Oncogene (2008) 27, 2276–2288

www.nature.com/onc

REVIEW

The FoxO code

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The FoxO family of Forkhead transcription factors plays an important role in longevity and tumor suppression by upregulating target genes involved in stress resistance, metabolism, cell cycle arrest and apoptosis. FoxO transcription factors translate a variety of environmental stimuli, including insulin, growth factors, nutrients and oxidative stress, into specific gene-expression programs. These environmental stimuli control FoxO activity primarily by regulating their subcellular localization, but also by affecting their protein levels, DNA-binding properties and transcriptional activity. The precise regulation of FoxO transcription factors is enacted by an intricate combination of post-translational modifications (PTMs), including phosphorylation, acetylation and ubiquitination, and binding protein partners. An intriguing possibility is that FoxO PTMs may act as a 'molecular FoxO code' read by selective protein partners to rapidly regulate gene-expression programs. The effective control of FoxO activity in response to environmental stimuli is likely to be critical to prevent aging and age-dependent diseases, including cancer, neurodegenerative diseases and diabetes.

Oncogene (2008) **27,** 2276–2288; doi:10.1038/onc.2008.21

Keywords: FoxO transcription factors; post-translational modifications; high molecular weight complex; chromatin; aging; cancer

Introduction

The FoxO subfamily of Forkhead transcription factors is conserved from *Caenorhabditis elegans* to mammals. Invertebrates have one FoxO gene whereas mammals have four FoxO family members: FoxO1 (FKHR), FoxO3 (FKHRL1), FoxO4 (AFX) and FoxO6. Intriguingly, FoxO transcription factors extend longevity in invertebrates (Lin *et al*., 1997; Ogg *et al*., 1997; Henderson and Johnson, 2001; Giannakou *et al*., 2004; Hwangbo *et al*., 2004). In mammals, FoxO factors have a wide range of organismal functions: they promote tumor suppression and may also extend mammalian lifespan. They also regulate energy metabolism and development of a number of tissues (Nakae *et al*., 2002; Bluher *et al*., 2003; Holzenberger *et al*., 2003; Furuyama *et al*., 2004; Hosaka *et al*., 2004; Hu *et al*., 2004; Paik *et al*., 2007) (Figure 1).

FoxO proteins mainly act as potent transcriptional activators by binding to the conserved consensus core recognition motif TTGTTTAC (Furuyama *et al*., 2000; Xuan and Zhang, 2005). FoxO transcription factors promote cell cycle arrest, repair of damaged DNA, detoxification of reactive oxygen species, apoptosis and autophagy by upregulating specific gene-expression programs (Figure 1) (Brunet *et al*., 1999; Dijkers *et al*., 2000; Medema *et al*., 2000; Kops *et al*., 2002; Nemoto and Finkel, 2002; Tran *et al*., 2002; Lee *et al*., 2003; Murphy *et al*., 2003; Mammucari *et al*., 2007; Zhao *et al*., 2007). FoxO-dependent cell cycle arrest and apoptosis may be critical for the tumor-suppressive effect of these transcription factors, whereas FoxO-induced resistance to oxidative stress may participate in FoxO-dependent lifespan extension (Figure 1). FoxO proteins also regulate cell differentiation in blood, muscle and adipose tissue, which may contribute to their role in development (Hribal *et al*., 2003; Nakae *et al*., 2003; Bakker *et al*., 2004; Miyamoto *et al*., 2007; Tothova *et al*., 2007) (Figure 1). Finally, FoxO proteins control energy metabolism by promoting gluconeogenesis and by enhancing food intake (Puigserver *et al*., 2003; Kim *et al*., 2006; Kitamura *et al*., 2006; Matsumoto *et al*., 2006, 2007) (Figure 1). As FoxO cellular functions are diverse and in some cases antagonistic, it is likely that the activity of these transcription factors is differentially controlled in specific tissues in response to various types or intensities of external stimuli.

FoxO transcription factors are regulated by a wide range of external stimuli, such as insulin, insulin-like growth factor (IGF-1), other growth factors, neurotrophins, nutrients, cytokines and oxidative stress stimuli. These stimuli control FoxO protein levels, subcellular localization, DNA-binding and transcriptional activity. FoxO regulation is achieved by changes in post-translational modifications (PTMs) on the FoxO proteins, including phosphorylation, acetylation, mono- and polyubiquitination and possibly other modifications yet to be identified. An attractive model is that FoxO PTMs create a 'FoxO code' that is read by protein partners specifying the level and activity of FoxO transcription factors within cells. FoxO PTMs may act by both affecting FoxO conformation and creating

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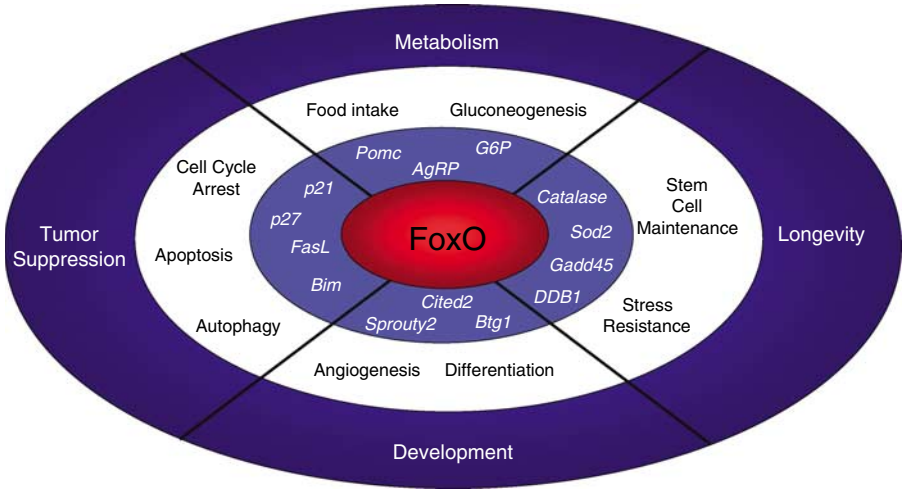


Figure 1 Roles of FoxO transcription factors in cells and in the organism. FoxO transcription factors trigger a variety of cellular processes by upregulating a series of target genes (in italics). The cellular responses elicited by FoxO affect a variety of organismal processes, including tumor suppression, longevity, development and metabolism. Note that some cellular processes may not be exclusive to one organismal function (e.g. cell cycle arrest).

specific binding motifs for FoxO binding partners to modulate FoxO function. In this review, we will discuss the various levels of FoxO regulation and how the combinatorial action of FoxO PTMs and protein partners specifies FoxO-dependent gene-expression programs in response to environmental stimuli in cells and in organisms.

