The Many Forks in FOXO's Road

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The FOXO family of transcription factors constitutes an evolutionarily conserved subgroup within the larger family known as winged helix or Forkhead transcriptional regulators. Building upon work in the nematode, researchers have uncovered a role for these proteins in a diverse set of cellular responses that include glucose metabolism, stress response, cell cycle regulation, and apoptosis. At the organismal level, FOXO transcription factors are believed to function in various pathological processes ranging from cancer and diabetes to organismal aging. A number of studies have also shed light on the signaling pathways that regulate FOXO activity in response to external stimuli and have identified multiple FOXO target genes that mediate this varied set of biological responses.

Introduction

The winged helix or Forkhead box (FOX) class of transcription factors constitute a family of structurally related transcriptional activators that have been identified in species ranging from yeast to human. The first member of this transcription factor class was identified in 1989 as a nuclear homeotic gene involved in *Drosophila melanogaster* embryonic development (*1*). Subsequent work by Weigel and Jackle identified the forkhead box DNA binding domain (*2*), which is now the defining trait of the FOX class of transcription factors. The FOXO family of forkhead transcription factors represents a subfamily within the larger grouping of FOX transcription factors, and in mammals consists of three members: FOXO1 (also called FKHR), FOXO3a (also called FKHRL1), and FOXO4 (also called AFX) (*3-6*). Further clarification of the nomenclature of individual FOX genes can be found at http://www.biology.pomona.edu/fox.html. The FOXO factors all function as transcriptional activators and bind as monomers to the consensus DNA sequence TTGTTTAC (*7*). Interestingly, this core consensus sequence is also recognized by other FOX transcriptional regulators (*8*); however, the relation between these different FOX factors remains largely uncharacterized. FOXO family members participate in various cellular functions, including programmed cell death, cell cycle progression, and stress detoxification. Here, we present an overview of our current understanding of how signaling pathways regulate FOXO function. We also review recent studies that have begun to identify the transcriptional targets of FOXO activity. In particular, we attempt to reconcile the diverse functions of FOXO family members in cell cycle control, stress responses, and apoptosis at the cellular level with possible effects on aging and tumor formation at the organismal level.

Molecular Mechanisms Controlling FOXO Activity

The Trail That Leads to FOXO. Although FOXO factors appear to serve as a point of convergence for multiple signaling pathways, the molecular mechanisms associated with FOXO regulation have largely been characterized in response to growth factor signaling via the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway. The PI3K-Akt pathway is an evolutionarily conserved signaling cassette that functions in mammals to transduce survival signals in response to growth factor stimulation (*9*). Binding of growth factors to their cognate tyrosine kinase receptors on the cell surface leads to the intracellular recruitment and activation of PI3K, a lipid kinase that phosphorylates the 3′ position of phosphoinositides. Active PI3K, in turn, generates a local increase in the 3′-phosphorylated phosphoinositides phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and phosphatidylinositol-3,4-bisphosphate (PIP_2) at the plasma membrane (*10*). PI3K activity is opposed in the cell by the product of the tumor suppressor gene *PTEN*, which acts as a lipid 3′-phosphatase (*11*). The accumulation of phosphorylated lipid second messengers recruits the serine-threonine kinase Akt (also called protein kinase B or PKB) to the plasma membrane, where Akt becomes fully activated by phosphorylation at two critical residues, Thr^{308} and Ser^{473} (*9*).

Once activated, Akt serves as a general mediator of cell survival, suppressing the death of a wide range of cell types challenged by any one of a number of apoptotic stimuli (*9*). The study of Akt function has been greatly aided by the description of a consensus sequence for phosphorylation by Akt (RXRXXS/T) (*12*), which has permitted the identification of a number of Akt substrates. Indeed, Akt mediates survival in part by phosphorylating and inactivating a number of cytoplasmic substrates, including the pro-apoptotic Bcl-2 family member Bad (*13*), the cell death protease caspase 9 in humans (*14*), and the metabolic regulator glycogen synthase kinase-3 (GSK-3) (*15*).

