are mainly regulated via reversible changes in subcellular localization, the degradation of FOXO protein represents an additional and irreversible level of regulation of this family of transcription factors. FOXO protein degradation often accompanies cell transformation (Hu *et al*., 2004; Huang *et al*., 2005), suggesting that this mechanism of regulation may be a critical initiation step towards tumorigenesis.

The degradation of FOXO transcription factors is mediated by the ubiquitin–proteasome pathway (Matsuzaki *et al*., 2003; Plas and Thompson, 2003; Aoki *et al*., 2004; Hu *et al*., 2004; Huang *et al*., 2005). Akt activity is necessary for ubiquitin-mediated degradation of FOXO3 and FOXO1 (Plas and Thompson, 2003; Huang *et al*., 2005). Among the three Akt phosphorylation sites, phosphorylation of Ser256 is the primary event that triggers FOXO1 ubiquitination (Huang *et al*., 2005). FOXO degradation is dependent not only on phosphorylation by Akt but also on proper localization of FOXO proteins. A mutant of FOXO1 in which all three Akt sites were replaced by alanines but which is forced to localize to the cytoplasm through a mutation in the NLS displays a reduction in ubiquitination. In addition, when phosphorylated FOXO1 is forced into the nucleus by a mutation in the NES, FOXO1 ubiquitination is decreased (Huang *et al*., 2005). Thus, FOXO1 needs to be present in the cytoplasm to be successfully ubiquitinated by an E3 ubiquitin ligase and subsequently degraded.

Recent evidence has identified the E3 ubiquitin ligase complex that catalyses FOXO1 ubiquitination. FOXO1 binds to the F-box protein Skp2, a subunit of the SCF (Skp1/Cul1/F-box) E3 ubiquitin ligase protein complex and this interaction is responsible for the degradation of FOXO1 (Huang *et al*., 2005). Interestingly, Skp2 has been found to interact with FOXO1, but not with FOXO3 or FOXO4 (Huang *et al*., 2005). This result raises the possibility that specific E3 ubiquitin ligase complexes may control the degradation of different FOXO family members.

I kappaB kinase β (IKKβ) also causes the proteasome-dependent degradation of FOXO factors (Hu *et al*., 2004). IKKβ is known to activate the transcription factor NF-κB through the phosphorylation and subsequent degradation of IκB, which normally serves as a negative regulator of NF-κB (for a review, see Karin *et al*., 2002). IKKβ induces the phosphorylation of FOXO3 at Ser644, in the extreme C-terminal portion of the molecule (Figure 2). This phosphorylation results in the ubiquitination and subsequent degradation of FOXO3 (Hu *et al*., 2004). Since IKKβ-induced

tumorigenesis can be suppressed by overexpression of FOXO3 (Hu *et al*., 2004), the regulation of FOXO protein degradation by IKKβ may play an important role in tumorigenesis. However, Ser644 is not conserved in other FOXO isoforms and is not present in worms and flies. Thus, whether IKKβ phosphorylates and controls the other FOXO isoforms remains to be determined. It is possible that the degradation of FOXO isoforms is regulated by different protein kinases via independent mechanisms.

FOXO1 and FOXO3 protein degradation is regulated by Akt and, at least for FOXO3, by IKKβ. However, whether FOXO4 and FOXO6 protein degradation is also actively regulated, and if so, whether the mechanisms of regulation are similar, still remains to be established. One major difference between the FOXO family members is that they display overlapping but different patterns of expression. While these differences may be partly due to mRNA expression (see above), it is possible that protein degradation also plays an important role in the distinction between FOXO isoforms *in vivo*. As tumorigenesis appears to be associated with a loss in FOXO proteins, understanding the regulation of FOXO expression will likely give important insight into mechanisms that govern tumor suppression.

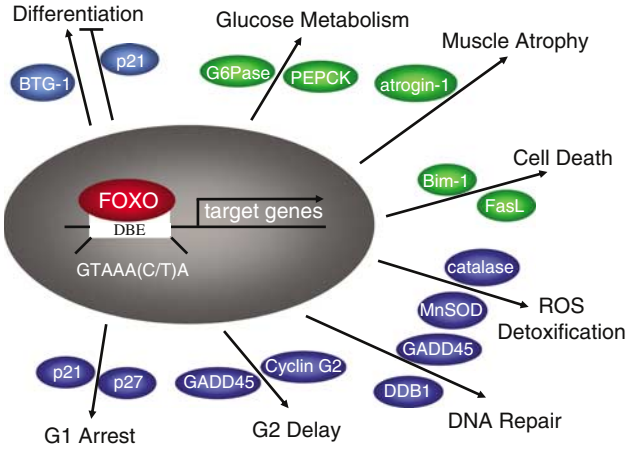

Figure 3 FOXO target genes and cellular roles. FOXO transcription factors induce the transcription of a wide array of target genes in dividing cells (blue) and in postmitotic cells (green). Note that this figure does not include all FOXO target genes. BTG-1, B-cell translocation gene 1; p21, cyclin-dependent kinase inhibitor 1A; p27, cyclin-dependent kinase inhibitor 1B; MnSOD, manganese superoxide dismutase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; FasL, Fas ligand; GADD45, growth arrest and DNA damage-inducible protein 45; DDB1, damage-specific DNA-binding protein 1; DBE, DAF-16 family member-binding element

FOXO functions in mammalian cells

FOXO DNA binding and transcriptional abilities

In the nucleus, FOXO proteins bind to DNA as monomers via the Forkhead box, a 110 amino-acid region located in the central part of the molecule (Weigelt *et al*., 2001). The core motif of the consensus recognition site for FOXO on DNA is GTAAA(C/T)A (termed the DBE for DAF-16 family member-binding element) (Furuyama *et al*., 2000; Biggs and Cavenee, 2001). Bioinformatics evidence indicates that a large number of genes contain FOXO-binding sites (DBEs) in their promoters (Xuan and Zhang, 2005). When present in the nucleus and bound to DNA, FOXO factors typically act as potent transcriptional activators (Brunet *et al*., 1999; Kops and Burgering, 1999). The transactivation domain of FOXO factors is located in the C-terminal region of the molecule. Gene array analyses have indicated that FOXO proteins can also act as transcriptional repressors (Ramaswamy *et al*., 2002). Thus, this family of transcription factors, depending on the promoter context and extracellular conditions, may activate or repress transcription.

FOXO cellular functions: a balance between stress resistance and death function

The characterization of FOXO target genes has, in many cases, been concomitant with or allowed the identification of the cellular functions of these transcription factors. In this section, we will discuss the different cellular functions of FOXO factors as well as the main FOXO target genes that mediate these functions (Figure 3 and Table 2).