Regulation of FoxO subcellular localization

The major mechanism how FoxO transcription factors are regulated in response to external stimuli is by changes in subcellular localization. The precise control of FoxO subcellular localization is achieved via multiple layers of PTMs, particularly phosphorylation and monoubiquitination (Figures 2 and 3).

Relocalization of FoxO from the nucleus to the cytoplasm

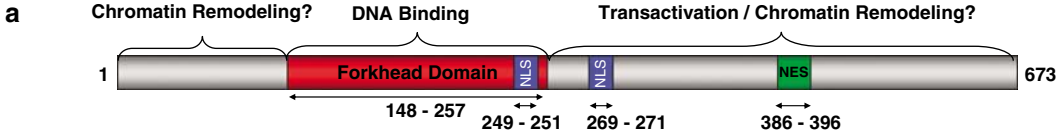
Phosphorylation by Akt and SGK in response to insulin and growth factors

The FoxO family is negatively regulated by the PI3K–Akt signaling pathway in response to insulin, insulin-like growth factors, growth factors and neurotrophic factors (Lin *et al*., 1997; Ogg *et al*., 1997; Brunet *et al*., 1999; Guo *et al*., 1999; Jackson *et al*., 2000; Medema *et al*., 2000; Yellaturu *et al*., 2002; Zheng *et al*., 2002). Phosphorylation of FoxO factors at three conserved sites by the protein kinases Akt and SGK (serum and glucocorticoid-induced kinase) causes the sequestration of FoxO factors in the cytoplasm, thereby preventing FoxO factors from transactivating their target genes (Figures 2 and 3) (Biggs *et al*., 1999; Brunet *et al*., 1999, 2001; Kops *et al*., 1999; Nakae *et al*., 1999). A notable exception is FoxO6, which is not regulated by nucleo-cytoplasmic shuttling (Jacobs *et al*., 2003). The fact that FoxO6 is phosphorylated at only two of the three phosphorylation sites underscores the importance of phosphorylation at all three sites in the regulation of FoxO subcellular localization.

The cytoplasmic sequestration of FoxO proteins is mediated by a combination of binding to protein partners and changes in the physico chemical properties of FoxO. The phosphorylation of FoxO by Akt and SGK at the first and second phosphorylation sites (T32 and S253 in FoxO3) creates binding sites for the chaperone protein 14-3-3 (Brunet *et al*., 1999; Obsilova *et al*., 2005; Rinner *et al*., 2007; Li *et al*., 2007a). 14-3-3 binds to FoxO factors in the nucleus and allows their active export, probably by helping expose FoxO nuclear export sequence (Brunet *et al*., 2002) (Figure 3). The binding of 14-3-3 also affects the flexibility of FoxO nuclear localization signal (Obsilova *et al*., 2005), thereby further preventing FoxO re-entry into the nucleus (Figure 3). In addition, phosphorylation of the second site (S256 in FoxO1) prevents FoxO re-entry into the nucleus by introducing a negative charge in the basic stretch of residues that forms the nuclear localization signal (Figure 3) (Rena *et al*., 2001). Thus, the cytoplasmic sequestration of FoxO proteins is the result of enhanced FoxO nuclear export and decreased FoxO nuclear entry.

Phosphorylation by other growth factor-activated protein kinases

The phosphorylation of FoxO factors at additional sites (S249, S322 and S325 in human FoxO1) contributes to FoxO cytoplasmic sequestration in response to growth factor stimulation (Figure 2). The phosphorylation of FoxO1 by Akt at S319 creates a consensus sequence for the binding of the protein kinase casein kinase 1,



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Phosphorylation

Enzyme	FoxO1	FoxO3	FoxO4	FoxO6	Effect
Akt/SGK	T24 S256 S319	T32, S253 S315	T28 S193 S258	T26 S184	-
CK1	S322 S325	S318, S321	S261 S264		-
DYRK1	S329	S325	S268		-
CDK2	S249				-
IKKβ		S644			-
AMPK		*T179* *S399* S413 *S555* *S588* *S626*			+
JNK			T447 T451		+
MST1	S207				+
PP2A					-/+

Acetylation

Enzyme	FoxO1	FoxO3	FoxO4	FoxO6	Effect
CBP/ P300	**K242** **K245** **K262**	*K242* *K245* *K259*	***K186*** ***K189*** ***K407***	*K173* *K176* *K190*	-
PCAF	*K294*	K290	*K237*	*K229*	-
? Oxidative Stress	*K274* *K559*	K271 K569	*K215*	*K202*	
SIRT1	*K274* *K294*	K271 K290	*K215* *K237*	*K202* *K229*	-/+ ?

Ubiquitination

Enzyme	FoxO1	FoxO3	FoxO4	FoxO6	Effect
?			K199 K211		Mono +
USP7/ HAUSP			K199 K211		-
Skp2	?				Poly -

Figure 2 FoxO post-translational modifications. (**a**) Schematic of the domains in FoxO3. NLS, nuclear localization signal; NES, nuclear export sequence. Amino acids are for human FoxO3. (**b**) Post-translational modifications of FoxO. The site numbers are for human FoxO family members. Note that mouse FoxO family members may have different numbering. (−), Inhibition of FoxO; (+), activation of FoxO; black, PTM verified in cells; blue, expected site based on sequence alignment with other FoxO family members; green, PTM verified in cells for the mouse FoxO, numbering for human FoxO based on sequence alignment; red, removal of PTM. Italics—PTM identified *in vitro*, not verified in cells.

which sequentially phosphorylates FoxO1 at two sites S322 and S325 (Rena *et al*., 2002). The phosphorylation of S322 and S325 potentiates FoxO1 export to the cytoplasm in response to growth factors by directly increasing the interaction between FoxO and the export machinery (Ran and Exportin/Crm1) (Rena *et al*., 2002; Zhao *et al*., 2004) (Figure 3). Cdk2 phosphorylation of FoxO1 at S249 also results in the sequestration of FoxO1 in the cytoplasm (Huang *et al*., 2006), although the mechanism by which phosphorylation at this site impacts FoxO localization is still unclear.

Interaction with Melted at the plasma membrane

FoxO cytoplasmic sequestration may be enhanced by the interaction of FoxO with the pleckstrin homology domain-containing protein Melted in *Drosophila*. Melted recruits FoxO to the plasma membrane, bringing it in close proximity to activated Akt (Teleman *et al*., 2005). The recruitment of FoxO to the plasma membrane may be important to fully inactivate FoxO proteins (Figure 3), as well as to position FoxO in close proximity with other signaling modules, such as the TOR pathway (Teleman *et al*., 2005).

Phosphorylation of FoxO at multiple sites: fail-safe or fine-tuning mechanism?

