JB Minireview

J. Biochem. **141,** 769–774 (2007)
doi:10.1093/jb/mvm104

FOXO Transcription Factors in the Regulatory Networks of Longevity

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Received October 2, 2006; accepted November 1, 2006

FOXO (Forkhead box O) transcription factors constitute an evolutionally conserved subgroup within the large Forkhead family of transcription regulators. FOXO factors are important regulators of the cell cycle, apoptosis, DNA repair, metabolism, oxidative stress resistance and longevity. Genetic studies of *Caenorhabditis elegans* demonstrated that FOXO factors are major targets of the insulin-like signalling implicated during the regulation of glucose metabolism and lifespan extension. Recently, emerging evidence has also shed light on signalling pathways under oxidative stress that regulate FOXO activity by various post-translational modifications, including phosphorylation, acetylation and ubiquitination. Thus, FOXO factors react to external stimuli by altering their modifications, modulating target gene expression that may be involved in longevity.

Key words: FOXO, lifespan regulation, oxidative stress, post-translational modification, transcription.

Forkhead box O (FOXO) transcription factors belong to the large Forkhead family of proteins, which are characterized by a conserved DNA-binding domain termed the 'forkhead box (FOX)' (*1*). Forkhead subgroups of the O class (FOXO) contain four members in mammals: FOXO1, FOXO3, FOXO4 and FOXO6 (*2*). FOXO subgroups have also been identified in other organisms including *Caenorhabditis elegans* and *Drosophila melanogaster* (*2*). FOXO factors are associated with a wide range of biological processes, including cell cycle arrest, apoptosis, DNA repair, muscle atrophy, glucose metabolism, anti-oxidative stress and longevity (*3*, *4*). One of the most intriguing functions of FOXO transcription factors is their conserved ability to increase lifespan (*2*, *5–7*), which is correlated with the detoxification of reactive oxygen species (ROS) and the repair of damaged DNA (*8–11*). In this review, we briefly summarize the current understanding of the roles of FOXO factors, paying special attention to their implication in oxidative stress response and lifespan regulation.

REGULATION OF FOXO ACTIVITY BY PHOSPHORYLATION

A crucial mechanism that strictly regulates FOXO activity is a shuttling system, which confines FOXO factors to either the nucleus or the cytoplasm. Shuttling of FOXO factors requires protein phosphorylation within several domains and association with 14-3-3 proteins and the nuclear transport machinery (*12*). Among the numerous transduction cascades, the first-identified and primary regulation signal for FOXO factors is the PI3K-Akt signalling pathway that responds to insulin/IGF-I and several other growth factors (*13*).

Insulin-like Signalling Pathway—The initial evidence that FOXO might be a key downstream effecter of the PI3K-Akt pathway was derived from genetic studies in *C. elegans* (*14*, *15*). Inactivating mutations in the worm insulin receptor (*daf*-2) or PI3K (*age*-1) resulted in up to 3-fold extended longevity (*16–19*). This lifespan extension was reverted when the worm FOXO ortholog, *daf-16* was mutated (*14*, *15*). These genetic studies revealed that the insulin-like signalling pathway is a major determinant of lifespan and that the FOXO factors comprise a critical downstream modulator that is negatively regulated by its upstream components. Moreover, DAF-16-dependent longevity in worms was reported to account for the detoxification of ROS by up-regulating the expression of free radical scavenging enzyme, such as Mn superoxide dismutase (MnSOD, SOD3 in worms) (*20*).

Biochemical studies in mammalian cells, on the other hand, have shown that Akt (also called PKB) directly phosphorylates the FOXO transcription factors FOXO1, FOXO3 and FOXO4 at three regulatory sites (The24, Ser256 and Ser319 in the human FOXO1 sequence) that are both putative Akt recognition motifs (RXRXX(S/T) and highly conserved between nematodes and mammals (*13*, *21*, *22*). In the absence of insulin/IGF-I or other growth factors, when Akt is inactive, FOXO factors are predominantly localized to the nucleus. When the PI3K-Akt cascade is activated by growth factors, the FOXO proteins are directly phosphorylated, resulting in their nuclear export and cytoplasmic retention via binding to 14-3-3 proteins (*13*, *23*). 14-3-3 proteins are a family of cellular chaperones that interact with their protein ligands in a phosphorylation-dependent manner (*24*). In the nucleus, the interaction between 14-3-3 proteins and FOXO factors is dependent on the phosphorylation of two consensus sites by Akt (*13*, *23*). Several mechanisms have been suggested