Cell cycle arrest In cells that have the capacity to divide, the main effect of the expression of active forms of FOXO family members is to promote cell cycle arrest at the G1/S boundary (Medema *et al*., 2000). Target genes that mediate FOXO-induced cell cycle arrest are the Cdk inhibitor p27KIP1 (Medema *et al*., 2000) and the Rb family member p130 (Kops *et al*., 2002b). FOXO factors' ability to induce G1 arrest is diminished in p27/p130-deficient fibroblasts (Kops *et al*., 2002b), suggesting that p27 and p130 are both critical to mediate FOXO-dependent G1 arrest. In the presence of TGFβ, FOXO factors also bind to the promoter of p21, a cell cycle inhibitor, and induce cell cycle arrest at the G1/S transition (Seoane *et al*., 2004). Interestingly, FOXO factors can also promote cell cycle arrest by repressing the expression of cyclin D1 and D2, two cell cycle positive regulators (Ramaswamy *et al*., 2002; Schmidt *et al*., 2002). Thus, FOXO factors play a major role in G1 arrest by both upregulating cell cycle inhibitors (p21 and p27) and by repressing cell cycles activators (cyclin D1/D2). Akt allows cell proliferation by sequestering FOXO transcription factors in the cytoplasm and preventing them from inducing a G1 arrest.

FOXO factors also play a role at other cell cycle checkpoints. Cells in which FOXO3 is activated in the S phase display a delay in their progression through the G2 phase of the cell cycle (Tran *et al*., 2002). Microarray analyses led to the identification of several FOXO3 target genes that may mediate FOXOs' effect at the G2/M boundary, such as cyclin G2 and growth arrest

Table 2 FOXO targets gene functions

	Gene	*Function*
Cell Death	BIM-1	Bcl-2 proapoptotic family member
	bNIP3	Bcl-2 proapoptotic family member
	Bcl-6	Repressor of BCL-XL
	FasL	Triggers apoptosis through Fas
	Trail	Cytokine that induces apoptosis in transformed and tumor cells
Atrophy	atrogin-1	One of four subunits of an SCF E3 ubiquitin ligase complex
Metabolism	G6Pase	Converts glucose 6 phosphate to glucose
	PEPCK	Converts oxaloacetate to phosphoenolpyruvate
G1 cell cycle arrest	p21CIP1	Binds to and inhibits cyclin E–CDK2 and cyclin D–CDK4 complexes
	p27KIP1	Binds to and inhibits cyclin E–CDK2 and cyclin D–CDK4 complexes
	p130	Rb family protein which inhibits E2F4 to affect cell cycle regulation
G2 cell cycle arrest	GADD45	Responds to environmental stresses to promote G2 arrest and mediate DNA repair
	Cyclin G2	Inhibits cell cycle progression
DNA repair	GADD45	Responds to environmental stresses to promote G2 arrest and mediate DNA repair
	DDB1	Functions in nucleotide-excision repair
Detoxification	MnSOD	Converts superoxide byproducts to hydrogen peroxide and oxygen
	catalase	Converts hydrogen peroxide to water and oxygen
	PA26	Encodes a member of the sestrin family and acts as an antioxidant
Differentiation	p21CIP1	Binds to and inhibits cyclin E–CDK2 and cyclin D–CDK4 complexes
	BTG1	Negative regulator of cell proliferation, member of antiproliferative family

FasL, Fas ligand; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; GADD45, growth arrest and DNA damage-inducible protein 45; DDB1, damage-specific DNA-binding protein 1; MnSOD, manganese superoxide dismutase; PA26, sestrin 1; BTG-1, B-cell translocation gene 1

and DNA damage-inducible protein 45 (GADD45) (Furukawa-Hibi *et al*., 2002; Tran *et al*., 2002). Thus, FOXO factors mediate cell cycle arrest at the G1/S and G2/M transitions, two checkpoints that are critical in the cellular response to stress. FOXO-induced cell cycle arrest may allow time for repair of damaged DNA and for detoxification of cells.

DNA repair and detoxification: role in stress resistance Consistent with FOXO factors' role in promoting cell cycle arrest at the G1/S and G2/M boundaries, the expression of active forms of FOXO proteins upregulates several genes involved in DNA repair (Ramaswamy *et al*., 2002; Tran *et al*., 2002). GADD45 may mediate part of FOXO3-induced DNA repair since FOXO3-induced DNA repair is diminished in GADD45-deficient fibroblasts (Tran *et al*., 2002). In addition, FOXO proteins have been reported to allow detoxification of reactive oxygen species (ROS) by upregulating the free radical scavenging enzymes, including Mn superoxide dismutase (MnSOD) and catalase (Kops *et al*., 2002a; Nemoto and Finkel, 2002; Ramaswamy *et al*., 2002; Tran *et al*., 2002). Thus, FOXO transcription factors control two aspects of the cellular resistance to stress: repair of the damages caused by ROS and detoxification of ROS.

Cell differentiation In differentiating cells, FOXO factors have been implicated in inhibiting and promoting differentiation, depending on the cell type and the FOXO isoform. In adipocytes and myoblasts, the expression of a constitutively active form of FOXO1 inhibits differentiation in *in vitro* differentiation assays (Hribal *et al*., 2003; Nakae *et al*., 2003). The specific mechanisms underlying this inhibition are not fully characterized yet, but in adipocytes, FOXO1 directly upregulates the expression of the cell cycle inhibitor p21CIP1, without altering p27KIP1 or the C/EBP dimerization partner Chop10 (Nakae *et al*., 2003). These results suggest that in adipocytes, p21 may mediate FOXO's ability to prevent differentiation, even though p21 has been found to promote differentiation in other cellular contexts.

In contrast with FOXO1's role in adipocytes and myoblasts, FOXO3 appears to potentiate erythroid differentiation, in part by inducing B-cell translocation gene 1 (BTG1). BTG1 in turn modulates protein arginine methylation activity which causes erythroid differentiation (Bakker *et al*., 2004). Since FOXO isoforms play opposite roles in the regulation of differentiation in different cell types, it will be important to determine if other molecules are involved in specifying FOXO function in differentiating precursors.

Cell death The expression of constitutively nuclear forms of FOXO proteins trigger cell death, particularly in neurons and in lymphocytes (Brunet *et al*., 1999; Zheng *et al*., 2000; Dijkers *et al*., 2002; Gilley *et al*., 2003). Thus, one way by which Akt and SGK promote cell survival is by sequestering FOXO factors away from death genes. FOXO target genes that mediate apoptosis include BIM, a proapoptotic Bcl-2 family member (Dijkers *et al*., 2000b). Apoptosis induced by the inactivation of the PI3K–Akt/SGK pathway is reduced in lymphocytes from Bim-deficient mice, indicating that BIM may be an important target gene of FOXO factors to relay cell death. Microarray analysis has identified another proapoptotic member of the Bcl-2 family, bNIP3, as a FOXO target gene (Tran *et al*., 2002). In addition, FOXO4 indirectly downregulates the

expression of the prosurvival Bcl2 family member Bcl-xL by inducing the transcriptional repressor Bcl-6 (Tang *et al*., 2002). Thus, one way by which FOXO factors trigger apoptosis is by modulating the ratio of prodeath and prosurvival members of the Bcl-2 family.