The phosphorylation of multiple FoxO sites that contribute to FoxO nuclear export in different ways may serve as 'fail-safe' mechanisms to ensure the complete sequestration and inactivation of FoxO factors in response to insulin and growth factors. These multiple sites of phosphorylation may also be used differentially to control the kinetics at which FoxO proteins are inactivated or activated by changes in subcellular localization. In this capacity, they could help fine-tune the exact quantity of FoxO factor present in the nucleus. Finally, the sequential phosphorylation of FoxO may affect the rate at which other sites are modified. Indeed, one phosphorylation event could change the conformation of FoxO, thus allowing other enzymes that add PTMs to FoxO to bind more efficiently.

Relocalization of FoxO from the cytoplasm to the nucleus

Dephosphorylation by protein phosphatases

The regulation of FoxO by changes in subcellular localization implies that there is a pool of phosphorylated

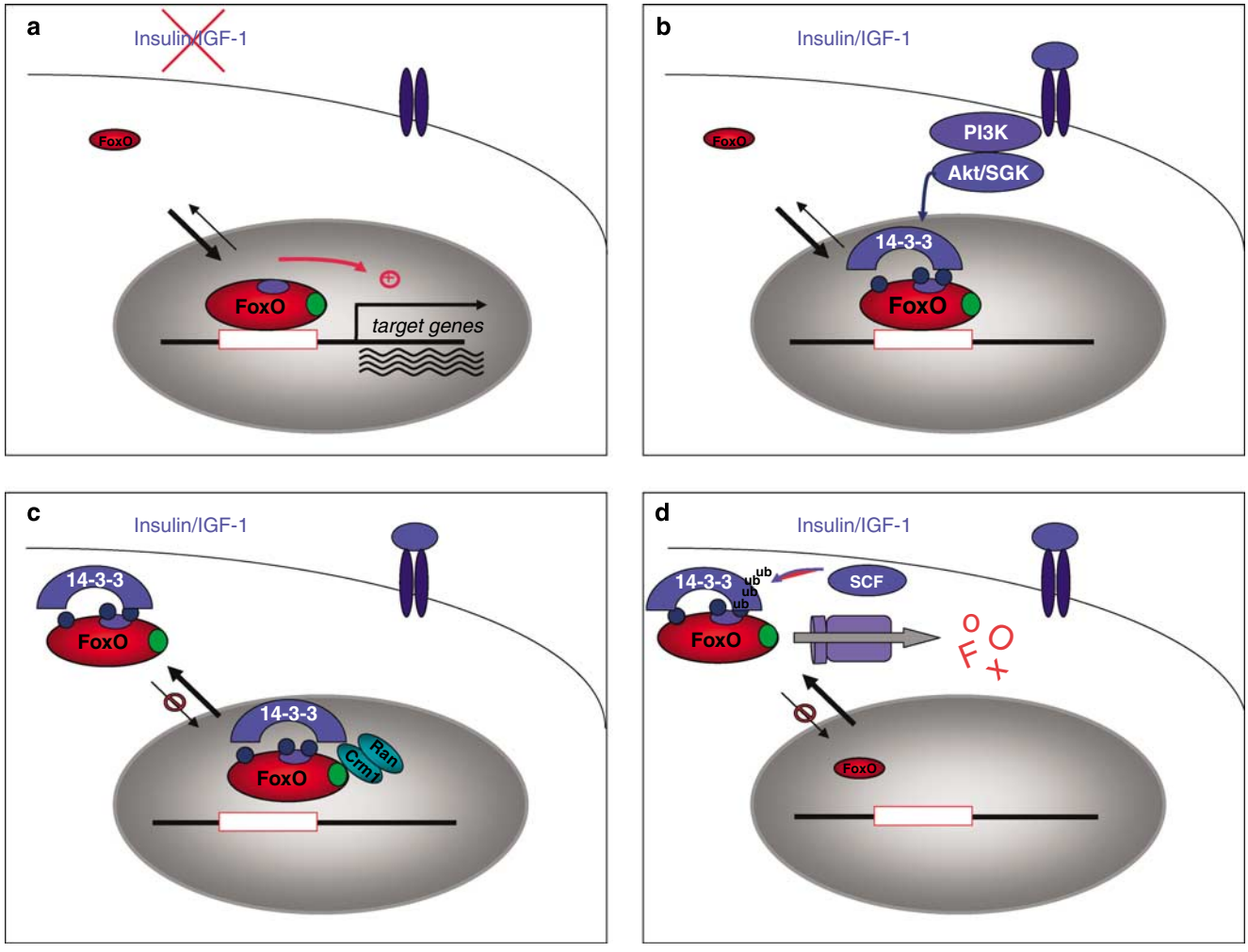

Figure 3 Model for the sequential inhibition of FoxO transcription factors in response to insulin/growth factors. FoxO factors are constantly shuttling between the cytoplasm and the nucleus (Brownawell *et al*., 2001). (**a**) In the absence of insulin/growth factors, FoxO are mostly localized in the nucleus. (**b**) Activation of the PI3K–Akt/SGK pathway by insulin and growth factors triggers the phosphorylation of FoxO in the nucleus, the binding of the 14-3-3 and the release of FoxO from their DNA-binding sites. (**c**) The binding of 14-3-3 may lead to the exposure of NES and facilitate the interaction between FoxO and Ran/Crm1 at the nuclear pore. (**d**) In the cytoplasm, phosphorylated FoxO is degraded by proteasome-dependent degradation. NES, nuclear export sequence.

and inactivated FoxO in the cytoplasm, poised to be activated. The protein phosphatases that are responsible for FoxO dephosphorylation at the Akt/SGK sites have not been entirely characterized yet. Protein phosphatase 2A was identified as a possible FoxO3 binding partner in a purified FoxO3-containing protein complex, suggesting that protein phosphatase 2A may be one of the phosphatases that dephosphorylates FoxO (Rinner *et al*., 2007). In worms, SMK-1 is a co-activator of FoxO/DAF-16, which encodes the regulatory subunit of PPH-4.1 (protein phosphatase 4), raising the possibility that this phosphatase could also participate in FoxO regulation in worms and other species (Wolff *et al*., 2006). The subcellular localization of the FoxO phosphatases could provide another layer of spatial control of FoxO activity. In addition, the rate at which the phosphatases remove the phosphate group from each phosphorylated site of FoxO proteins may affect the kinetics of localization of these transcription factors.

