Dynamic FoxO transcription factors

Haojie Huang[1,*] and Donald J. Tindall[2]

[1]Cancer Center and Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455, USA
[2]Departments of Urology and Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN 55905, USA
*Author for correspondence (e-mail: huang253@umn.edu)

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Summary

Forkhead box O (FoxO) transcription factors FoxO1, FoxO3a, FoxO4 and FoxO6, the mammalian orthologs of *Caenorhabditis elegans* DAF-16, are emerging as an important family of proteins that modulate the expression of genes involved in apoptosis, the cell cycle, DNA damage repair, oxidative stress, cell differentiation, glucose metabolism and other cellular functions. FoxO proteins are regulated by multiple mechanisms. They undergo inhibitory phosphorylation by protein kinases such as Akt, SGK, IKK and CDK2 in response to external and internal stimuli. By contrast, they are activated by upstream regulators such as JNK and MST1 under stress conditions. Their activities are counterbalanced by the acetylases CBP and p300 and the deacetylase SIRT1. Also, whereas polyubiquitylation of FoxO1 and FoxO3a leads to their degradation by the proteasome, monoubiquitylation of FoxO4 facilitates its nuclear localization and augments its transcriptional activity. Thus, the potent functions of FoxO proteins are tightly controlled by complex signaling pathways under physiological conditions; dysregulation of these proteins may ultimately lead to disease such as cancer.

Key words: Akt, CDK2, FoxO, Cancer, Phosphorylation, Ubiquitylation

Introduction

Forkhead transcription factors are a superfamily of proteins. Since the identification of the *fork head* gene in *Drosophila melanogaster*, the founding member of this family whose mutation results in the development of a forkhead-like appearance (Weigel et al., 1989), more than 100 structurally related forkhead transcription factors have been identified. Proteins in this family share a conserved 100-residue DNA-binding domain, the so-called forkhead (FKH) domain. Crystal structure analysis of hepatocyte nuclear factor 3 γ (HNF-3γ), one member of this family, indicates that this domain contains three major α-helices and two large wing-like loops (Clark et al., 1993) (see Fig. 1A). Therefore, these proteins are also called winged helix transcription factors. Owing to the use of multiple names and classification systems in the literature, a new nomenclature classifying these proteins as forkhead box (Fox) transcription factors has been adopted (Kaestner et al., 2000). Mammalian FoxO proteins belong to the O ('other') class of the Fox superfamily (Barthel et al., 2005; Kaestner et al., 2000), which reflects the fact that FoxO proteins form the most divergent subfamily of the Fox family because of sequence differences within their DNA-binding domains.

The presence of highly conserved sites for phosphorylation by the survival kinase Akt [a downstream target of PI3-kinase (PI3K) signaling] within and nearby their forkhead domains is the other distinguishing feature of FoxO proteins. Genetic studies in *Caenorhabditis elegans* have demonstrated that activation of the PI3K/Akt pathway by insulin or insulin-like growth factor 1 (IGF-1) suppresses activity of the DAF-16 forkhead transcription factor, the nematode ortholog of mammalian FoxO proteins (Kimura et al., 1997; Lin et al., 1997; Ogg et al., 1997). Analysis of the DAF-16 sequence reveals three consensus Akt sites [RXRXX(S/T)] (Alessi et al., 1996; Brunet et al., 1999). These sites have been found conserved in all the members of the mammalian FoxO family, which include FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX) and FOXO6 in humans (Biggs, 3rd et al., 1999; Brunet et al., 1999; Jacobs et al., 2003; Kops et al., 1999; Rena et al., 1999; Tang et al., 1999). Akt-phosphorylated FoxO proteins bind to 14-3-3 chaperone proteins and become sequestered in the cytoplasm, where they are unable to regulate gene expression.

FOXO1 was originally called FKHR (forkhead in rhabdomyosarcomas). The gene was identified in studies of the t(2,13)(q35;q14) and t(1,13)(p36;q14) chromosomal translocations commonly found in alveolar rhabdomyosarcoma, a skeletal-muscle tumor that is prevalent in children (Galili et al., 1993). Interestingly, two other members of this family, FOXO3a and FOXO4, also lie at sites of chromosomal translocations that occur in human tumors. FOXO3a [also called FKHR-like protein 1 (FKHRL1)] was cloned by cDNA library screening as a relative of FKHR (Anderson et al., 1998) and, independently, identified following characterization of a chromosomal translocation [t(6;11)(q21;q23)] from an acute leukemia patient (Hillion et al., 1997). While defining genes that fused with the *MLL* gene as a consequence of chromosomal translocations in acute leukemias, three groups identified *FOXO4*, a novel forkhead-box-containing gene initially designated *AFX* (acute leukemia fusion gene located in chromosome X), at the chromosomal translocation site t(X;11)(q13;q23) (Borkhardt et al., 1997; Corral et al., 1993; Parry et al., 1994). FoxO6 is the latest member of the FoxO family to be cloned and shares significant sequence similarity with the other members of the family (Jacobs et al., 2003).