FOXO-induced apoptosis also appears to be dependent on the induction of death cytokines, including Fas ligand and TRAIL (Brunet *et al*., 1999; Modur *et al*., 2002). Fas ligand, TRAIL, and other death cytokines may amplify FOXO factors ability to induce apoptosis by triggering death pathways in neighboring cells.

Atrophy In fully differentiated skeletal and cardiac muscle cells, expression of a constitutively active form of FOXO3 causes atrophy (Sandri *et al*., 2004; Stitt *et al*., 2004; Skurk *et al*., 2005). Importantly, FOXO-induced muscular atrophy is not due to apoptosis, but rather caused by a decrease in cell size (Sandri *et al*., 2004). This reduction in cell size is accompanied by a decrease in global protein levels and appears to be mediated by FOXO-dependent increase in the gene encoding atrogin-1 (Sandri *et al*., 2004; Stitt *et al*., 2004). Expression of atrogin-1, a muscle-specific ubiquitin ligase, enhances protein degradation and muscle atrophy. Consistent with these observations, transgenic mice which overexpress FOXO1 in muscle display a decrease in size of type I and type II muscle fibers (Kamei *et al*., 2004). Similarly, in cardiac muscle, FOXO3 prevents cardiac hypertrophy, an increase in cardiac muscle size that occurs in response to exercise (pressure, stretch, growth factors) (Skurk *et al*., 2005). Thus, the Akt signaling pathway, by repressing FOXO activity on the one hand and by activating mTOR on the other hand, acts to increase cell size through a concomitant increase in protein synthesis and decrease in protein degradation.

Glucose metabolism Finally, FOXO transcription factors also play an important role in upregulating genes that control glucose metabolism. FOXO factors elicit gluconeogenesis by upregulating glucose 6 phosphatase (G6Pase), which is responsible for converting glucose 6 phosphate to glucose, and phosphoenolpyruvate carboxykinase (PEPCK), which converts oxaloacetate to phosphoenolpyruvate (Schmoll *et al*., 2000; Nakae *et al*., 2001; Yeagley *et al*., 2001; Puigserver *et al*., 2003). Thus, insulin effects on glucose metabolism are mediated in part through the repression of FOXO factors by the PI3K–Akt pathway. For a more complete description of the role of FOXO transcription factors in metabolism, please refer to Barthel *et al*. (2005).

Since PI3K–Akt/SGK pathway negatively regulates FOXO transcription factors, it is not surprising that many functions of FOXO factors are opposite to the roles of Akt and SGK in cells. A large number of Akt and SGK critical functions in cells may be mediated by the repression of FOXO transcription factors.

It is important to note, however, that most studies have addressed whether FOXO factors are sufficient for a particular function, using the constitutively active mutants of FOXO proteins in which all three Akt sites have been replaced by alanine residues. These constitutively nuclear FOXO mutants may not entirely mimic the effect of activated wild-type FOXO factors. Complementary studies testing whether FOXO are necessary, using RNAi and gene disruption approaches, will be an important step in deciphering the cellular functions and target genes of FOXO transcription factors.

Specificity FOXO transcription factors promote a variety of cellular responses, including cell cycle arrest, resistance to oxidative stress, apoptosis, and atrophy by modulating a series of specific target genes. Whether yet undiscovered target genes of FOXO proteins, which may be specific to certain tissues or cell types, also mediate these cellular functions still remains to be uncovered. In addition, whether FOXO1, FOXO3, FOXO4, and FOXO6 have different subsets of target genes or share similar target genes is still not understood. In light of the observation that FOXO transcription factors trigger different, even opposite, functions (e.g. stress resistance and cell death), it will be important to determine the molecular mechanisms by which FOXO transcription factors specify cellular functions. Phosphorylation of FOXO proteins by Akt, which appears to mostly turn FOXO's activity on and off, may not be sufficient to explain the selectivity of cellular responses to extracellular stimuli.

FOXO factors in the whole organism: a conserved role in longevity?

Role of FOXO factors in the response to stress and organismal longevity in invertebrates

The identification of FOXO roles in whole organisms has been pioneered by genetic studies in worms and, more recently, in flies. One of the most intriguing functions of FOXO transcription factors is their conserved ability to increase longevity (Kenyon, 2005). In worms, mutations in the insulin receptor or PI3K result in an extended longevity by up to threefold (Johnson, 1990; Kenyon *et al*., 1993; Morris *et al*., 1996; Kimura *et al*., 1997). This lifespan extension is reverted when the worm FOXO orthologue (DAF-16) is mutated (Lin *et al*., 1997; Ogg *et al*., 1997). Thus, the FOXO orthologue DAF-16 plays a crucial role downstream of the insulin-signaling pathway to regulate longevity.

The target genes that mediate DAF-16's ability to increase longevity in worms include MnSOD (SOD3 in worms) (Honda and Honda, 1999), heat–shock proteins, and antimicrobial agents (Lee *et al*., 2003; Murphy *et al*., 2003; McElwee *et al*., 2004). DAF-16 appears to induce a program of genes that coordinately regulate longevity, by promoting resistance to oxidative stress, protection of protein structure, and resistance to pathogens. Consistent with the notion that resistance to oxidative stress is correlated with longevity, all the long-lived worm mutants that lead to the activation of DAF-16 also display resistance to oxidative stress, heat shock, and UV radiations (Henderson and Johnson,

2001). These findings suggest that one way in which DAF-16 activity leads to an increase in organismal lifespan is by increasing the resistance of cells to various stresses.

Similarly, in drosophila, dFOXO plays an important role in conferring stress resistance (Junger *et al*., 2003). Interestingly, expression of a wild-type form of dFOXO is sufficient to increase longevity in flies (Giannakou *et al*., 2004; Hwangbo *et al*., 2004) and prevent the age-associated decline of cardiac functions (Wessells *et al*., 2004). These findings suggest that FOXO factors promote longevity and reduce age-dependent diseases in invertebrates.

Distinct but overlapping roles of FOXO isoforms in mammals

While the roles of mammalian FOXO transcription factors in cultured cells are well established, their roles in the whole organism are just beginning to be unraveled. The deletions of FOXO1, FOXO3, and FOXO4 individual genes have recently been achieved in mice and give insights into the organismal functions of this family of transcription factors (Table 1).