Phosphorylation by MST1 and JNK1 in response to oxidative stress stimuli

Interestingly, stress stimuli trigger the relocalization of FoxO proteins in the nucleus, even in the presence of growth factors (Brunet *et al*., 2004; Kitamura *et al*., 2005; Frescas *et al*., 2005). This observation suggests that oxidative stress may allow FoxO to enter the nucleus even in the absence of FoxO dephosphorylation at the Akt/SGK sites. Indeed, in response to oxidative stress, the protein kinase MST1 (mammalian Ste20-like kinase) phosphorylates FoxO3 at Ser207, a conserved site localized in the DNA-binding domain (Figure 2). MST1 phosphorylation of FoxO3 disrupts 14-3-3 binding, thereby triggering the relocalization of FoxO3 from the cytoplasm to the nucleus (Lehtinen *et al*., 2006). JNK (c-Jun kinase), another stress-activated protein kinase, phosphorylates FoxO4 at Thr447 and Thr451 and triggers the relocalization of FoxO family members from the cytoplasm to the nucleus

(Essers *et al*., 2004; Oh *et al*., 2005) (Figure 2). JNK also phosphorylates 14-3-3, which helps release FoxO factors from their 14-3-3 anchors (Sunayama *et al*., 2005). Thus, the oxidative stress-activated MST1 and JNK pathways directly oppose the insulin/growth factor-activated PI3K–Akt/SGK pathway: Akt/SGK sequesters FoxO in the cytoplasm in mammalian cells and inhibits lifespan extension in worms, while JNK and MST1 promote FoxO nuclear localization in mammalian cells and extend lifespan in worms (Oh *et al*., 2005; Lehtinen *et al*., 2006).

FoxO monoubiquitination in response to oxidative stress stimuli

Oxidative stress stimuli trigger the relocalization of FoxO4 into the nucleus and the subsequent activation of FoxO-dependent transcription by inducing the monoubiquitination of FoxO4 at K199 and K211 (van der Horst *et al*., 2006) (Figure 2). The exact mechanism by which monoubiquitination triggers the relocalization of FoxO to the nucleus is still unclear. As monoubiquitination affects lysines that could be acetylated, the interplay between monoubiquitination and other PTMs may play an important role in the regulation of FoxO localization (see below). In this regard, the deubiquitination of FoxO4 by the deubiquitinase USP7/HAUSP could help control the status of FoxO acetylation and localization (van der Horst *et al*., 2006).

FoxO acetylation/deacetylation in response to oxidative stress stimuli: targeting to PML bodies

FoxO acetylation in response to oxidative stress stimuli also affects FoxO subcellular localization. FoxO acetylation levels are modulated by the opposing action of protein acetylases—CBP (CREB-binding protein), p300 and PCAF (p300/CBP-associated factor)—and protein deacetylases, including members of the Sir2/Sirt family of deacetylases (Fukuoka *et al*., 2003; Brunet *et al*., 2004; Daitoku *et al*., 2004; Motta *et al*., 2004; Van Der Horst *et al*., 2004; Kitamura *et al*., 2005; Wang *et al*., 2007). For example, FoxO1 is acetylated by CBP at K242, K245 and K262 in response to oxidative stress stimuli (Daitoku *et al*., 2004) (Figure 2). In the β cells of the pancreas, FoxO1 acetylation induces the interaction between FoxO1 and PML (promyelocytic leukemia protein) and the relocalization of FoxO1 to PML nuclear bodies (Kitamura *et al*., 2005). In this way, acetylation increases the local concentration of FoxO in nuclear subcompartments that are densely packed with proteins, which may influence FoxO regulation and function.

Hierarchy of FoxO PTMs

These examples illustrate the hierarchy of control of FoxO transcription factors in which one set of PTMs (phosphorylation by MST1 and JNK or monoubiquitination) overrides the effects of another set of PTMs (phosphorylation by Akt and SGK). The need to dephosphorylate all the FoxO sites that sequester this family of transcription factors in the cytoplasm is bypassed, which could explain why FoxO factors respond quickly to stress stimuli. As active FoxO proteins elicit cellular and organismal stress resistance, a rapid response to oxidative stress stimuli may play an adaptive role in regulating homeostasis during an organism's lifespan.

Regulation of FoxO protein levels

Although the major mechanism of FoxO regulation is by changes in subcellular localization, altering FoxO protein levels can also have dramatic effects in the organism. Indeed, overexpression of wild-type FoxO in worms and flies can extend lifespan (Henderson and Johnson, 2001; Giannakou *et al*., 2004; Hwangbo *et al*., 2004). In contrast, loss of FoxO is associated with increased cancer in mammals (Borkhardt *et al*., 1997; Paik *et al*., 2007). Changes in FoxO protein levels in cells are the consequence of at least three possible events: (1) FoxO protein degradation; (2) FoxO transcription; and (3) mutation in the FoxO genes. In contrast to changes in subcellular localization, which are rapidly reversible, changes in FoxO protein levels are more permanent, which may have profound impacts on FoxO functions.

Regulation of FoxO protein stability

While FoxO transcription factors are relatively stable proteins, they can still be degraded in a proteasome-dependent manner in response to insulin and growth factors (Matsuzaki *et al*., 2003; Plas and Thompson, 2003; Aoki *et al*., 2004; Hu *et al*., 2004; Huang *et al*., 2005). Insulin/growth factor-mediated FoxO protein degradation is triggered by the phosphorylation of FoxO proteins by Akt (Matsuzaki *et al*., 2003; Plas and Thompson, 2003; Aoki *et al*., 2004; Huang *et al*., 2005). The E3 ubiquitin ligase that is responsible for FoxO1 polyubiquitination and proteasome degradation in response to insulin is the SCF^{skp2} (Skp1/Cul1/F-box) polyubiquitination complex (Huang *et al*., 2005) (Figure 2). Thus, the insulin–PI3K–Akt signaling pathway elicits both the cytoplasmic sequestration and the degradation of FoxO proteins (Figure 3). How insulin affects the balance between cytoplasmic sequestration and degradation of FoxO is not known yet. One possibility is that in response to mild or transient increases in insulin signaling, FoxO factors may be temporarily sequestered into the cytoplasm, such that they may be re-activated without *de novo* synthesis (Figure 4). In contrast, upon potent or chronic insulin stimulation, FoxO factors may be degraded (Figure 4). The permanent removal of FoxO from cells, via increased FoxO degradation, could lead to cell transformation and tumorigenesis (Hu *et al*., 2004; Huang *et al*., 2005).