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to explain how the interaction of 14-3-3 proteins and FOXO factors promotes the exclusion of FOXO factors from the nucleus. A possible explanation is that 14-3-3 protein binding to FOXO factors may induce an interaction between Exportin/Crm1 and the FOXO nuclear export signal (NES) (*23*) and also prevent re-entry of FOXO factors into the nucleus by masking the FOXO nuclear localization signal (NLS) (*25*, *26*). The interaction of 14-3-3 proteins and FOXO factors appears to be a determinant of FOXO subcellular localization by altering the relative rate of FOXO nuclear import and export.

JNK Signaling—Longevity is known to be coupled with increased oxidative stress resistance in a variety of organisms (*27*). It is therefore feasible that FOXO factors respond to oxidative stress stimuli via certain signalling pathways and thus allow an adaptive response to stress. A series of recent experiments indicated that JNK, a member of the MAPK superfamily, is a critical upstream modulator of FOXO factors in several organisms (*28–30*). The JNK signalling pathway is triggered by external stimuli, including UV radiation and oxidative stress, and the JNK cascade is evolutionary conserved. The first clues to the mechanism of JNK-dependent FOXO regulation came from a *Drosophila* study suggesting that JNK signalling is an important genetic factor for lifespan regulation (*31*). Further genetic analysis in flies established that JNK requires the fly FOXO ortholog dFOXO for lifespan extension (*30*). JNK promotes the nuclear translocation of dFOXO and induces the expression of small heat shock proteins, preventing the accumulation of protein aggregates caused by oxidative stress. In addition, JNK systemically antagonizes the insulin-like signalling pathway by activating dFOXO and repressing the expression of *dilp2*, a peptide that closely resembles human insulin (*30*). Whether JNK directly phosphorylates dFOXO in flies is not yet known. In *C. elegans*, the worm JNK ortholog JNK-1 binds and phosphorylates DAF-16 in response to heat stress, and this phosphorylation was implicated in the nuclear translocation of DAF-16 (*29*). Genetic studies have suggested that the JNK pathway acts in parallel but opposite fashion to the insulin-like signalling that regulates lifespan, and both pathways converge onto DAF-16 (*29*). On the other hand, human FOXO4 is phosphorylated by JNK at residues The447 and The451 via the oxidative stress-dependent activation of the small GTPase Ral (*28*). Although FOXO4 phosphorylation by JNK does not disrupt any interaction with 14-3-3 proteins in the cytoplasm, JNK has also been shown to phosphorylate 14-3-3 proteins directly, thereby releasing 14-3-3 substrates (*32*). Indeed, JNK activity promotes the nuclear translocation of FOXO4 and up-regulates the expression of MnSOD (*28*). The physiological relevance of the JNK-mediated FOXO4 phosphorylation has not yet been addressed in mammals. Taken together, the Akt and JNK pathways appear to finely modulate FOXO activity; specifically, Akt prevents FOXO nuclear localization and suppresses longevity while JNK accelerates FOXO nuclear localization and extends lifespan.

MST1 Signalling—Recently, the protein kinase MST1 was found to be a mediator of FOXO transcription factors and to be required for oxidative stress-induced cell death in primary mammalian neurons (*33*). MST1 phosphorylates FOXO factors at a site within the forkhead domain of these proteins that is highly conserved from nematodes to mammals (*33*). In mammalian neurons, oxidative stress-activated MST1 phosphorylates FOXO3, which in turn disrupts the 14-3-3/FOXO3 protein complex in the cytoplasm, promoting nuclear translocation of FOXO3 and thereby inducing the expression of the pro-apoptotic FOXO target gene BIM (*33*). Genetic analysis in worms further demonstrated that knockdown of the nematode MST1 ortholog CST-1 accelerated aging and reduced lifespan, while overexpression of CST-1 prolonged lifespan and delayed aging (*33*). Importantly, the *cst-1*-induced lifespan extension occurs in a *daf-16*-dependent manner (*33*). These findings present an intimate and evolutionarily conserved signaling link between the MST1 and FOXO transcription factors that regulates cellular responses to oxidative stress in mammals and induces longevity in nematodes.