FOXO1-null mice die at embryonic day 10.5, from defects in angiogenesis (Furuyama *et al*., 2004; Hosaka *et al*., 2004). FOXO1 heterozygote mutant mice are viable and they rescue the diabetic phenotype of the insulin receptor mutant mice (Nakae *et al*., 2002). This finding provides an important genetic confirmation that FOXO1 is a physiological substrate of the insulin-signaling pathway that relays insulin effects on glucose metabolism in mice.

FOXO3-null mice are viable (Castrillon *et al*., 2003; Hosaka *et al*., 2004). The main defect of FOXO3-null mice is an age-dependent female infertility (Castrillon *et al*., 2003), due to the premature activation of the ovarian follicles. FOXO3 mutant mice exhibit defects in glucose uptake (Castrillon *et al*., 2003), consistent with a role for FOXO family members in glucose metabolism. FOXO3-null mice also display overproliferation of helper T cells, in line with FOXO3's role in promoting cell cycle arrest (Lin *et al*., 2004). Surprisingly, neutrophils from FOXO3-null mice show an increase in apoptosis, associated with an upregulation of FasL expression (Jonsson *et al*., 2005). These results are in contrast with the observation that active FOXO3 promotes cell death in several cell types, and that part of this cell death can be mediated by death cytokines, including FasL. This discrepancy may be an example of the difference between *in vivo* and cell culture studies. Alternatively, it is possible that additional coregulators are involved in controlling whether FOXO3 acts as a repressor or an activator of the Fas ligand promoter depending on the external stimuli or the cell type.

FOXO4-null mice are viable and do not appear to have an overt phenotype (Hosaka *et al*., 2004), and FOXO6-null mice have not been generated yet. Taken together, these results suggest that FOXO1, FOXO3, FOXO4, and possibly FOXO6 family members may have both distinct and overlapping functions in the organisms. Importantly, a functional compensation of one member by another member of the FOXO family may have occurred, thereby masking the function of individual FOXOs. The differences in phenotypes of FOXO-null mice may be due to the different patterns of expression of each FOXO isoform, but may also reflect specific regulations, protein partners, or target genes of these isoforms.

Evidence for a role of FOXO in stress resistance and longevity in mammals?

FOXO factors are pivotal downstream targets of the insulin/IGF-1 pathway and mice that are deficient for either the insulin receptor or the IGF-1 receptor are long lived and resistant to oxidative stress stimuli (Bluher *et al*., 2003; Holzenberger *et al*., 2003). In addition, FOXO transcription factors induce stress resistance in mammalian cells and some FOXO target genes involved in stress resistance are conserved between worms and mammals (e.g. MnSOD). These observations raise the exciting possibility that FOXO transcription factors may also regulate lifespan in mammals. Initial evidence indicates that FOXO3 mutant mice do not display a defect in lifespan (Castrillon *et al*., 2003). However, it has to be noted that in flies or worms, deletion of the FOXO gene only have a minor effect on lifespan in normal organisms (Lin *et al*., 1997; Ogg *et al*., 1997). The effects of FOXO on lifespan are revealed in contexts where the insulin signaling is deficient. Thus, analysing the effects of FOXO on lifespan in mammals may require crossing FOXO mutant mice with mice that bear mutations in the insulin or the IGF-1 receptors. In addition, while worms and flies have only one FOXO isoform, mammals have four FOXO isoforms, which may functionally compensate for each other in single knockout mouse models, thereby masking a potential role of the FOXO family in longevity. Unraveling the role of FOXO in stress resistance and longevity in mammals will benefit from a more sophisticated set of mouse models in which several FOXO members are disrupted specifically in adults.

FOXO regulation in response to stress stimuli

FOXO phosphorylation in response to stress stimuli

FOXO transcription factors have multiple cellular and organismal roles, ranging from stress resistance and longevity to apoptosis and atrophy. The diversity of FOXO functions raises the possibility that FOXO transcription factors integrate other external stimuli in addition to insulin and growth factors. As longevity is coupled with increased oxidative stress resistance, an enticing possibility is that FOXO factors are themselves regulated in response to oxidative stress stimuli, thus allowing an adaptive response to stress. Consistent with this possibility, a variety of stress stimuli, including oxidative stress, heat shock, and UV radiation, induce the phosphorylation of FOXO factors at eight novel

phosphorylation sites (Brunet *et al*., 2004). In addition, a series of recent experiments indicate that JNK, a MAPK family member activated by stress stimuli, is responsible for FOXO regulation in several organisms (Essers *et al*., 2004; Oh *et al*., 2005; Wang *et al*., 2005). The regulation of FOXO by JNK modulates organismal longevity, at least in invertebrates.

JNK directly phosphorylates human FOXO4 and worm DAF-16 *in vitro* (Essers *et al*., 2004; Oh *et al*., 2005). FOXO4 is phosphorylated by JNK at Thr447 and Thr451 (Essers *et al*., 2004). These sites do not appear to be conserved in FOXO1, FOXO3, or DAF-16, even though these proteins are phosphorylated by JNK *in vitro*. Thus, JNK likely phosphorylates FOXO factors at other regulatory sites that remain to be identified. In worms, flies, and mammalian cells, stress stimuli trigger the relocalization of FOXO to the nucleus (Henderson and Johnson, 2001; Lin *et al*., 2001; Wang *et al*., 2003; Brunet *et al*., 2004; Essers *et al*., 2004). In mammalian cells, the phosphorylation of FOXO4 by JNK also results in the translocation of FOXO from the cytoplasm to the nucleus (Essers *et al*., 2004). In the nucleus, FOXO4 can upregulate MnSOD and catalase, thereby triggering detoxification of ROS in response to stimuli, which would normally cause ROS to accumulate. This adaptive response to stress stimuli may play an important role in regulating homeostasis at the organismal level and therefore contribute to longevity.

Growth factor-activated Akt and stress-activated JNK have opposing effects on FOXO subcellular localization, via phosphorylation of FOXO proteins at different sites. Interestingly, stress stimuli appear to override the sequestration of FOXO by growth factors in mammalian cells (Brunet *et al*., 2004). These results are corroborated by genetics experiments in drosophila: in cells with high insulin signaling levels, overexpression of the drosophila JNK upstream kinase is sufficient to cause the relocalization of dFOXO to the nucleus (Wang *et al*., 2005). The mechanism by which stress stimuli and JNK allow the relocalization of FOXO to the nucleus are beginning to be identified. Akt's effect on FOXO subcellular localization is mediated by 14-3-3 binding. In contrast, 14-3-3 binding appears to be unaffected by mutations at Thr447 and Thr 451, which are phosphorylated by JNK (Essers *et al*., 2004). However, JNK appears to phosphorylate 14-3-3 proteins, thereby releasing 14-3-3 substrates, including FOXO factors (Tsuruta *et al*., 2004). These results suggest that JNK regulates FOXO subcellular localization by a mechanism that is different than Akt, but that also involves 14-3-3 proteins (Figure 4).