FoxO proteins function primarily as transcription factors in the nucleus and bind as monomers to their cognate DNA targeting sequences. The co-crystal structure of HNF-3γ with DNA shows that there are 14 protein-DNA contacts distributed

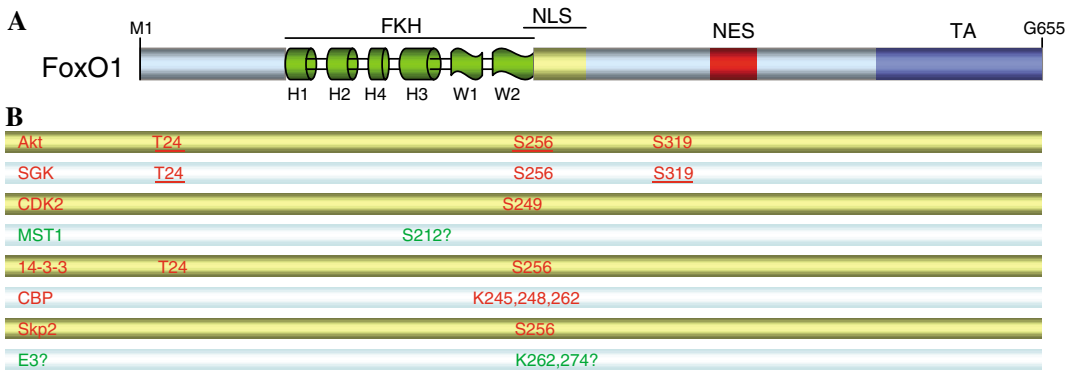

Fig. 1. Domain structure of the FoxO1 protein (A) and the major post-translational modifications or protein interactions within and near the forkhead (FKH) domain and the nuclear localization signal (NLS) (B). NES, nuclear export signal; TA, the transactivation domain. The secondary structures predicted from the FKH domain of HNF-3γ – four α-helices (H1, H2, H3 and H4) and two winged-loops (W1 and W2) – are included. Residues representing the identified or potential post-translational modifications, and the site for interaction with Skp2, that inhibit FoxO1 are indicated in red; those that promote FoxO1 activity are in green. Preferred Akt- and SGK-phosphorylation sites are underlined.

throughout the forkhead domain but the primary DNA-recognition site is at α-helix H3 (Clark et al., 1993). Both winged loops also make important interactions with DNA (Boura et al., 2007; Clark et al., 1993). Notably, most post-translational modifications (see below) occur in these regions (see Fig. 1A,B). Although the molecular basis of the DNA-binding specificity of FoxO transcription factors is poorly understood, high-affinity DNA-binding studies have identified a consensus FoxO-recognized element (FRE) as (G/C)(T/A)AA(C/T)AA (Biggs, 3rd et al., 1999; Furuyama et al., 2000; Gilley et al., 2003). Indeed, functional FRE sites that match this consensus sequence have been identified in the promoters of genes encoding Fas ligand (FasL), insulin-like growth factor binding protein 1 (IGFBP1), the apoptotic regulator BIM, and many other proteins (for reviews, see Accili and Arden, 2004; Greer and Brunet, 2005). Additional putative FoxO-target genes and their potential cis-regulatory binding sites have been predicted by systematic bioinformatic approaches (Xuan and Zhang, 2005). Thus, FoxO transcription factors appear to be involved in various signaling pathways and control a wide range of biochemical processes.

Functions of FoxO transcription factors

FoxO proteins can regulate cell fate by modulating the expression of genes involved in apoptosis, cell cycle transitions, DNA repair, oxidative stress and longevity, and control of muscle growth (Figs 2 and 3), as well as cell differentiation and glucose metabolism [see reviews elsewhere (Accili and Arden, 2004; Barthel et al., 2005; Greer and Brunet, 2005)]. Below we examine some of these processes and key target genes.

Apoptosis

The link between activation of the FoxO proteins and cell death was first established by the finding that these proteins can be phosphorylated and inhibited by Akt, a regulator of cell survival (Biggs, 3rd et al., 1999; Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999; Tang et al., 1999). Moreover, three consensus FRE sequences were identified in the promoter of the pro-apoptotic gene Fas ligand (*FasL*) (Fig. 3) (Brunet et al., 1999), which encodes a protein that activates the death receptor Fas/CD95/APO-1 and promotes mitochondria-independent apoptosis. Importantly, expression of a phosphorylation-resistant form of FoxO3a triggers apoptosis in cerebellar

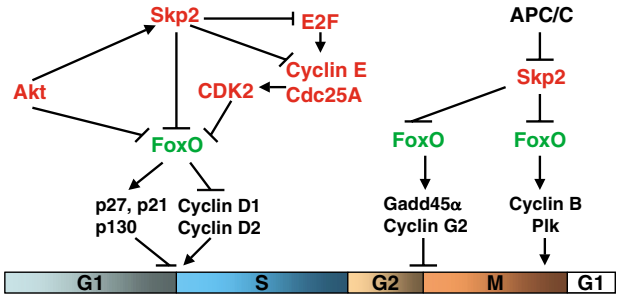

Fig. 2. Transcriptional activity and regulation of FoxO transcription factors during the cell cycle. Negative regulators of the G1/S transition of the cell cycle, such as $p27^{KIP1}$, $p21^{WAF1}$ and p130, are upregulated by the FoxO transcription factors. Moreover, expression of positive regulators, such as cyclin D1 and D2, is repressed by FoxO proteins. Activation of FoxO proteins also induces the expression of Gadd45α and cyclin G2, resulting in cell cycle arrest at G2/M. By contrast, activation of the transcriptional activity of FoxO proteins induces expression of cyclin B and polo-like kinase (Plk), two key genes during mitosis. These FoxO-dependent transcriptional programs appear to be tightly controlled by various signaling pathways during the cell cycle. Upon stimulation with growth factors, quiescent (G0) cells re-enter G1 phase, and Akt is activated. Activation of Akt can lead to the phosphorylation and inhibition of FoxO proteins. Moreover, activated Akt also induces the expression of Skp2, with which it works in concert to promote the degradation of FoxO proteins, at least in the case of FoxO1. With the progression of cells into S phase, CDK2 is highly activated owing to E2F-dependent expression of cyclin E and Cdc25A, two activators of CDK2. Activated CDK2 can phosphorylate and inhibit FoxO proteins, such as FoxO1 and FoxO6. At the end of DNA synthesis, E2F and cyclin E can be degraded through a Skp2-dependent mechanism and, therefore, CDK2-mediated inhibition of FOXO1 is diminished. It is possible that the inhibitory function of FoxO proteins at G2/M is under the control of Skp2 only until anaphase, when Skp2 is targeted for degradation by the anaphase-promoting complex/cyclosome (APC/C) E3 ligase.

granule neurons through a mechanism that depends, at least in part, on FasL (Brunet et al., 1999). Furthermore, an unbiased gene profiling study revealed that tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL), another death receptor ligand, is FoxO-regulated in prostate cancer cells (Modur et al., 2002). Thus, FoxO proteins regulate cell survival by modulating the expression of death receptor ligands that function in autocrine and paracrine pathways.