Several key observations suggested a potential link between the Akt pathway and transcriptional regulation. First, apoptosis induced by growth factor withdrawal in neurons was found to be blocked by transcriptional and translational inhibitors (*16*, *17*), suggesting that de novo gene expression is necessary for cell death to occur. Akt also translocates to the nucleus within 30 min of activation by growth factors, raising the possibility that key Akt substrates might reside in the nucleus (*18*, *19*). Additional evidence that directly implicated Forkhead family transcription factors came from genetic analyses performed in the nematode *Caenorhabditis elegans*. DAF-16, a forkhead transcription factor in the worm, functions to extend life-span as a downstream target of the growth factor-PI3K-Akt signaling pathway (*20*, *21*). Epistatic analysis indicated that transcriptional activity of DAF-16 is repressed by the activity of the PI3K-Akt pathway, and subsequent analysis of the DAF-16 sequence revealed four sites that correspond to the consensus site for Akt phosphorylation. This pathway is conserved in mammalian cells, where Akt directly phosphorylates all three members of the FOXO family, leading to the nuclear export of FOXO and inhibition of FOXO-dependent transcription (*22-27*). Thus, growth factor signaling to FOXO family members through PI3K and Akt represents an evolutionarily conserved mecha-

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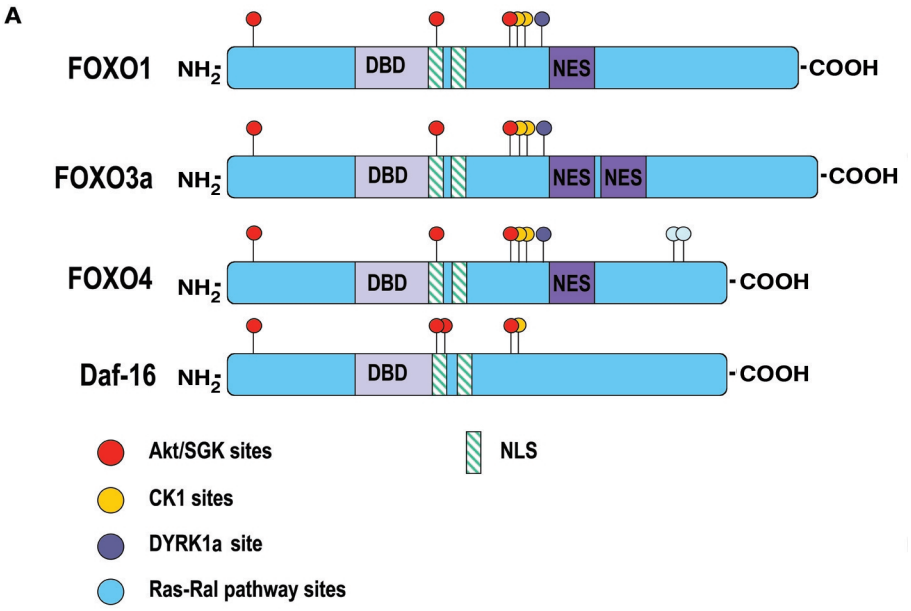
nism for FOXO phosphorylation and inhibition.

The sites of FOXO phosphorylation by Akt have been mapped to three key regulatory residues that are conserved within the FOXO family (Fig. 1). In FOXO3a, these three sites are phosphorylated by both Akt and the related serum and glucocorticoid-induced kinase (SGK), which is also activated in response to extracellular stimuli and has been suggested to play a role in cell cycle progression and sodium homeostasis control (*28-32*). Although Thr^{32} is robustly phosphorylated by both kinases, Akt preferentially phosphorylates Ser^{253} whereas SGK preferentially phosphorylates Ser^{315}. Mutation of these three regulatory sites renders FOXO3a refractory to Akt-mediated inhibition, even in the presence of growth factors that activate the PI3K-Akt pathway. Additionally, phosphorylation of these sites appears to control FOXO subcellular localization because mutant forms of FOXO that cannot be phosphorylated at the three regulatory sites are exclusively localized to the nucleus under all conditions tested (*22*, *27*) (Fig. 2). Thus, phosphorylation of FOXO family members at these three sites in response to growth factor stimulation negatively regulates FOXO activity by relocalizing FOXO from the nucleus (where FOXO acts to activate or, in some cases, may repress target genes) to the cytoplasm (where it is sequestered away from target promoters).