JNK activation extends the lifespan and stress resistance in worms, and JNK's effect on longevity is mediated by DAF-16 (Oh *et al*., 2005). The expression of JNK further extends the lifespan of Akt mutant worms, indicating that JNK's ability to regulate lifespan is dependent on FOXO factor but independent of Akt (Oh *et al*., 2005). JNK's ability to prolong lifespan in a FOXO-dependent manner is conserved in flies (Wang *et al*., 2003, 2005). Flies that display an increased JNK

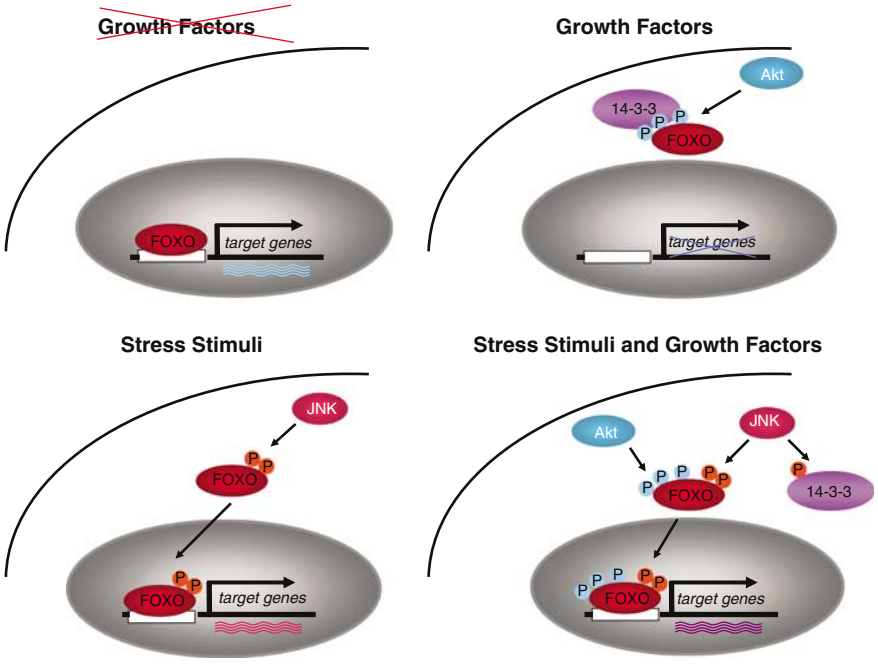

Figure 4 FOXO regulation by growth factors and stress stimuli. In response to growth factors, the PI3K/Akt pathway inhibits FOXO-dependent transcription through the phosphorylation and subsequent sequestration of FOXO factors in the cytoplasm. Stress stimuli are sufficient to overcome the cytoplasmic sequestration of FOXO factors. In response to stress stimuli, JNK phosphorylates FOXO factors, which causes the nuclear translocation of FOXO proteins. Although FOXO phosphorylation by JNK does not directly inhibit the binding of 14-3-3 proteins, JNK can phosphorylate 14-3-3 directly, thus releasing 14-3-3 substrates. JNK activity is sufficient to overcome Akt inhibition of FOXO factors and causes FOXO factors to transcribe a program of genes involved in stress resistance. We propose that a specific subset of target genes is transcribed under each condition. These specific transcriptional programs may dictate whether cells undergo cell cycle arrest, stress resistance, or apoptosis

activity, because they are heterozygous for the JNK-specific phosphatase termed Basket, live longer than wild-type flies and this lifespan extension is reverted by the ablation of one dFOXO allele (Wang *et al*., 2005). Thus, the Akt and the JNK pathways appear to be in a tight balance to regulate FOXO: Akt prevents FOXO nuclear localization and inhibits longevity, while JNK promotes FOXO nuclear localization and extends lifespan. Whether JNK affects lifespan in mammals is not known yet.

FOXO regulation by acetylation and deacetylation: role of the Sir2 family of deacetylases

FOXO transcription factors are tightly regulated by an opposite interplay of phosphorylation events. However, this balance is not sufficient by itself to account for all the different actions of FOXO transcription factors. Indeed, an additional level of regulation of FOXO transcription factors is their binding to coactivator or corepressor complexes and the subsequent changes in FOXO acetylation levels. FOXOs bind to the transcriptional coactivator CBP (CREB-binding protein) and to p300 (Figure 5a), thus providing a connection between these transcriptional regulators and the basal transcriptional machinery (Nasrin *et al*., 2000; Fukuoka *et al*., 2003; Van Der Horst *et al*., 2004). FOXO1 has also been found to bind to the transcriptional coactivator peroxisome proliferative-activated receptor-γ coactivator 1 (PGC-1), a coactivator known to regulate nuclear receptors (Puigserver *et al*., 2003). PGC-1 binding to FOXO1 results in the potentiation of FOXO1-dependent transcription and the upregulation of a program of genes involved in gluconeogenesis (Puigserver *et al*., 2003). CBP, p300, and PCAF also directly acetylate FOXO transcription factors at several conserved lysine residues (Figure 2) (Fukuoka *et al*., 2003; Brunet *et al*., 2004; Motta *et al*., 2004; Van Der Horst *et al*., 2004). For example, CBP acetylates mouse Foxo4 at K186, K189, and K408 (Fukuoka *et al*., 2003). FOXO-binding to coactivators and FOXO acetylation by these coactivators may actually have opposing effects on FOXO function. While binding of FOXO to coactivators tend to potentiate FOXO-dependent transcription, the acetylation of FOXO1 and FOXO4 by CBP has been found to actually suppress these transcription factor activity in a manner that is independent from CBP's effect on chromatin (Fukuoka *et al*., 2003; Daitoku *et al*., 2004). Since many FOXO acetylation sites are located in the DNA-binding domain of the molecule (Figure 2), it is possible that acetylation of FOXO interferes with FOXO binding to DNA and thereby prevents FOXO-dependent transcription. Thus, acetylation may represent a way to regulate nuclear FOXO factors. Acetylation may affect FOXO function differently on different promoters and control FOXO specificity.