In addition to the death receptor ligands, FoxO proteins have been shown to be involved in the transactivation of *BIM*, a gene that encodes a member of the pro-apoptotic BH3-only subgroup of BCL-2 family proteins, which functions in the 'intrinsic', mitochondrial apoptotic pathway. FoxO proteins were first implicated in the induction of *BIM* in hematopoietic cells deprived of growth factors (Dijkers et al., 2000a; Stahl et al., 2002). Two functional FRE sites are present in the *BIM* promoter (Gilley et al., 2003). Thus, FoxO transcription factors can induce cell death through mitochondria-dependent and -independent mechanisms.

Cell cycle transitions and DNA repair

Whereas forced expression of FoxO1 induces apoptosis in certain cancer cell types, such as prostate cancer cells (Huang et al., 2004; Li et al., 2003; Modur et al., 2002; Nakamura et al., 2000), it induces G1 arrest in renal cell carcinoma and glioma cells lacking the tumor suppressor PTEN (phosphatase and tensin homologue deleted on chromosome ten) by upregulating the cyclin-dependent kinase inhibitor $p27^{KIP1}$ (Figs 2 and 3) (Nakamura et al., 2000). The activity of the *p27*^{KIP1} promoter is significantly increased by the expression of a constitutively active form of FoxO4 – as measured by luciferase reporter gene assays (Medema et al., 2000). FoxO-induced G1 arrest can also be attributed, at least in part, to induction of the retinoblastoma protein (RB)-related protein p130, another negative regulator of the G1/S transition (Kops et al., 2002b). Moreover, in response to the stimulation of transforming growth factor-β (TGF-β), FoxO proteins form a protein complex with activated Smad proteins to induce expression of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ and G1-phase arrest of the cell cycle (Seoane et al., 2004).

FoxO-induced G1 arrest has also been linked to the downregulation of cyclin D1 and D2 (Ramaswamy et al., 2002; Schmidt et al., 2002). Because a FoxO1 mutant (H215R) that cannot bind to the FRE sequence can be recruited to the cyclin