REGULATION OF FOXO ACTIVITY BY ACETYLATION/DEACETYLATION

A series of recent studies in mammalian cells indicates that the reversible acetylation of FOXO proteins by nuclear coactivators and corepressors provides another layer of regulation of nuclear FOXO transcriptional factors.

CBP/p300—The acetyltransferase protein, CBP (CREB-binding protein) and p300 have been shown to act as global transcriptional coactivators by associating with numerous transcription factors (*34*). Our group and others have demonstrated that CBP/p300 directly binds and acetylates FOXO proteins in mammalian cells (*35–38*). We have further shown that CBP modulates the transactivation function of FOXO proteins via its acetyltransferase activity (*37*, *38*), but the consequence of acetylation on FOXO activity (activation or repression) is a still a matter of debate (*39*, *40*). We hypothesize that CBP coactivates FOXO-mediated transcription, presumably by acetylating chromosomal histones surrounding target genes, thereby forming the pre-initiation complex; subsequent acetylation of FOXO, however, leads to its transcriptional attenuation (*38*). Because CBP-induced acetylation sites are primarily located in the forkhead domain of FOXO proteins, we considered that acetylation could interfere with FOXO binding to target DNA and thereby prevent FOXO-mediated transcription. Indeed, acetylation by CBP at the positively charged basic residues in the FOXO1 DNA-binding domain diminished its ability to interact with target gene DNA (*41*). Interestingly, acetylation promotes the phosphorylation of FOXO1 via the PI3K-Akt signaling pathway (*41*). These findings suggest a mechanism by which the acetylation of FOXO1 destabilizes the FOXO1-DNA complex, and hence Akt readily phosphorylates FOXO1 within the DNA-binding domain.

SIR2 (SIRT1)—SIR2 and its ortholog SIRT1, belong to a family of NAD-dependent protein deacetylases, called sirtuins, which respond to metabolic changes in the cellular environment, including the availability of nutrients/energy and cellular stress (*42*). A series of genetic studies has demonstrated that increased Sir2 activity extends the lifespan of (*43*), as well as the longevity

Table 1. **The mediators of FOXO proteins.**

Mediator	Modification	Function	Lifespan
Akt	Phosphorylation	Nuclear export	Decrease
JNK	Phosphorylation	Nuclear import	Increase
MST1	Phosphorylation	Inhibition of cytoplasmic retention Nuclear import	Increase
CBP/p300	Acetylation	Transcriptional coactivation Reduction of DNA-binding ability	–
SIRT1	Deacetylation	Gene-specific transcriptional modulation	Increase
Skp2	Polyubiquitination	Proteasomal degradation	–
Unknown	Monoubiquitination	Nuclear import?	–
USP7	Deubiquitination	Nuclear export	–
β-catenin	–	Transcriptional coactivation	Increase
SMK-1	–	Gene-specific transcriptional modulation	Increase
14-3-3	–	Cytoplasmic retention Transcriptional coactivation	Increase