A series of recent studies have revealed a connection between FOXO transcription factors and the Sir2 family of deacetylases (Brunet *et al*., 2004; Daitoku *et al*., 2004; Motta *et al*., 2004; Van Der Horst *et al*., 2004; Frescas *et al*., 2005; Yang *et al*., 2005b). The Sir2 family encodes class III deacetylases which use NAD^+ as a cofactor (Imai *et al*., 2000). In yeast, worms, and flies, expression of Sir2 extends longevity (Kaeberlein *et al*., 1999; Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004; Wood *et al*., 2004). In mammalian cells, FOXO factors and SIRT1 interact in response to oxidative stress. SIRT1 directly catalyses the deacetylation of FOXO factors *in vitro* and participates in the deacetylation of these factors within cells (Brunet *et al*., 2004; Daitoku *et al*., 2004; Motta *et al*., 2004; Van Der Horst *et al*., 2004; Frescas *et al*., 2005; Yang *et al*., 2005b). The sites of FOXO1 that appear to be primarily deacetylated by SIRT1 are K242, K245, and K262 (Daitoku *et al*., 2004). The effects of SIRT1 on FOXO function vary depending on FOXO target genes. However, a consensus that emerges from these studies is that SIRT1, by deacetylating FOXO factors, activates FOXO's ability to induce a subset of its target genes, including stress-resistance genes (Brunet *et al*., 2004; Van Der Horst *et al*., 2004). This result is consistent with the observation that CBP inhibits FOXO4 function (Fukuoka *et al*., 2003). On the other hand, SIRT1 also appears to prevent FOXO factors from inducing apoptosis (Brunet *et al*., 2004; Motta *et al*., 2004). Consistent with a role of SIRT1 in repressing FOXO-dependent transcription

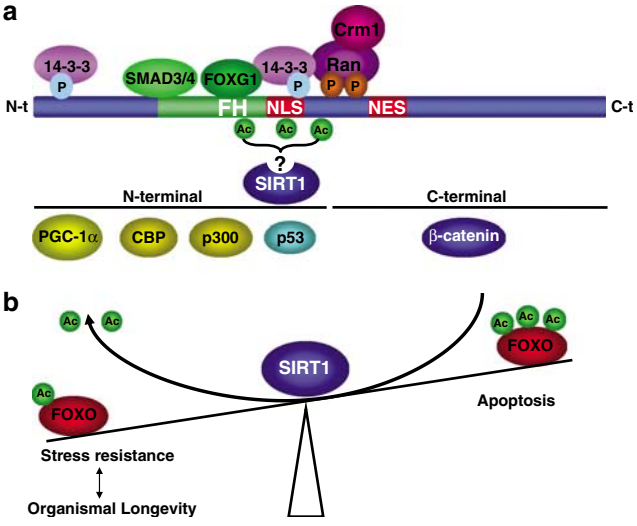

Figure 5 FOXO-binding partners. (**a**) A range of proteins has been found to interact with FOXO transcription factors. The specific regions of interaction for most proteins still need to be determined. The domains of FOXO factors that have been found to bind to specific protein partners are depicted. Proteins for which the exact region of interaction has not yet been determined are represented at the bottom of the figure. Ran, Ras-related nuclear protein; Crm1, exportin 1; PGC-1α, peroxisome proliferative-activated receptor-γ coactivator 1; CBP, CREB-binding protein; p300, E1A-binding protein p300. (**b**) SIRT1 belongs to the Sir2 family of protein deacetylase, which promotes longevity in invertebrates. SIRT1 binds to FOXO proteins and deacetylates several sites of FOXO factors. SIRT1 tips the balance of FOXO function towards stress resistance and away from apoptosis. As stress resistance is highly coupled with organismal longevity, one way by which SIRT1 may trigger longevity is by changing FOXO factors acetylation 'signature' and allowing stress resistance

under some circumstances, SIRT1-deficient mice display an increase in the expression of IGF-BP1, a target of FOXO transcription factors (Lemieux *et al*., 2005). Whether SIRT1's effect on FOXO-induced cell death is direct or whether it is due to increased stress resistance is not known yet. Nevertheless, SIRT1 may play a crucial role in tipping the balance of FOXO functions away from cell death towards stress resistance. The mechanism by which SIRT1 differentially affects FOXO functions is not known. It is possible that binding of SIRT1 to FOXO factors and deacetylation of FOXO proteins by SIRT1 have different functions. One attractive possibility is that SIRT1, by changing the 'acetylation signature' on FOXO factors, allows the recruitment of FOXO factors to the subset of stress-resistance promoters (Figure 5b). As increased stress resistance is closely correlated with lifespan extension (Kirkwood and Austad, 2000), the ability of SIRT1 to tip FOXO functions towards stress resistance may explain why the Sir2 family of proteins extends longevity. Indeed, in nematodes, the ability of Sir2 to prolong lifespan is dependent on DAF-16 (Tissenbaum and Guarente, 2001). Thus, the connection between SIRT1 and FOXO factors may play an important conserved role in controlling organismal lifespan.

FOXO transcription factor network in cancer and aging

FOXO factors in cancer development: potential tumor suppressors

In mammals, FOXO factors' ability to induce cell cycle arrest, DNA repair, and apoptosis makes them attractive candidates as tumor suppressors. Loss of FOXO function may lead to a decreased ability to induce cell cycle arrest, leading to tumor development. A decreased ability to repair damaged DNA due to the absence of FOXO factors may result in genomic instability. Finally, in the absence of FOXOs, abnormal cells that would normally die may instead survive, resulting in tumor expansion. Several lines of evidence indicate that FOXO factors are likely to play a significant role in cancer regulation: (1) FOXO3 is dysregulated in breast cancer. (2) Expression of active forms of FOXO factors reduces tumorigenicity in nude mouse paradigms. (3) FOXO proteins functionally or physically interact with tumor suppressors or oncogenes. (4) FOXO factors are found at chromosomal translocations in human tumors.

Expression of FOXO factors reduces tumorigenicity The idea that FOXO family members may serve as tumor suppressors is underscored by evidence in human cancer tissue samples (Hu *et al*., 2004). The presence of cytoplasmic FOXO3 in breast cancer sections highly correlates with poor survival of breast cancer patients. In addition, cell proliferation and tumorigenicity in nude mice induced by IKKβ expression can be overriden by the expression of an active form of FOXO3 (Hu *et al*., 2004). Similarly, the expression of a constitutively active form of FOXO4 reduces the tumor onset as well as tumor size and progression in nude mice transplanted with cells expressing the HER2 oncogene (Yang *et al*., 2005a). HER2 activation triggers the activation of the PI3K–Akt pathway. These results indicate that an active form of FOXO4 can suppress tumor formation.

Finally, cells that are deficient for PTEN, the phosphatase which normally counteracts the activity of the PI3K–Akt pathway, induce tumorigenesis in nude mice. These tumors are decreased by the expression of a constitutively active form of FOXO1 (Ramaswamy *et al*., 2002). Thus FOXO1, FOXO3, and FOXO4 can prevent tumor progression.

FOXO factors trigger a variety of functions that may participate in tumor suppression (cell cycle arrest, repair of damaged DNA, apoptosis), raising the question of which functions of FOXO factors are responsible for FOXOs' ability to inhibit tumor progression. Tumor progression induced by PTEN-deficient cells in nude mice is also inhibited by a form of FOXO1 that has a mutation in the DNA-binding domain, which prevents binding to some promoters (BIM), but not to others (cyclin D2) (Ramaswamy *et al*., 2002). These findings suggest that FOXO-induced tumor suppression, at least in these PTEN-deficient cells, may not be mediated by FOXO proapoptotic targets but rather by FOXO cell cycle arrest targets, including cyclin D2.