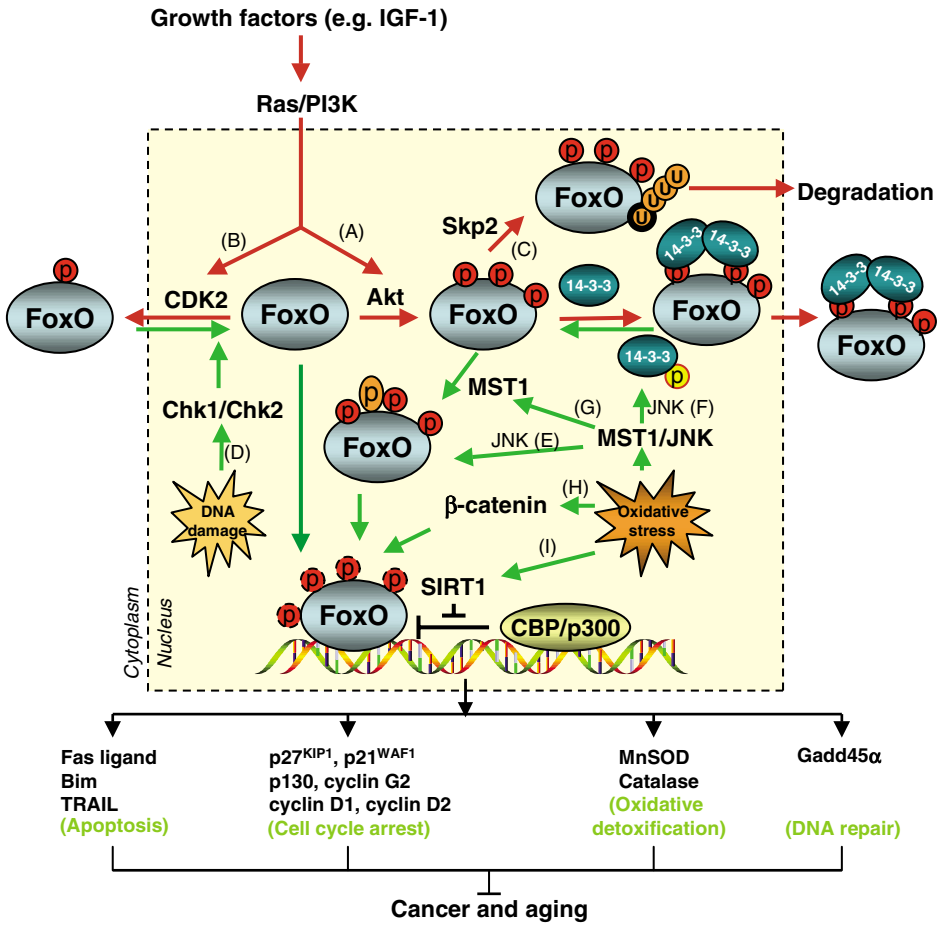

Fig. 3. Regulation of FoxO proteins in response to external and internal stimuli. Treatment of cells with growth factors such as IGF-1 and insulin leads to the activation of Akt (A) and CDK2 (B) through the Ras- and PI3K-dependent pathways. This in turn results in hyperphosphorylation of FoxO transcription factor. Akt-mediated phosphorylation allows FoxO proteins to bind to the chaperone proteins 14-3-3 and be exported into the cytoplasm in a CRM1-dependent manner. The substrate-binding F-box protein Skp2 of the SCF^{Skp2} E3 ligase also interacts with and ubiquitylates FoxO1 (C). This interaction requires Akt-mediated phosphorylation of FoxO1 at serine 256. CDK2-mediated phosphorylation of FoxO1 also leads to cytoplasmic localization of FOXO1 through a mechanism that appears not to be affected by 14-3-3 binding. Upon DNA damage, however, CDK2-dependent phosphorylation and cytoplasmic localization of FoxO1 is abolished; this depends on activation of Chk1 and Chk2 (D). Similarly, FoxO proteins translocate to the nucleus in response to oxidative stress. Oxidative-stress-promoted nuclear localization of FoxO proteins is likely to be because of their phosphorylation by JNK (E), JNK-dependent phosphorylation of 14-3-3 proteins (F) or direct phosphorylation of FoxO proteins by MST1 (G). Oxidative stress also enhances the interaction of FoxO proteins with β-catenin and thus their activity (H). Expression of the histone acetyl-transferases CBP and p300 has been shown to enhance the transcriptional activity of FoxO proteins. Interestingly, it has been shown that FoxO1 can be acetylated by CBP at three lysine residues. Acetylation of FoxO proteins by CBP/p300 inhibits their transcriptional activity. Thus, CBP/p300-induced increase in FoxO transcriptional activity appears to be mediated by general histone acetylation. This effect can be overcome by activation of the deacetylase SIRT1 under oxidative-stress conditions (I). P, phosphate; U, ubiquitin. Red arrows indicate negative regulation; green arrows indicate positive regulation. Bars show inhibitory effects.

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D1 and D2 promoters, FoxO proteins may repress their expression through crosstalk with other transcriptional regulators or have FRE-independent FoxO-binding elements in the promoters of these genes (Ramaswamy et al., 2002; Schmidt et al., 2002). Moreover, the BCR-ABL inhibitor STI571, FoxO3a and the transcriptional repressor BCL6 all repress transcription of the *cyclin D2* gene through a STAT5/BCL6 site in the promoter (Fernandez de Mattos et al., 2004). Inhibition of BCR-ABL is thought to be part of a signaling pathway that leads to FoxO3a activation, which in turn induces the expression of *BCL6* and thereby inhibits the transcription of cyclin D2.

The activity of FoxO transcription factors is also important for other cell cycle transitions (Fig. 2). Expression of FoxO3a in Rat-1 fibroblasts synchronized in S phase delays progression from early to late stages of mitosis (Tran et al., 2002). This result is consistent with gene array results showing that activation of FoxO3a induces expression of growth arrest and DNA damage-inducible protein 45 alpha (Gadd45α) and cyclin G2, both of which can mediate G2/M arrest by modulating the function of the G2-specific kinase complexes such as Cdc2-cyclin B1 (Tran et al., 2002). Expression of an active form of FoxO4 also activates the *Gadd45* promoter and induces G2/M arrest in unsynchronized C2C12 mouse myoblasts and HeLa cells (Furukawa-Hibi et al., 2002). Interestingly, cells expressing a constitutively active form of the catalytic subunit of PI3K (p110CAAX) accumulate in telophase, and this correlates with inactivation of FoxO3a in G2/M phase (Alvarez et al., 2001). Expression of an active form of FoxO3a in G2 phase overcomes the effect of p110CAAX and facilitates the transition from M phase to G1. Accordingly, the expression levels of cyclin B and polo-like kinase (Plk), two key factors for cytokinesis, increase in these cells (Alvarez et al., 2001). Thus, the function of FoxO proteins apparently undergoes a prompt shift from blocking G2/M transition to promoting the M/G1 transition during mitosis (Fig. 2) – the regulatory mechanism involved is unclear.

Expression of FoxO1 in alveolar rhabdomyosarcoma cells that contain very low levels of endogenous FoxO1 protein induces rapid apoptosis (Bois et al., 2005). By contrast, cells resistant to apoptosis primarily arrest in G2/M phase. At present, it is unknown whether FoxO proteins induce apoptosis in a cell-cycle-dependent manner or whether cells in G2/M phase are resistant to FoxO-induced cell death.

Oxidative stress resistance and longevity

Caloric restriction is known to increase the lifespan of *C. elegans*. This is thought to occur through a reduction in the levels of reactive oxygen species (ROS) produced during respiration. The silencing information regulator (*Sir2*) gene in yeast is required for caloric-restriction-induced increase in lifespan (Lin et al., 2000). The ability of Sir2 to extend lifespan in worms requires function of the FoxO ortholog DAF-16 (Tissenbaum and Guarente, 2001). One of the human orthologs of Sir2, SIRT1, forms a complex with FoxO3a in response to oxidative stress (Brunet et al., 2004). Interestingly, SIRT1-mediated activation of FoxO3a induces cell-cycle arrest and oxidative-stress resistance but inhibits FoxO3a-induced cell death, although the molecular basis for such biased effects of SIRT1 on the function of FoxO3a is currently unknown (Brunet et al., 2004).

Activated FoxO proteins promote stress resistance by binding to the promoters of the genes encoding manganese superoxide dismutase (MnSOD) and catalase, two scavenger proteins that play essential roles in oxidative detoxification in mammals (Balaban et al., 2005; Kops et al., 2002a; Nemoto and Finkel, 2002) (Fig. 3). FoxO-mediated oxidative-stress resistance is influenced by multiple other pathways. β-catenin binds directly to FoxO proteins and enhances their transcriptional activity in mammalian cells (Essers et al., 2005). This interaction is enhanced in cells exposed to oxidative stress (Fig. 3). The β-catenin ortholog in *C. elegans,* BAR-1, is required for oxidative-stress-induced expression of the DAF-16 target gene *sod-3* and for resistance to oxidative damage (Essers et al., 2005).