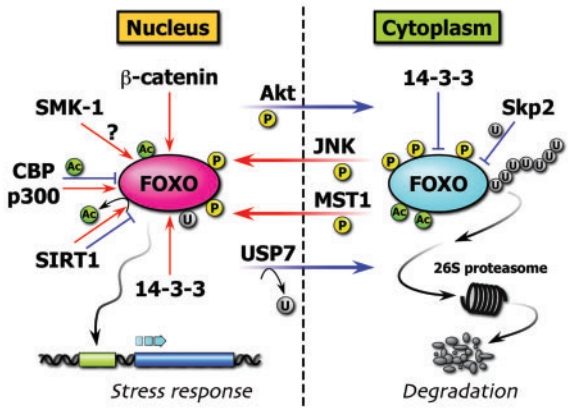

Fig. 1. **Model of FOXO regulation, especially during oxidative stress.** In response to oxidative stress, JNK (*28–30*) and MST1 (*33*) phosphorylate FOXO proteins, causing the nuclear translocation of FOXO factors. In the nucleus, SIRT1 binds and deacetylates FOXO proteins at residues acetylated by CBP/p300 (*38, 46–49*). β-catenin (*57*) and 14-3-3 (*59, 60*) proteins interact with FOXO factors and enhance their transactivating functions. Oxidative stress stimuli also induce the monoubiquitination and USP7-dependent deubiquitination of FOXO proteins, which controls the intracellular shuttling of FOXO factors (*56*). SMK-1 constitutively remains in the nucleus and genetically correlates with FOXO factors, but physical interactions have not yet been tested (*58*). Nuclear FOXO factors induce the expression of anti-oxidative stress genes (*8–11*). Conversely, Akt-mediated phosphorylation of FOXO proteins leads to their nuclear exit and cytoplasmic retention by 14-3-3 (*13*). Thereafter, Skp2-containing E3 ubiquitin ligase polyubiquitinates FOXO proteins, resulting in their proteasomal degradation (*52–54*). P (yellow), phosphorylation; Ac (green), acetylation; U (grey), ubiquitination.

of worms (*44*) and flies (*45*). In *C. elegans*, the ability of *sir-2* to prolong lifespan is entirely dependent on *daf-16* (*44*). Given that *daf-16* is required for the increased longevity of worms due to reduced insulin-like signalling activity, the genetic correlation between *sir-2* and *daf-16* suggests that SIR2 protein functionally interacts with one or more components of the insulin-like signalling pathway, including *daf-16*. As expected, several recent articles have shown that the human Sir2 ortholog SIRT1 binds and deacetylates FOXO proteins at lysine residues that are acetylated by CBP/p300 (*38*, *46–49*). Importantly, the interaction between SIRT1 and FOXO factors occurs in response to oxidative stress (*39*, *46*, *48*, *49*). Although the effects of SIRT1 on FOXO function vary depending on FOXO target genes, the consensus that emerges from these studies is that SIRT1 may play a crucial role in tipping the balance of FOXO functions away from cell death and towards stress resistance (*40*). More recently, extensive genetic analysis with a newly developed *sir-2* deleted strain revealed that *sir-2* is required for lifespan extension due to calorie restriction, but is completely independent of the insulin-like signalling pathway (*50*). These genetic data suggests a model in which *sir-2* and *daf-16* have both overlapping and distinct functions with respect to the regulation of the lifespan of *C. elegans* (*50*).

The reversible acetylation of FOXO1 is also involved in protection against pancreatic β-cell failure (*51*). Chronic exposure to elevated glucose concentrations, the so-called 'glucose toxicity' that is commonly seen in type 2 diabetes, causes continual oxidative stress and consequently impairs insulin gene transcription in β-cells. Acetylation serves the dual purposes of increasing FOXO1 stability and targeting FOXO1 to PML nuclear bodies, where it is deacetylated by SIRT1 to enhance expression of the *Ins2* gene transcription factors NeuroD and MafA (*51*).

REGULATION OF FOXO ACTIVITY BY POLYUBIQUITINATION, MONOUBIQUITINATION AND DEUBIQUITINATION

In addition to chemical modification, including phosphorylation and acetylation, we and others have found that FOXO transcription factors can be regulated by irreversible polyubiquitination and reversible monoubiquitination in mammalian cells.

Skp2—In response to insulin and serum growth factors, the polyubiquitination and subsequent degradation of FOXO factors occurs through the 26S proteasome (*52–54*). The polyubiquitination of FOXO factors requires both Akt-dependent phosphorylation and cytoplasmic

retention (*52*). A recent report identified the E3 ubiquitin ligase complex that catalyses FOXO1 polyubiquitination (*55*). FOXO1 binds to the F-box protein Skp2, a subunit of the SCF (Skp1/Cul1/F-box) E3 ubiquitin ligase protein complex and consequently leads to the degradation of FOXO1 (*55*). The polyubiquitin-mediated degradation of FOXO factors represents an irreversible level of regulation.

USP7—Very recently, van der Horst *et al*. (*56*) revealed a novel regulatory mechanism of FOXO proteins via reversible monoubiquitination. Under oxidative stress, monoubiquitination of FOXO4 occurs rapidly and induces its nuclear localization and transcriptional activation. Interestingly, following rapid monoubiquitination, oxidative stress also results in the deubiquitination of FOXO4 by the deubiquitinating enzyme USP7. In contrast to the phospho-dependent polyubiquitination, this reversible monoubiquitination does not influence FOXO protein stability. Notably, monoubiquitination and acetylation compete, at least in part, for the same lysine residues of FOXO4. In view of that monoubiquitination of FOXO4 precedes oxidative stress-induced acetylation, the authors suggest a model of stress response of FOXO4 in which the initial activation of FOXO4 via monoubiquitination can be terminated by USP7-mediated deubiquitination and subsequent CBP/p300-mediated acetylation to prevent further monoubiquitination.