Cytokine stimulation also triggers the degradation of FoxO3. In response to tumor necrosis factor-α stimulation, I-kappa-B kinase β phosphorylates FoxO3 at S644, resulting in the ubiquitination and subsequent degradation of FoxO3 (Hu *et al*., 2004) (Figure 2). Because S644

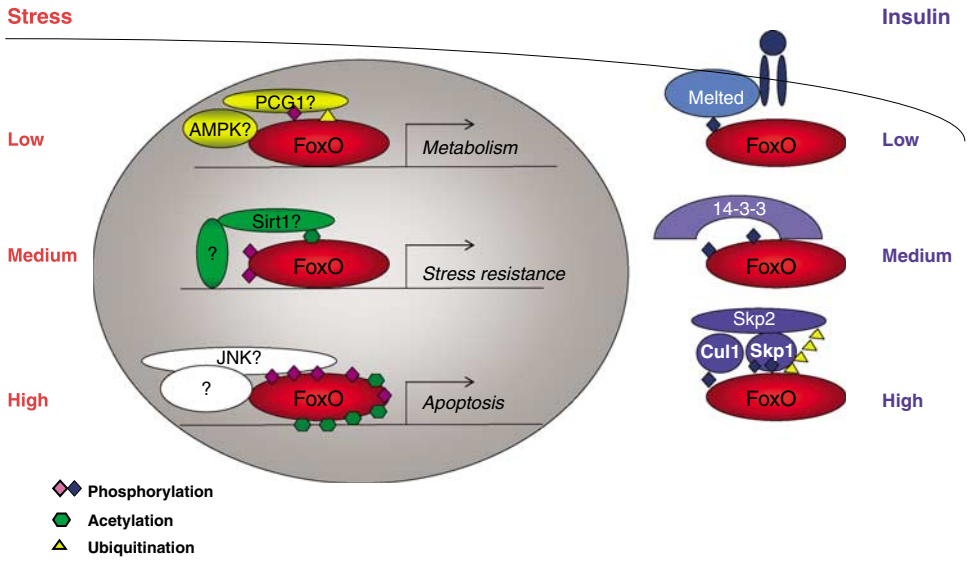

Figure 4 A FoxO code. FoxO PTM and association with specific binding partners can have a dramatic effect on FoxO function. A series of enzymes 'write' the FoxO PTM code (e.g. Akt, JNK, Sirt1, etc). FoxO protein partners may 'read' the FoxO code and cause export from the nucleus (e.g. 14-3-3), membrane targeting (e.g. Melted), protein degradation or target-gene specification. Some proteins likely act to both 'write and read' the PTM code in that they might bind to a specific PTM of FoxO, which would allow them to in turn add another PTM to FoxO. PTM, post-translational modification.

is only present in FoxO3, the degradation of the other FoxO family members may be regulated via independent mechanisms. Ectopic expression of FoxO3 in the context of I-kappa-B kinase β overexpression suppresses cellular transformation and tumor growth (Hu *et al*., 2004), underscoring the importance of FoxO levels in limiting tumorigenesis.

Conversely, oxidative stress stimuli increase FoxO1 stability in β cells of the pancreas. Stress-induced acetylation of FoxO1 appears to increase FoxO1 stability by preventing FoxO1 polyubiquitination (Kitamura *et al*., 2005). The mechanism by which FoxO1 acetylation interferes with polyubiquitination appears to be indirect, since the specific lysines that are acetylated in FoxO1 are not those that are polyubiquitinated. This example underscores how one type of modification can influence another type of modification, probably by modulating the FoxO interaction with specific protein partners.

Other mechanisms that control FoxO levels

FoxO levels can also be affected by transcription of the FoxO genes. The basal level of FoxO1 and FoxO3 mRNA is dependent on the presence of E2F-1, a transcription factor involved in cell cycle progression and apoptosis (Nowak *et al*., 2007). E2F-1 binds to specific sites in the FoxO1 and FoxO3 promoters (Nowak *et al*., 2007), indicating that these two FoxO genes are transcriptional targets of E2F-1. FoxO1/3 mRNA levels have been found to increase in muscle from rats that have been either fasted or calorically restricted for 48 h (Furuyama *et al*., 2002; Imae *et al*., 2003), suggesting that a nutrient deprivation-induced signaling cascade may also elicit the transcription of FoxO factor genes. However, the transcription factors responsible for the nutrient-inducible transcription of the FoxO genes have not been identified yet. Interestingly, FoxO3 and FoxO4 mRNAs are modulated as a function of age in rat muscle, peaking at 6 and 12 months respectively (Furuyama *et al*., 2002), raising the possibility that regulating FoxO levels may affect longevity in mammals. Whether the changes in FoxO mRNA levels in response to nutrients or age are due to changes in transcription or mRNA stability is still unknown.

The expression of FoxO in cells can also be affected by rearrangements at the *FoxO* gene loci. *FoxO1*, *FoxO3* and *FoxO4* genes are present at chromosomal translocation break points in cells of two types of pediatric tumors, rhabdomyosarcomas and acute myeloid leukemias. These chromosomal translocations result in chimeric transcription factors where the transactivation domain of FoxO factors is fused to the DNA-binding domain of other transcription factors (Pax3 or Pax7 for FoxO1 in rhabdomyosarcomas; MLL (mixed lineage leukemia) for FoxO3 and FoxO4 in acute myeloid leukemias). In addition to creating an aberrant chimeric transcription factor, these chromosomal break points also result in the loss of one correct allele of the *FoxO* gene (Galili *et al*., 1993; Davis *et al*., 1994; Parry *et al*., 1994; Borkhardt *et al*., 1997; Hillion *et al*., 1997). Thus, the tumor phenotype in these cancers is likely due to combination of the chimeric transcription factor and the haploinsufficiency and/or loss of heterozygosity of the *FoxO* locus. These findings, combined with mouse knockout and human studies illustrate the importance of FoxO levels in tumor suppression (Hu *et al*., 2004; Paik *et al*., 2007).

Regulation of FoxO DNA-binding activity

FoxO phosphorylation by Akt

While the major role of Akt/SGK phosphorylation is to sequester FoxO factors in the cytoplasm, it is becoming increasingly clear that the phosphorylation of FoxO by Akt also disrupts FoxO interactions with DNA. The phosphorylation of FoxO at the second of the three AKT/SGK sites (S256 for FoxO1) introduces a negative charge in the positively charged DNA-binding domain, thereby inhibiting DNA binding in *in vitro* binding assays (Nasrin *et al*., 2000; Zhang *et al*., 2002). However, in the case of FoxO4, the binding to the chaperone protein 14-3-3 is necessary for the complete inhibition of DNA binding (Obsil *et al*., 2003). 14-3-3 contributes to FoxO release from DNA by modifying the characteristics of FoxO DNA-binding domain (Boura *et al*., 2007) (Figure 3). These findings are consistent with the observation that a mutant of FoxO1 in which the nuclear export sequence is disrupted—and is therefore sequestered in the nucleus—is still inhibited by the PI3K–AKT/SGK pathway, presumably because it is released from DNA (Tsai *et al*., 2003). It will be important to examine whether the phosphorylation of S256 also affects the binding of FoxO1 in the context of chromatin *in vivo*. Interestingly, a subset of FoxO-binding sites in target-gene promoters may be more affected than others by the phosphorylation of FoxO by Akt/SGK at S256, which would provide another way of fine-tuning FoxO gene-expression programs in response to external stimuli.