As in yeast and worms, the lifespan of *Drosophila* can be increased under stress conditions, such as those that activate the stress-responsive Jun-N-terminal kinase (JNK)-dependent pathway. Activation of JNK causes nuclear localization of FoxO, induces stress defense genes and increases the lifespan of *Drosophila* (Wang et al., 2005). In mammalian cells, there is a marked increase in ROS in FoxO-deficient hematopoietic stem cells compared with wild-type hematopoietic stem cells (Tothova et al., 2007). Importantly, production of ROS in FoxO-null hematopoietic stem cells is correlated with changes in the expression of genes that regulate their synthesis. Thus, members of the FoxO family function as a key component of various signaling pathways that influence longevity by induction of genes that are directly involved in oxidative-stress detoxification.

Muscle growth and atrophy

Decreased activity of the IGF-1/PI3K/Akt signaling pathway leads to skeletal muscle atrophy. Moreover, activation of Akt in rat muscles prevents denervation-induced atrophy. However, the direct link between these two events was not uncovered until recently. When cultured myotubes are undergoing atrophy, the activity of the PI3K/Akt pathway decreases, leading to the activation of FoxO transcription factors and the induction of atrogin-1 (MAFbx), an F-box protein component of a muscle-specific ubiquitin E3 ligase complex (Sandri et al., 2004). Importantly, constitutively active FoxO3a acts directly on the atrogin-1 promoter to induce the expression of this gene and atrophy of myotubes and muscle fibers (Sandri et al., 2004). The IGF-1/PI3K/Akt pathway prevents induction of MAFbx and MuRF1, another muscle-specific ubiquitin E3 ligase; this inhibition might therefore be linked to the Akt-mediated inactivation of FoxO transcription factors (Stitt et al., 2004). In vivo studies in which FoxO1-transgenic mice have been shown to have decreased skeletal muscle mass and expression of type I (slow twitch/red muscle) fiber genes support this idea (Kamei et al., 2004).

Energy-rich exercising muscles that are resistant to atrophy express high levels of the peroxisome-proliferator-activated receptor γ coactivator 1 α (PGC-1α), an important coregulator for many transcription factors, including FoxO proteins. By contrast, expression of PGC-1α mRNA is largely decreased in skeletal muscles during atrophy induced by denervation as well as by cancer cachexia, diabetes and renal failure in an in vivo rodent model (Sandri et al., 2006). Transfection of PGC-1α into adult fibers decreases both FoxO3a-induced fiber atrophy and expression of the atrogin-1 gene (Sandri et al., 2006). This

provides an explanation for how exercise might combat muscle atrophy. FoxO3a also prevents both IGF-1 and stretch-induced hypertrophy in cultured rat neonatal cardiomyocytes (Skurk et al., 2005). It also stimulates the expression of multiple atrophy-related genes, 'atrogenes', including atrogin-1, and causes a significant reduction in cardiomyocyte size in the hearts of mice (Skurk et al., 2005). Thus, as in skeletal muscle, FoxO3a activates a transcription program of atrogenes that retards or prevents hypertrophy in cardiomyocytes.

In addition to the ubiquitin ligase pathways, a new mechanism that may play a crucial role in muscle atrophy is emerging (Hishiya et al., 2006). Expression of the zinc finger protein ZNF216, which binds directly to polyubiquitin chains through its N-terminal A20-type zinc finger domain and associates with the 26S proteasome, is increased during denervation- and fasting-induced muscle atrophy. Interestingly, expression of FoxO4 increases ZNF216 expression in C2C12 mouse myoblasts, and mice lacking ZNF216 are resistant to denervation-induced atrophy (Hishiya et al., 2006). Thus, FoxO proteins control muscle atrophy/hypertrophy through multiple pathways.

Post-translational modifications of FoxO proteins: it's all about nuclear localization

FoxO transcription factors are controlled by sophisticated signaling networks. In response to growth and survival factors such as insulin and IGF-1, they become phosphorylated and localized to the cytoplasm. By contrast, these proteins remain in the nucleus in response to various stress events even in the presence of growth factors. Most of these environmental stimuli result in post-translational modifications of FoxO factors, which include phosphorylation, ubiquitylation and acetylation.

Phosphorylation

The DAF-16 forkhead transcription factor in *C. elegans* is inhibited by the PI3K/Akt pathway (Lin et al., 1997; Ogg et al., 1997). Similarly, mammalian FoxO proteins are phosphorylated by Akt at three sites (threonine 24, serine 256 and serine 319 in the FOXO1 sequence) (Fig. 1B and Fig. 3) (Biggs, 3rd et al., 1999; Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999; Tang et al., 1999). Phosphorylated FoxO3a binds to 14-3-3 proteins and remains in the cytoplasm in cells stimulated with survival factors such as IGF-1 or transfected with constitutively active Akt (Fig. 3). As such, its function as a transcription factor is abrogated (Brunet et al., 1999). Although the majority of 14-3-3 proteins are localized to the cytoplasm, surprisingly they bind to FoxO proteins in the nucleus (Fig. 3) (Brunet et al., 2002), which is consistent with the finding that Akt localizes to the nuclei of cells exposed to survival factors, including IGF-1 (Andjelkovic et al., 1997). 14-3-3-bound FoxO proteins are then exported into the cytoplasm. These proteins remain in the cytoplasm through a mechanism depending, at least in part, on the 14-3-3-mediated masking of their nuclear localization signals (NLSs) (Fig. 1A,B) (Brunet et al., 2002; Zhao et al., 2004).