The overactivaction of the PI3K–Akt pathway is a hallmark of many human cancers (see review by Altomare and Testa in this issue of *Oncogene Reviews*), including glioblastomas, breast cancers, or prostate cancers. As FOXO factors are repressed by an active PI3K–Akt pathway, a potential strategy to fight these types of cancers would be to reactivate FOXO factors in tumor cells. Strategies targeting other components of the PI3K–Akt pathway are discussed in the reviews by Cheng *et al*. and Kumar and Madison in this issue.

Protein partners of FOXO that are tumor suppressors or oncogenes In response to stress stimuli or to nutrient deprivation, FOXO3 has been found to interact with the tumor suppressor p53 *in vitro* and within cells, at least when both proteins are overexpressed (Brunet *et al*., 2004; Nemoto *et al*., 2004). The interaction between p53 and FOXO appears to occur in the nucleus. The observation that FOXO and p53 interact, combined with the findings that p53 and FOXO share similar target genes (p21, GADD45, WIP1, PA26), suggests that these two proteins may coordinate tumor suppression.

In addition, FOXO factors form a complex with SMAD transcription factors (Seoane *et al*., 2004) (Figure 5a). SMADs can act as tumor suppressors to mediate the cytostatic effect of transforming growth factor β (TGF-β). The interaction between FOXO transcription factors and the SMAD transcriptional regulators occurs on the p21 promoter in response to TGF-β stimulation. The FOXO/SMAD complex elicits the upregulation of p21 and subsequent G1 arrest. In glioblastomas, FOXO factors are repressed by an overactive PI3K–Akt pathway. In this context, another FOX family member, the oncogene FOXG (also known

as c-Qin), appears to prevent FOXO from binding to the p21 promoter, thereby enhancing tumor progression (Seoane *et al*., 2004).

Finally, the oncogene β-catenin has been shown to bind to FOXO factors (Essers *et al*., 2005) (Figure 5a). The binding of β-catenin to FOXO factors enhances the ability of FOXO proteins to inhibit cell cycle progression (Essers *et al*., 2005). Since β-catenin, in combination with T-cell factor (TCF), has been implicated in cancer progression, in particular in colon cancer, it is possible that FOXO factors could counteract tumor progression by sequestering β-catenin away from TCF, thereby inhibiting cell cycle progression.

Chromosomal translocations involving FOXO factors

An independent clue that FOXOs may play a role in tumorigenesis comes from the initial observations that FOXO1, FOXO3, and FOXO4 are present at chromosomal breakpoints in human tumors (rhabdomyosascomas for FOXO1, and acute myeloid leukemias for FOXO3 and FOXO4) (Table 1). These chromosomal translocations all result in a chimeric protein in which the C-terminal domains of FOXO factors are fused to the N-terminal domain of other transcriptional regulators. (Pax3 or Pax7 for FOXO1 and Mixed Lineage Leukemia gene (MLL) for FOXO3 and FOXO4) (Galili *et al*., 1993; Davis *et al*., 1994; Parry *et al*., 1994; Borkhardt *et al*., 1997; Hillion *et al*., 1997; Anderson *et al*., 1998). These translocations all occur at a breakpoint in the same large intron (intron 2) of the FOXO family members. Although the fusion proteins still retain two of the three Akt phosphorylation sites (Ser 253 and Ser315 for FOXO3), these fusions are no longer controlled by Akt and are constitutively present in the nucleus (del Peso *et al*., 1999). The reciprocal translocation does occur, fusing the N-terminal domain of FOXO family members with the C-terminal portion of PAX3/7 or MLL, but these products are expressed at low levels, at least in the case of PAX3 (Galili *et al*., 1993). In addition, the expression of PAX3-FOXO1 or MLL-FOXO3 in cells results in changes in the expression profile of PAX3/7 or MLL target genes (Khan *et al*., 1999; Keller *et al*., 2004b), consistent with the fact that these fusions retain the DNA-binding domain of PAX3/7 or MLL. Thus, it is likely that the PAX3-FOXO1 or MLL-FOXO3 fusions are mostly responsible for the types of cancer phenotypes observed. Nevertheless, the absence of a proper FOXO allele may potentiate the tumorigenicity of the chimeric proteins.

The idea that the loss of one FOXO allele contributes to tumor suppression has not been supported yet by evidence in mouse models. Mouse model experiments indicate that the expression of human PAX3-FOXO1 fusion proteins in transgenic mice or knock-in is not sufficient to promote cancer (Anderson *et al*., 2001; Lagutina *et al*., 2002; Keller *et al*., 2004a). Knocking out one allele of FOXO1 failed to increase cancer incidence in the mice expressing the PAX3/FOXO1 fusion (Keller *et al*., 2004a). These data suggest that the haploinsufficiency of one FOXO family member may not be sufficient to increase tumor incidence. However, mimicking human rhabdomyosarcomas in mice may be difficult regardless of FOXO status. Thus, more experiments are required to understand if the loss of FOXO alleles in FOXO translocations plays a role in tumor progression *in vivo*.

So far the analysis of FOXO null mouse models has not revealed a direct role of FOXO family members in cancer (Castrillon *et al*., 2003; Hosaka *et al*., 2004). However, it is possible that the lack of cancer phenotype in individual FOXO knockout mice is due to the compensation by the other FOXO family members. In addition, the FOXO1 knockout has not been examined with respect to cancer because of its early embryonic lethality. Finally, it is possible that FOXO factors only induce tumor suppression under stress conditions. FOXO factors may only play a role in specific types of cancer. Mouse models in which the FOXO family as a whole is disrupted in an inducible manner will be extremely helpful to assess FOXO's role in tumorigenicity. In addition, crossing of FOXO mutant mice with mice that are prone to tumors or submitted to stress stimuli may also unmask the effects of FOXO on tumor suppression.