FoxO acetylation by CBP/p300 and deacetylation by Sirt1

FoxO DNA-binding capacity is also regulated by acetylation. Acetylation of FoxO1 and FoxO3 interferes with DNA binding (Matsuzaki *et al*., 2005). The 2.7 Å crystal structure of the FoxO3 DNA-binding domain bound to DNA revealed that K245 directly interacts with the phosphate group of DNA, raising the possibility that the acetylation moiety prevents this chemical interaction (Tsai *et al*., 2007). FoxO1 acetylation also enhances the phosphorylation at S253 by Akt, which further decreases DNA binding (Matsuzaki *et al*., 2005). This example illustrates the interplay between two types of PTMs, acetylation and phosphorylation, in regulating FoxO DNA binding. Interestingly, the Sir2/Sirt deacetylases extend longevity in a range of organisms (Kaeberlein *et al*., 1999; Tissenbaum and Guarente, 2001; Rogina and Helfand, 2004). The ability of Sir2/Sirt1 to deacetylate FoxO factors and regulate FoxO DNA binding at specific target genes may contribute to lifespan extension.

Regulation of FoxO-dependent transcription: role of PTMs

FoxO phosphorylation in response to insulin (independent of Akt)

Insulin stimulation inhibits FoxO-dependent transcription not only by sequestering FoxO factors in the cytoplasm and by preventing their binding to DNA, but also by directly inhibiting FoxO intrinsic transcriptional activity. Indeed, insulin still represses the activity of a fusion protein between the Gal4 DNA-binding domain and the C-terminal transactivation domain of FoxO1, even though this fusion protein is constitutively bound to DNA (Perrot and Rechler, 2003). The repressive action of insulin on FoxO1 is mediated by phosphorylation at S319 and S499 in mouse FoxO1 (Perrot and Rechler, 2003). The exact mechanism by which phosphorylation at these sites inhibits FoxO1 transactivation properties and the protein kinases responsible for the phosphorylation of these sites is not known yet, but S319 is the equivalent of human S322 that is phosphorylated by casein kinase 1. Thus, insulin signaling blocks FoxO action in multiple, and possibly sequential, ways (Figure 3): it suppresses FoxO transcriptional activity, it inhibits FoxO binding to DNA, it promotes FoxO nuclear export, it sequesters FoxO into the cytoplasm and it induces FoxO degradation. This quintuple layer of FoxO inactivation by insulin highlights the importance of completely shutting-off FoxO, perhaps to ensure a tight metabolic response to insulin.

FoxO phosphorylation by AMPK in response to nutrient stress

FoxO-dependent transcription is also affected by nutrients. In response to nutrient deprivation, AMP-activated protein kinase (AMPK) phosphorylates FoxO3 at six sites *in vitro* (T179, S399, S413, S355, S588, S626) and at least two sites in cells (S413 and S588) (Greer *et al*., 2007b) (Figure 2). AMPK appears to activate FoxO3 transcriptional activity, independent of FoxO3 localization or FoxO3 DNA-binding activity (Greer *et al*., 2007b), although it may not affect FoxO1 in the same manner (Barthel *et al*., 2002; Dixit *et al*., 2007). Genome-wide microarray analysis revealed that AMPK phosphorylation of FoxO3 induces changes in the expression of specific target genes, including energy metabolism and stress resistance genes (Greer *et al*., 2007b). Thus, AMPK phosphorylation of FoxO3 may recruit this transcription factor to specific genes to achieve a gene-expression program that allow cells to adapt to changes in energy levels. Intriguingly, AMPK activation does not affect all FoxO target genes in the same manner, suggesting that AMPK-induced phosphorylation sites on FoxO3 may be involved in target gene specification. The mechanism by which AMPK specifies FoxO target genes is still unknown but may involve one or several of FoxO binding partners described below. In worms, AMPK also phosphorylates FoxO/DAF-16 and extends lifespan in a FoxO/DAF-16-dependent manner in response to one method of caloric restriction (Greer *et al*., 2007a). These results raise the possibility that the regulation of FoxO by AMPK in response to nutrient deprivation might play an important conserved role in longevity.

Regulation of FoxO-dependent transcription: interaction with co-activator and co-repressor protein partners

Recruitment of protein acetylases

The physical interaction between FoxO and co-activators is pivotal in controlling FoxO transcriptional activity. FoxO1 recruits the p300/CBP/SRC co-activator complex in the vicinity of the *IGFBP-1* (IGF binding protein 1) promoter in liver cells (Nasrin *et al*., 2000) and the *AgRP* (Agouti related protein) promoter in hypothalamic cells (Kitamura *et al*., 2006). Thus, while the acetylation of FoxO factors by p300/CBP inhibits FoxO function by preventing DNA binding (see above) (Furuyama *et al*., 2002), the interaction between FoxO and p300/CBP allows the recruitment of a co-activator complex to the promoters of specific genes and initiates transactivation of these genes. This antagonistic role of p300/CBP on FoxO DNA binding and transcriptional activities could be used to fine-tune the levels of expression of FoxO target genes. Alternatively, the inhibitory effect of p300 acetylation of FoxO could occur at later time points than the interaction between these two proteins and might be involved in terminating FoxO-dependent transcription of specific genes after stimulation has occurred. Interestingly, the interaction between FoxO and p300/CBP is disrupted by erythropoietin stimulation during erythropoiesis (Mahmud *et al*., 2002), raising the possibility that the regulation of this interaction could play an important role in the regulation of differentiation by FoxO factors.

Sequestration of common co-activators

In response to oxidative stress, FoxO factors bind to β-catenin, a co-activating factor that normally functions in the Wnt signaling pathway by binding to and activating the TCF (T-cell factor) transcription factor. The FoxO–β-catenin interaction is conserved from *C. elegans* to mammals and increases the transactivation potential of FoxO factors in response to oxidative stress stimuli (Essers *et al*., 2005; Almeida *et al*., 2007). Interestingly, in mammalian osteoblastic cell lines, oxidative stress causes the activation of FoxO-dependent transcription and the inhibition of the TCF-dependent transcription. A plausible model for these observations is that FoxO sequesters β-catenin away from TCF (Almeida *et al*., 2007). As some FoxO target genes are upregulated while Wnt target genes are downregulated during aging in mice, this mechanism could account, in part, for age-related decreases in bone density (Almeida *et al*., 2007). The interaction of FoxO and β-catenin illustrates how FoxO factors may affect other signaling pathways by sequestering shared co-activators.

Similarly, FoxO4 binds to myocardin, a transcriptional co-activator of smooth muscle genes (Liu *et al*., 2005). The physical interaction between FoxO4 and myocardin inhibits myocardin activity, thereby preventing smooth muscle differentiation. The mechanism of action of FoxO4 on myocardin is still unclear, but may also involve the sequestration of myocardin away from other transcription factors involved in the upregulation of genes involved in smooth muscle differentiation.