FoxO proteins are also phosphorylated by serum- and glucocorticoid-inducible kinases (SGKs) (Fig. 1B) (Brunet et al., 2001). SGKs are serine/threonine kinases that are related to Akt. In common with Akt, these proteins are activated by the PI3K pathway and translocate to the nucleus in cells stimulated with survival factors. SGK1 phosphorylates FoxO3a at the same sites as those phosphorylated by Akt, likewise leading to the cytoplasmic localization and inhibition of FoxO3a. However, SGK1 preferentially phosphorylates serine 319 whereas Akt prefers serine 256 (Fig. 1B) (Brunet et al., 2001). SGK1 is a direct transcriptional target of the activated glucocorticoid receptor (GR), and induction of SGK1 is correlated with GR-mediated protection against apoptosis triggered by growth factor depletion (Mikosz et al., 2001). Activation of GR leads to the phosphorylation and inactivation of FoxO3a, and this effect requires GR-mediated induction of SGK-1 (Wu et al., 2006). Importantly, FoxO3a-induced apoptosis in SK-BR-3 breast cancer cells is inhibited by GR activation (Wu et al., 2006). Therefore, glucocorticoid-induced cell survival is likely to involve SGK1-mediated phosphorylation of FoxO3a. SGK3 can also directly phosphorylate and inactivate FoxO1 (Liu et al., 2000).

Surprisingly, FoxO3a remains in the cytoplasm in some tumors even in the absence of active Akt (Hu et al., 2004). This observation led to the discovery that IκB kinase (IKK) interacts with and phosphorylates FoxO3a at serine 644, thereby inhibiting its transcriptional activity in an Akt-independent manner (Hu et al., 2004). The effect is apparently specific to human and mouse FoxO3a, because this site is not conserved in other members of the FoxO family. Phosphorylation of FoxO3a by IKK also leads to its cytoplasmic localization, although the underlying export mechanism remains to be determined (Hu et al., 2004).

The dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK) phosphorylates FoxO1 at a novel phosphorylation site, serine 329 (Woods et al., 2001). This site is conserved from worms to human, and phosphorylation decreases the transcriptional activity of FoxO1. Interestingly, when this site is converted to alanine, the mutated FoxO1 becomes predominantly nuclear in 90% of cells, whereas wild-type protein is nuclear only in 75% of cells (Woods et al., 2001). Several serine residues near serine 329 can also be phosphorylated, and phosphorylation of these sites together with serine 329 helps to promote FoxO1 nuclear export by stabilizing interaction with Ran, a nuclear export protein (Rena et al., 2002).

FoxO1 can be phosphorylated by CDK2 primarily at serine 249 in vitro and in vivo (Fig. 1B and Fig. 3) (Huang et al., 2006). This phosphorylation site lies in a CDK consensus phosphorylation sequence [(K/R)(S/T)PX(K/R)] also identified in substrates of CDK2, such as RB and histone H1B. Although this site is not conserved throughout the entire FoxO subfamily, sequence analysis reveals its presence in human and mouse FoxO1 and FoxO6 (Huang et al., 2006). CDK2-mediated phosphorylation of FoxO1 not only decreases the basal transcriptional activity of FoxO1 but also overcomes PTEN-induced activation of FoxO1 (Huang et al., 2006), which suggests that CDK2 negatively regulates FoxO1 independently of Akt. Indeed, treatment of prostate cancer cells with the CDK inhibitor roscovitine and the PI3K inhibitor LY294002 synergistically induces the expression of the FoxO1 target gene *BIM* (Huang et al., 2006). Mutation of a three-arginine motif (residues 251-253) in one of the NLSs blocks the nuclear localization of FoxO proteins induced by survival factor withdrawal or inhibition of the PI3K/Akt pathway (Brunet et al., 2002). Phosphorylation within this NLS might therefore promote the cytoplasmic localization of FoxO

proteins either by increasing negative charge or masking the NLS as a consequence of 14-3-3 binding. Indeed, the CDK2 phosphorylation site in FoxO1 (serine 249) is adjacent to the three-arginine motif (Fig. 1A,B) (Huang et al., 2006), and co-transfection of FOXO1 with cyclin E and CDK2 leads to the cytoplasmic and perinuclear localization of FOXO1 in DU145 prostate cancer cells. Consistent with the finding that 14-3-3 prefers to bind to the phosphorylation sites at threonine 24 and serine 256 (Brunet et al., 1999) is the observation that phosphorylation of serine 249 does not affect 14-3-3 binding (Huang et al., 2006).

Whereas many signaling pathways drive FoxO proteins out of the nucleus, others can keep FoxO molecules in the nucleus. FoxO proteins localize to the cytoplasm in cells treated with growth factors. However, they remain in the nucleus under stress conditions, such as oxidative and genotoxic stresses, even in the presence of growth factors (Fig. 3) (Brunet et al., 2004; Essers et al., 2004; Huang et al., 2006). This suggests an additional layer of regulation that can counteract the signaling that leads to cytoplasmic localization of FoxO proteins. In response to DNA damage, for example, CDK2-mediated inhibition of FoxO1 is abolished in a manner dependent on activation of the checkpoint kinases Chk1 and Chk2 (Fig. 3) (Huang et al., 2006).

The oxidative-stress-induced nuclear localization of FoxO proteins has been linked to the JNK pathway (Essers et al., 2004). In the presence of low levels of oxidative stress generated by treatment of cells with H_2O_2, FoxO4 becomes activated and remains in the nucleus. This effect appears to involve phosphorylation of FoxO4 by JNK at threonine 447 and threonine 451 (Fig. 3) (Essers et al., 2004). The effect of JNK activation seems to be evolutionally conserved, because JNK can antagonize insulin/IGF-1 signaling in *Drosophila* and promote FoxO nuclear localization (Wang et al., 2005). Given that the JNK phosphorylation sites in FoxO4 are found in a region with a low degree of sequence similarity to other members of the FoxO family (Van Der Heide et al., 2004), additional mechanisms might mediate the JNK-dependent nuclear localization of FoxO proteins. Activation of JNK in vitro leads to phosphorylation of 14-3-3ξ at serine 184, which in turn causes dissociation of FoxO3a from 14-3-3 in the cytoplasm, resulting in nuclear localization of FoxO3a (Fig. 3) (Sunayama et al., 2005). Whether oxidative-stress-induced nuclear localization of FoxO proteins in cells is mediated by JNK-dependent phosphorylation and cytoplasmic sequestration of 14-3-3 remains to be investigated.