REGULATION OF FOXO ACTIVITY BY OTHER MEDIATORS

As described earlier, the activation of several signalling cascades in response to various external stimuli converges on FOXO factors in the form of multiple post-translational modifications, including phosphorylation, acetylation and ubiquitination, thus regulating stress-resistance activities of FOXO factors. In this section, we focus on new mediators of longevity that regulate FOXO function directly but independent of post-translational modifications.

β-*catenin*—β-catenin is a multifunctional protein that mediates Wnt signalling by associating with TCF transcription factors. The molecular link between FOXO factors and β-catenin was shown by the observation that *C. elegans* mutants lacking the function of BAR-1, a β-catenin ortholog, exhibit defects in starvation-induced dauer formation (*57*). Because DAF-16 is required for both dauer development and normal longevity, Essers *et al*. (*57*) investigated whether BAR-1 is involved in DAF-16 function. As expected, genetic studies showed that BAR-1 is required for DAF-16 signalling during dauer development and lifespan regulation. Moreover, mammalian β-catenin binds to FOXO4 and augments its transcriptional activity in response to oxidative stress. These findings provide evidence that β-catenin plays an essential role in the regulation of the FOXO-mediated detoxification response in worms and mammalian cells, particularly under conditions of oxidative stress.

SMK-1—Although the transcriptional targets of DAF-16 include a large number of genes involved in dauer formation, reproduction, heat-stress response, detoxification of oxidative stress and resistance to bacterial infection, the mechanism by which DAF-16 defines these different processes is unknown. Wolff *et al*. (*58*) searched for cofactors of DAF-16 that act specifically to regulate the aging process in *C. elegans* and identified a single gene, *smk-1*. Genetic, molecular, and physiological analysis showed that SMK-1 is indispensable for DAF-16-dependent longevity but does not regulate dauer formation or the reproductive functions of DAF-16. During the aging process, SMK-1 serves as a transcriptional coregulator specific for the regulation of oxidative stress, UV, and innate immunity, but is not required for the thermal response functions of DAF-16. Thus, SMK-1 appears specific to the regulation of insulin-like signaling-mediated longevity in *C. elegans*. Whether orthologs of SMK-1 proteins in diverse eukaryotic organisms such as yeasts, flies, worms, and mammals affect longevity and the stress response is not yet known.

14-3-3—As described in the 'Insulin-like Signaling Pathway' section, mammalian 14-3-3 proteins are known to form a complex with FOXO proteins via Akt-mediated phosphorylation, and the complex serves as a chaperone molecules that escorts FOXO proteins out of the nucleus. Therefore, it was proposed that 14-3-3 proteins are negative modulators of FOXO functions, including longevity. Surprisingly, however, two recent genetic studies in worms revealed that an increased dosage of 14-3-3 orthologs, PAR-5, or FTT-2, extends lifespan, and this extension requires DAF-16 (*59*, *60*). Following heat stress, SIR2 interacts with DAF-16 in a 14-3-3-dependent manner. Notably, SIR2-mediated longevity can be canceled by the reduction of 14-3-3 proteins, but SIR2 and 14-3-3 proteins have no effect on the increased lifespan due to reduced insulin-like signaling activity. Finally, the authors have proposed the existence of a stress-dependent pathway in which 14-3-3 proteins positively regulate lifespan by cooperating with both SIR2 and DAF-16.

CONCLUDING REMARKS

This review highlights recent discoveries of FOXO transcription factor regulatory networks that have primarily emerged from genetic studies of lifespan regulation in worms and flies. Alternatively, biochemical studies have continued to elucidate a complex regulatory code, including phosphorylation, acetylation, ubiquitination and other post-translational modifications, and how FOXO factors can specify precise programs of gene expression in response to diverse environmental cues. Further studies of the FOXO regulatory networks, using both genetic and biochemical strategies, will help us understand the complex molecular mechanism of this key molecule and provide important insight into the mechanisms of longevity.

Works in the author's laboratory is supported, in part, by Grant-in-Aid for Scientific Research in Priority Areas 'A nuclear system to DECODE' from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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