The sequestration of a common co-activator by FoxO might also play a role in the regulation of gluconeogenesis in the liver. FoxO1 interacts with the co-activator PGC-1 (peroxisome proliferative activated receptor-γ co-activator) and this interaction is important for the expression of gluconeogenic genes in liver cells (Puigserver *et al*., 2003). Insulin signaling inhibits gluconeogenesis by disrupting this interaction between FoxO1 and PGC-1 and/or by inhibiting PGC-1 (Schilling *et al*., 2006; Li *et al*., 2007b). Thus, in response to excess glucose, insulin may suppress the interaction between FoxO and PGC-1, thereby freeing up PGC1 to interact with other partners such as peroxisome proliferator-activated receptor-γ. Thus, the coordinated regulation of each transcription factor and co-activator may help redirect gene expression in an orchestrated manner.

Recruitment of protein deacetylases

FoxO interacts with the class III histone deacetylase Sirt1 in response to oxidative stress stimuli (Brunet *et al*., 2004; Daitoku *et al*., 2004; Motta *et al*., 2004; Van Der Horst *et al*., 2004). Intriguingly, Sirt1 appears to differentially regulate diverse target genes of FoxO: Sirt1 increases the expression of cell cycle arrest and stress resistance genes, but inhibits the ability of FoxO to induce apoptotic genes (Brunet *et al*., 2004; Daitoku *et al*., 2004; Motta *et al*., 2004; Van Der Horst *et al*., 2004). The difference in FoxO target-gene expression in response to Sirt1 may be due to the dual ability of Sirt1 to bind to FoxO and to deacetylate FoxO. Sirt1 binding to FoxO could silence chromatin at the vicinity of FoxO DNA-binding sites in some promoters by deacetylating histones, while directly increasing FoxO binding to DNA at other promoters. It is also possible that the number of acetylated sites in FoxO acts as a 'molecular code' to recruit FoxO to different target promoters and/or to different protein complexes on promoters (Figure 4). For example, high levels of oxidative stress stimuli could trigger the recruitment of FoxO at promoters of apoptosis target genes whereas low levels of oxidative stress stimuli could trigger the recruitment of FoxO at promoters of DNA repair and reactive oxygen species detoxification target genes (Figure 4). Sirt1, by controlling the number of acetylated lysines on FoxO factors, may play a key role in the ability of FoxO to act as a rheostat in response to oxidative stress levels (Figure 4). The interaction between FoxO and Sirt1 is itself modulated by FHL2 (four and a half LIM 2), a LIM domain-containing protein. FHL2 binds to FoxO in prostate cancer cells and increases the affinity of Sirt1 for FoxO, leading to FoxO deacetylation and the inactivation of FoxO-dependent apoptosis (Yang *et al*., 2005). This example illustrates how FoxO protein partners can affect the PTMs of these transcription factors, thereby modulating their activity in cancer cells.

Regulation of FoxO-dependent transcription: interaction with transcription factor protein partners

FoxO interacts with a number of specific transcription factors that help control the expression of FoxO target genes and may participate in specifying target-gene activation. In most cases, these interactions are fostered by the close proximity of FoxO-binding sites and other response elements in the promoters of specific genes (Figure 5).

Interactions that lead to the activation of target-gene expression

FoxO cooperates with several transcription factors that potentiate FoxO target-gene expression. For example, FoxO and RUNX3, a Runt domain-containing transcription factor, interact and bind concomitantly to the promoter of *BIM*, a pro-apoptotic Bcl-2 family member, which contains one FoxO binding site and two RUNX3-binding domains in close proximity (Figure 5). FoxO and RUNX3 cooperate to upregulate this pro-apoptotic gene and consequently promote apoptosis in gastric cancer cells (Yamamura *et al*., 2006). FoxO factors also interact with SMAD3 and SMAD4 in response to transforming growth factor-β signaling, thereby upregulating the cell cycle inhibitory gene $p21^{cip1}$ as well as other common target genes (Seoane *et al*., 2004; Gomis *et al*., 2006). The FoxO/SMAD interaction is mediated by the binding of these factors to response elements that are in close proximity within the *p21* promoter (Seoane *et al*., 2004).

Interestingly, the interaction between FoxO and specific transcription factors may positively regulate some genes but not others. During erythrocyte differentiation, FoxO3 interacts with the STAT5 transcription factor to upregulate the expression of *Cited 2* (CBP/p300-interacting transactivator with ED-rich tail 2), a gene that promotes differentiation. In contrast, the interaction between STAT5 and FoxO does not affect the expression of *Btg1*, another FoxO target gene that is normally negatively regulated by FoxO3. Instead, *Btg1* is regulated by CREB via the CREB-binding element located in close proximity to the FoxO-binding element in the promoter of the *Btg1* gene. Thus, the expression of two different FoxO target genes (*Cited 2* and *Btg1*) during erythrocyte differentiation appears to be controlled by alternate complex composition on the promoter of these genes (Bakker *et al*., 2004).

Finally, FoxO binding to other transcription factors may upregulate gene expression by releasing transcriptional repression at specific promoters (Nemoto *et al*., 2004). For example, the tumor suppressor p53 normally inhibits Sirt1 expression by binding to two sites in the promoter region of the *Sirt1* gene (Figure 5). In response to nutrient deprivation, FoxO releases p53-dependent repression of the *Sirt1* gene, thereby allowing the upregulation of Sirt1 expression. This repression appears to be mediated by the direct interaction between FoxO and p53 independent of the presence of FoxO-binding site in the *Sirt1* promoter (Nemoto *et al*., 2004).

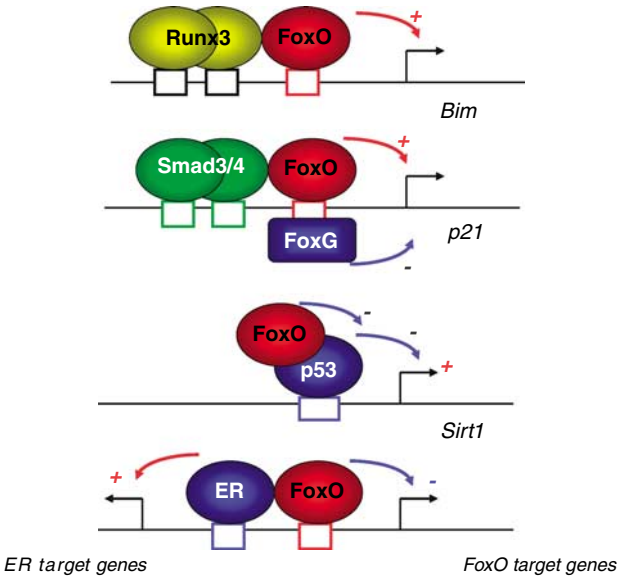

Figure 5 Interaction between FoxO factors and other transcription factors. Examples of FoxO interaction with transcription factors at gene promoters to specify gene-expression programs.