Another JNK-related mechanism has also been suggested for the nuclear localization of FoxO3a in cells under oxidative stress (Lehtinen et al., 2006). The yeast protein kinase Sterile 20 (Ste20) plays an important role in H_2O_2-induced cell death (Ahn et al., 2005), and the mammalian Ste20-like kinase-1 (MST1) can phosphorylate FoxO3a at serine 207 (Lehtinen et al., 2006). This blocks the interaction of FoxO3a with 14-3-3β and thereby induces the nuclear localization of FoxO3a (Fig. 3). Phosphorylation of serine 207 is also required for H_2O_2-induced nuclear localization of FOXO3a, and a mutation that converts serine 207 to alanine completely abolishes the nuclear localization of FoxO3a in cells treated with H_2O_2 (Lehtinen et al., 2006). This site is located in the FKH domain and is evolutionarily conserved throughout the FoxO family; FoxO1 is also likely to be regulated by MST1 (Fig. 1B). Notably, MST-FoxO interactions are also observed in *C. elegans* (Lehtinen et al., 2006). Interestingly, MST1 also activates the JNK pathway in mammalian cells (Graves et al., 1998). Thus, MST1 might regulate the activity of FoxO proteins through JNK-dependent pathways (Fig. 3).

Ubiquitylation

FoxO proteins are also regulated by the ubiquitin proteasome system. Steady-state levels of FoxO1 and FoxO3a are reduced in FL5.12 murine pro-B lymphocytes stably expressing Akt, and this effect is largely attenuated by treatment of cells with proteasome inhibitors (Plas and Thompson, 2003). Levels of FoxO1 also decrease in HepG2 cells following insulin treatment (Matsuzaki et al., 2003). Similarly, treatment of chicken embryo fibroblasts with platelet-derived growth factor (PDGF) decreases levels of FoxO1. This effect is inhibited by lactacystin (a proteasome inhibitor) or LY294002 (a PI3K inhibitor) (Aoki et al., 2004), which suggests that proteasome-mediated degradation of FoxO1 depends on Akt signaling. Moreover, phosphorylation by Akt is required for the polyubiquitylation of FoxO1 (Matsuzaki et al., 2003).

Ubiquitin-dependent degradation of FoxO1 requires its interaction with the F-box protein Skp2, the substrate-binding component of the Skp1/culin 1/F-box protein (SCF^{Skp2}) E3 ligase complex (Fig. 3) (Huang et al., 2005), which targets various proteins, including $p27^{KIP1}$, for degradation in the nucleus (Reed, 2002). Skp2-dependent polyubiquitylation of FoxO1 requires phosphorylation at serine 256 by Akt (Fig. 1B) (Huang et al., 2005). Skp2 is a nuclear protein (Sutterluty et al., 1999), Akt translocates into the nucleus upon activation by survival factors (Andjelkovic et al., 1997) and Akt phosphorylates FoxO proteins in the nucleus (Brunet et al., 2002). Thus, Skp2-dependent polyubiquitylation of FoxO1 probably occurs in the nucleus (Fig. 3). This notion is further supported by the finding that polyubiquitylation of FoxO1 is regulated by the promyelocytic leukemia protein Pml in the nuclei of pancreatic β cells (Kitamura et al., 2005). IKK-mediated phosphorylation of FoxO3a also leads to its ubiquitylation and degradation (Hu et al., 2004). However, the E3 ligase responsible for this event is unknown.

Whereas polyubiquitylation of FoxO1 and FoxO3a results in their degradation, FoxO4 is monoubiquitylated (van der Horst et al., 2006). Monoubiquitylation of FoxO4 is augmented in response to oxidative stress, leading to nuclear localization of FoxO4 and an increase in its transcriptional activity (van der Horst et al., 2006). Two conserved lysine residues, K199 and K211, which are located in the NLS at the C-terminus of the FKH domain of FoxO4, are targeted for monoubiquitylation (see Fig. 1B). Although it is unclear which E3 ligase is involved in this process, deubiquitylation of FoxO4 is known to be catalyzed by the deubiquitylating enzyme herpesvirus-associated ubiquitin-specific protease (HAUSP/USP7) (van der Horst et al., 2006). USP7-mediated deubiquitylation of FoxO4 results in the relocalization of FoxO4 from the nucleus to the cytoplasm. Therefore, oxidative-stress-mediated monoubiquitylation, at least for FoxO4, provides another means for cells to manipulate FoxO nuclear localization and thus its activity.

Acetylation

The nuclear proteins CBP and p300 and their associated

proteins, such as p300- and CBP-associated factor (PCAF), possess intrinsic histone acetyl-transferase (HAT) activity. These proteins play essential roles promoting transcription by acetylating histones and integrating signaling from enhancer and promoter regions. They also directly acetylate transcription factors through FAT (transcription factor acetyl-transferase) activity (Li et al., 2002). DAF-16 and its human ortholog FoxO1 recruit CBP to the *IGFBP1* promoter in HepG2 cells (Nasrin et al., 2000). CBP interacts physically with and acetylates mouse FoxO1 in vitro and in vivo (Daitoku et al., 2004). Expression of wild-type CBP, but not the mutant that lacks acetyl-transferase activity, enhances FoxO1 transcriptional activity (Daitoku et al., 2004). Surprisingly, substitutions in the CBP-acetylated sites in FoxO1 augment FoxO1 transcriptional activity (Daitoku et al., 2004). Thus, CBP appears to play a dual role in FoxO-mediated gene transcription: it can facilitate FoxO-mediated transcription by acetylating chromosomal histones but also promotes acetylation and regulation of FoxO proteins themselves (Fig. 1B and Fig. 3).