A regulatory network between FOXO, SIRT1, and p53

FOXOs' ability to induce cell cycle arrest, DNA repair, and apoptosis are reminiscent of the functions of the tumor suppressor protein p53. In that respect, it is interesting to note that genes such as GADD45, WIP1, p21, and PA26 that are induced in response to FOXOs have also been found to be regulated by p53. These observations raise the possibility that FOXOs and p53 may under some circumstances function in a cooperative manner. Consistent with this possibility, p53 and FOXO are both phosphorylated and acetylated in response to oxidative stress stimuli and UV radiations (Vousden and Lu, 2002; Brunet *et al*., 2004). In addition, both p53 and FOXOs bind to SIRT1 deacetylase (Luo *et al*., 2001; Vaziri *et al*., 2001). In contrast, p53 and FOXO factors appear to have opposite effects on organismal longevity. The contrasting roles of FOXOs and p53 in aging suggest that there exists a fine-tuned regulatory network orchestrating the actions of these two proteins.

p53 appears to indirectly inhibit FOXO function by inducing the protein kinase SGK, which results in the phosphorylation of FOXO3 and in the relocalization of FOXO3 from the nucleus to the cytoplasm (You *et al*., 2004). Reciprocally, FOXO3 has been found to prevent p53 from repressing SIRT1 gene expression. FOXO-induced repression of p53 appears to be mediated by the direct interaction between FOXO3 and p53 (Nemoto *et al*., 2004). That FOXO factors induce SIRT1 expression is consistent with the observation that SIRT1 expression is increased in rodent tissues when insulin and IGF-1 are low (Cohen *et al*., 2004). In turn, SIRT1 binds to and deacetylates p53 and FOXO transcription factors, controlling their activity. Thus, several loops of regulation appear to coexist in cells, which may allow several levels of feedback control.

Do p53 and FOXO coordinately orchestrate longevity and tumor suppression? Mice harboring a truncation in the p53 gene which results in the activation of the other allele of p53 display an average 19% reduction of lifespan and exhibit signs of premature aging (e.g. excessive curvature of the spine) (Tyner *et al*., 2002). Similarly, the expression of p44, an N-terminally truncated form of p53, which also leads to the activation of endogenous p53, elicit accelerated aging in mice (Maier *et al*., 2004). Interestingly, while activation of p53 in these mouse models reduces lifespan, p53 activation still allows an increased resistance to cancer (Tyner *et al*., 2002; Maier *et al*., 2004), suggesting that p53 causes tumor suppression at the expense of longevity.

The role for p53 in accelerating aging is in direct contrast with that of FOXO factors, which extend lifespan at least in worms and flies. Thus, active p53 and active FOXO might act synergistically to promote tumor suppression or act antagonistically to control longevity. The systematic elucidation of other protein partners of FOXO and p53 will provide important insights into the roles of FOXO factors and p53 at the interface between cancer and aging.

We propose two working frameworks to explain the dichotomy between p53 and FOXO in tumor suppression and in longevity. In one model, FOXO factors and p53 would both prevent tumor suppression in mammals at the expense of longevity. Indeed, even though FOXO promotes an extension of longevity in worms and flies, these organisms are mostly postmitotic. In mammals, the presence of tissue stem cells, which continue to proliferate throughout the lifespan, places the organisms at risk for cancer. It is therefore possible that in mammals, the organismal functions of FOXO transcription factors have been tipped towards tumor prevention. Preventing tumor formation requires a significant amount of energy and may be achieved at the detriment of overall longevity, in a process termed antagonistic pleiotropy (Campisi, 2005). However, the fact that insulin- and IGF-1 receptor-deficient mice display an extension of lifespan strongly suggests that FOXO factors promote longevity in mammals as well as in invertebrates.

In another model (Figure 6), p53 would provide baseline prevention against cancer by promoting cell cycle arrest and apoptosis. Since p53 inhibits SIRT1 expression and because Sir2 family members increase longevity in invertebrates, p53's ability to shorten lifespan may be mediated by a decrease in SIRT1 expression. FOXO factors may also promote tumor suppression by inducing cell cycle arrest and apoptosis. On the other hand, FOXO factors may promote longevity by upregulating the SIRT1 gene as well as eliciting a program of stress-resistance genes. SIRT1 expression would further activate FOXO by tipping the balance of FOXO function towards stress-resistance genes. Thus, the activation of FOXO may be sufficient to override the repression of SIRT1 triggered by p53. In this model, SIRT1 would serve as a hinge molecule that tips the scales in favor of longevity. Understanding the interplay between FOXO factors, SIRT1, and p53 in longevity and tumor suppression will necessitate the generation of mouse models that lead to controlled activation or inactivation of these genes.

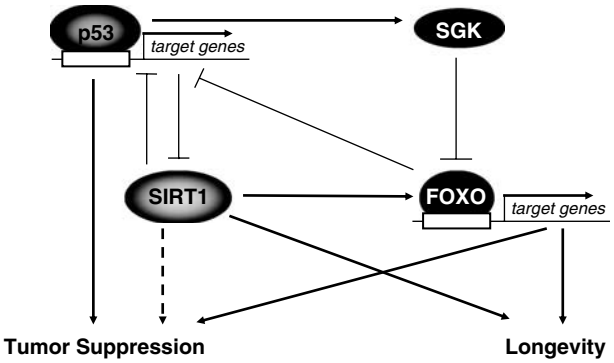

Figure 6 Feedback regulatory networks between FOXO factors, SIRT1, and p53 in longevity and tumor suppression. p53 is a tumor suppressor. FOXO factors have also been proposed to play a tumor suppressive role by inducing cell cycle arrest, apoptosis, and DNA repair. In addition, FOXO activity extends lifespan in invertebrates. In contrast, p53 has been shown to accelerate aging, in part by inhibiting FOXO factors activity and SIRT1 expression. The activation of FOXO factors in response to stress stimuli may overcome p53-dependent inhibition of SIRT1 and allow lifespan extension. SGK, serum and glucocorticoid inducible kinase

Conclusions

The ensemble of evidence presented in this review raises the exciting possibility that the FOXO family of transcription factors acts at the interface between aging and cancer. FOXO proteins play a pivotal role in cell fate decisions, including cell cycle withdrawal, differentiation, stress resistance, and apoptosis. Since these cellular responses are the major functions that go awry in cancer, the FOXO family is ideally positioned as a master regulator to control both individual cell integrity and the homeostasis of the organism. FOXO factors may serve as general gauges of organismal homeostasis, integrating environmental cues via a variety of different pathways to allow each cell to react appropriately. Since FOXO factors have been implicated in so many diverse cellular responses, it is not surprising that these factors are tightly controlled by a wide array of proteins, through phosphorylation, acetylation, ubiquitination, and protein–protein interactions. How FOXO factors specify precise programs of gene expression will be an exciting next step of discovery. In addition, the generation of novel mouse models, in which FOXO factors are deleted in a conditional and combinatorial manner in each tissue, will provide key clues on the specific function of each FOXO isoform in mammals. Based on genetic findings in worms and flies, a tantalizing possibility is that FOXO factors may act in specific tissues (e.g. the nervous system) to control general organismal functions, such as longevity or tumor suppression. Unraveling the complex network of pathways that involve FOXO factors will provide

important insights into the mechanisms that link aging and cancer. The analysis of FOXO transcription factor regulatory networks may also provide a molecular framework to understand the wide differences in longevity between species.

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