Similarly, in response to Notch signaling, FoxO1 binds to the transcription factor Csl and releases the repressive action of this transcription factor on the *Hes1* gene in muscle cells (Kitamura *et al*., 2007). In these situations, it is interesting to note that FoxO factors act as co-activators more than specific transcription factors, a function that may also be consistent with their ability to remodel chromatin (see below).

Interactions that lead to the inhibition of target-gene expression

In a more limited number of cases, FoxO binding partners can have an inhibitory effect on FoxO-dependent transcription. For example, the ability of FoxO/SMAD to upregulate *p21* expression can be shut off by FoxG, another Forkhead transcription factor that acts as a transcriptional repressor (Seoane *et al*., 2004) (Figure 5). FoxG, SMAD and FoxO form a ternary complex at the *p21* promoter, highlighting the possibility that FoxO functions in a high molecular weight complex at promoters.

Peroxisome proliferator-activated receptor-γ also inhibits FoxO-dependent transcription by directly binding to FoxO in mammalian cells (Dowell *et al*., 2003). The inhibitory effect of nuclear receptors on FoxO-dependent transcription extends to other members of this family. Indeed, FoxO interacts with the androgen receptor and the estrogen receptor (ERα), which leads in both cases to the repression of FoxO transactivation potential (Schuur *et al*., 2001; Li *et al*., 2003). Interestingly, the interaction between FoxO members and estrogen receptor-α inhibits FoxO-dependent transcription but increases estrogen receptor-dependent transcription, thus affecting two programs of gene expression with one interaction (Schuur *et al*., 2001) (Figure 5).

FoxO-containing complexes: 'coincidence' detectors for orchestrated responses

Although FoxO transcription factors interact with a number of proteins, whether FoxO proteins are present in large molecular weight complexes containing multiple proteins has not been thoroughly explored yet. The presence of FoxO in high molecular weight complexes may allow the orchestrated execution of complementary cellular responses. For example in the cytoplasm, Melted interacts with and increases the phosphorylation of both FoxO and Tsc1 (Teleman *et al*., 2005). This interaction leads to the inactivation of the FoxO pathway and the activation of the TOR pathway, which is involved in cell growth. It is therefore possible that FoxO and Tsc1 are in a complex together with Melted during the recruitment to the membrane, facilitating a rapid cellular response to coordinate cell cycle progression with cell growth.

Importantly, FoxO binding partners are themselves regulated by their own signaling cascades that may be controlled in concert with or independent of FoxO. For example, both FoxO factors and one of their co-activators, PGC-1, are phosphorylated by AKT and AMPK (Jager *et al*., 2007; Greer *et al*., 2007b; Li *et al*., 2007b). The regulation of both FoxO factors and their protein partners may act as a fail-safe mechanism or as 'co-incidence' detector for multiple changes in the environment.

A FoxO code?

An attractive possibility is that the combination of various FoxO PTMs represents a 'FoxO code' that specifies the cellular activities of the FoxO factors in response to variation in the environment. In this model, intra- and extracellular stimuli would lead to combinations of FoxO PTMs that would be 'written' by selective enzymes. This FoxO PTM code would be 'read' by specific binding partners of FoxO, which would then modulate the ability of FoxO to regulate programs of genes, thereby specifying the appropriate cellular responses (Figure 4). The type of cellular responses elicited would depend on the FoxO code, as well as on the localization of FoxO binding partners and their own PTMs.

The FoxO code may also affect chromatin properties more generally. Emerging evidence indicates that FoxO, similar to FoxA, has the capacity to bind to and remodel compacted chromatin (Cirillo *et al*., 2002; Hatta and Cirillo, 2007) (Figure 6). In this regard, it is interesting to speculate that the flexible loop immediately following FoxO DNA-binding domain may serve the same modulatory function as the histone tail that is heavily modified by PTMs. Thus, in addition to regulating the expression of specific genes, FoxO PTMs, similar to histone PTMs, may regulate overall chromatin structure and participate in the epigenetic regulation of gene expression.

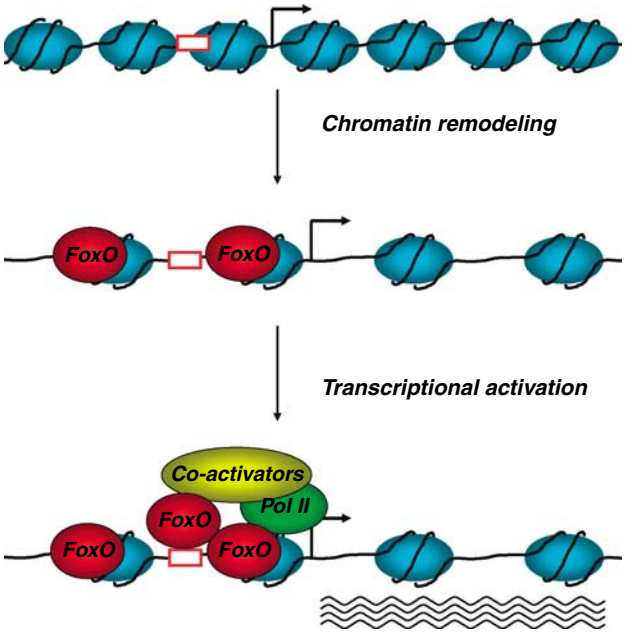

Figure 6 Possible model for a role of FoxO proteins as chromatin remodelers. In this model, FoxO proteins bind to compacted chromatin and open it by acting as chromatin remodelers (Hatta and Cirillo, 2007). FoxO factors can also bind to their binding sites in the promoters of specific genes to remodel chromatin in the vicinity of these sites as well to recruit a polymerase-containing complex to initiate gene transcription.

Conclusion

FoxO transcription factors represent a key node at the intersection of numerous signaling pathways. Because of the diverse roles of the FoxO factors, the fine-tuned regulation of FoxO activity is critical for eliciting appropriate cellular responses. While the control of FoxO proteins in response to insulin is beginning to be well understood, more research needs to be done to fully grasp how FoxO factors regulate gene-expression program in response to a variety of other environmental stimuli. It will also be important to examine if FoxO factors integrate other signaling pathways, if they are modified at other combinations of PTMs, and if they interact with other protein partners. Another tantalizing question will be to explore the possibility that FoxO factors have non-canonical functions in cells, independent of their roles as transcription factors, and if so, how are these non-canonical functions regulated by environmental stimuli. Finally, understanding how FoxO proteins are regulated and how they modulate gene-expression programs *in vivo* will likely be a critical step in the understanding of aging and age-dependent diseases, including cancer.

Acknowledgements

We thank members of the Brunet lab for helpful comments on the manuscript.

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