Acetylation of FoxO proteins by acetylases such as CBP and p300 increases in response to oxidative stress (Brunet et al., 2004; Frescas et al., 2005; Kitamura et al., 2005). Acetyl-FoxO proteins accumulate in the nucleus and associate with Pml bodies, which hinders their activity (Kitamura et al., 2005). Increased levels of FoxO acetylation in the nucleus suggests an additional layer of regulation by other pathways, such as those involving SIRT1, a nicotinamide adenine dinucleotide (NAD)-dependent histone deacetylase (Fig. 3). SIRT1 is localized in the nucleus in cells stimulated with growth factors (Langley et al., 2002; Vaziri et al., 2001). Upon stress stimuli, SIRT1 forms a complex with and deacetylates FoxO proteins in the nucleus (Brunet et al., 2004; Kitamura et al., 2005). Interestingly, expression of SIRT1 augments FoxO3a-induced cell cycle arrest by increasing expression of $p27^{KIP1}$ (Brunet et al., 2004). This is consistent with the fact that acetylation of FoxO proteins by CBP inhibits their activity (Fig. 3) (Daitoku et al., 2004). By contrast, FoxO3a-induced expression of apoptotic genes such as *BIM* is further enhanced by inhibition of SIRT1 by the class III histone deacetylase (HDAC) inhibitor nicotinamide and the class I/II HDAC inhibitor trichostatin A (Brunet et al., 2004). Moreover, expression of SIRT1 inhibits *BIM* promoter activity (Motta et al., 2004). These results are consistent with the findings that SIRT1 inhibits the transcriptional activity of p53 (Langley et al., 2002; Vaziri et al., 2001). SIRT1 thus appears to shift FoxO function from cell death to survival. The underlying mechanisms will require further investigation.

Perspectives and conclusion

Because FoxO proteins regulate expression of genes involved in almost every phase of the cell cycle (Fig. 2), their activity must be tightly regulated. In response to growth factor stimulation, FoxO proteins appear to be inhibited, presumably by Akt-mediated phosphorylation (Fig. 2). Activated Akt also leads to the induction of the Skp2 E3 ligase (Mamillapalli et al., 2001) and directly promotes ubiquitylation and degradation of FoxO1 (Huang et al., 2005). Growth factors also increase CDK2 activity, which inhibits FoxO1 (Huang et al., 2006). At the end of mitosis, Skp2 itself is targeted for degradation by another E3 ligase, the anaphase-promoting complex/cyclosome (APC/C) (Bashir et al., 2004; Wei et al., 2004). This in theory leads to FoxO-mediated expression of cyclin B and Plk, two key factors required for mitosis (Alvarez et al., 2001) (Fig. 2).

The CDK inhibitor $p27^{KIP1}$ blocks progression of cells from late G1 to S phase but has also been implicated in the regulation of apoptosis (Dijkers et al., 2000b; Wang et al., 1997; Wu et al., 1999). CDK2-mediated phosphorylation and inhibition of FoxO1 can be inhibited by $p27^{KIP1}$ (Huang et al., 2006), providing an additional control mechanism. Thus, $p27^{KIP1}$ may overcome CDK2-mediated phosphorylation of FoxO1 in cells and activate the FoxO1-dependent cell death program. We propose that, in cells susceptible to FoxO1-induced apoptosis, growth factors and cytokines activate both Akt- and CDK2-dependent pathways, which results in the hyperphosphorylation and inactivation of FoxO1 (Fig. 3). By contrast, inhibition of both pathways in response to genotoxic and oxidative stresses highly activates FoxO1, which in turn triggers cell cycle arrest and apoptosis.

Similarly in muscle, there is a dynamic balance between hypertrophic and atrophic processes. Hypertrophy is associated with the increased expression of IGF-1 (DeVol et al., 1990). Inhibition of the IGF-1/PI3K/Akt pathway leads to the activation of FoxO proteins and FoxO-dependent expression of the muscle-specific ubiquitin ligases atrogin-1/MAFbx and MuRF1, which promote muscle atrophy (Kamei et al., 2004; Sandri et al., 2006; Sandri et al., 2004; Stitt et al., 2004). Because both FoxO1 and FoxO3a are inhibited by androgens (Baron et al., 2004; Huang et al., 2004; Li et al., 2003), it will be important to determine whether androgen administration affects the activity of FoxO proteins in skeletal muscle and whether they affect the levels of the FoxO-regulated ubiquitin ligases in this tissue.

FoxO transcription factors are thus emerging as master signaling integrators, which translate various environmental stimuli into dynamic gene expression programs that influence many physiological and pathological processes, including cancer and aging (Fig. 3). The importance of FoxO proteins is also manifest by the fact that their functions are regulated at multiple levels, which include but are not limited to phosphorylation, ubiquitylation and acetylation. Interestingly, all of these activities affect nuclear/cytoplasmic trafficking of FoxO proteins. The functional redundancy of the FoxO proteins is well documented, but specific functions of each member of this family are starting to emerge (Hosaka et al., 2004; Paik et al., 2007). In the future, more post-translational mechanisms of FoxO transcription factors are expected to be defined and questions regarding the transcriptional regulation of FoxO genes and tissue-specific expression of different members of this gene family will be addressed. Thus, studies on cellular pathways that regulate or are regulated by the FoxO transcription factors should enhance our understanding of not only the roles of these proteins in the growth, differentiation and survival of normal cells but their contribution to pathological conditions such as cancer.

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