B

FOXO Phosphorylation sites

	Akt/SGK sites	CK1 sites	DYRK1a site	Ras-Ral pathway sites
FOXO1:	T24, S256, S319	S322, S325	S329	
FOXO3a:	T32, S253, S315	S318, S321	S325	
FOXO4:	T28, S193, S258	S261, S264	S268	T447, T451
Daf-16:	T54, S240, T242, S314	S317		

Fig. 1. Structure (**A**) and phosphorylation sites (**B**) of the FOXO transcription factors. DBD, DNA binding domain; NLS, nuclear localization sequence; NES, nuclear export sequence.

Other Signal Transduction Pathways That Regulate FOXO. FOXO transcription factors are also targets of posttranslational modifications by several other kinases, although the function of these modifications remains largely uncharacterized. FOXO1 is phosphorylated at Ser^{322} and Ser^{325} in vivo in response to growth factor stimulation, and phosphorylation of these sites is dependent upon the prior Akt-mediated phosphorylation of Ser^{319} (*33*). Casein kinase 1 (CK1) phosphorylates Ser^{322} and Ser^{325} in vitro and may mediate phosphorylation of these sites in vivo. Phosphorylation of Ser^{319} by Akt generates a consensus site for CK1 phosphorylation of Ser^{322}, which in turn primes Ser^{325} for phosphorylation by CK1. These phosphorylation events appear to accelerate FOXO translocation to the cytoplasm by increasing the interaction with the nuclear export machinery (see below). CK1 has been implicated in the phosphorylation and nuclear export of the NFAT family of transcriptional activators (*34*); however, the importance of CK1-mediated posttranslational modifications for FOXO function remains to be more fully characterized.

Phosphopeptide analysis of baculovirus-produced FOXO1 has also identified Ser^{329} as a site of phosphorylation (*35*). In extracts derived from rabbit skeletal muscle, the dual-specificity tyrosine phosphorylated regulated kinase 1a (DYRK1a) was identified as a Ser^{329} kinase. DYRK1a is a newly identified member of the mitogen-activated protein kinase (MAPK) family that phosphorylates FOXO1 at Ser^{329} in vitro and associates with FOXO1 in the nucleus. Although mutation of Ser^{329} increases FOXO1-mediated transcription and nuclear localization, the relevance of this modification for FOXO regulation is unclear because phosphorylation at this site is not known to be al-

tered by extracellular stimuli.

Finally, activation of the small guanosine triphosphatase (GTPase) Ras has also been implicated in FOXO regulation. Although activated Ras induces PI3K signaling, activated Ras also modulates FOXO activity through a PI3K-independent mechanism that involves the Ral GTPase (*23*). Upon Ras activation by mitogenic signaling, Ras associates with and activates several guanine nucleotide exchange factors for Ral (RalGEFs). Introduction of oncogenic Ras into cells suppresses FOXO transcriptional activity and induces FOXO phosphorylation, and these effects are at least in part dependent on Ral activation (*23*). Activation of the Ras-Ral pathway leads to phosphorylation of FOXO4 at Thr^{447} and Thr^{451}, two amino acid residues that are located within the COOH-terminal transactivation domain (*36*). Surprisingly, mutation of these residues renders FOXO4 transcriptionally inactive but still competent for Akt-mediated translocation to the cytoplasm. In addition, inhibition of endogenous Ral suppresses FOXO4-dependent transcription. Thus, under normal physiological conditions, signaling through the Ras-Ral pathway induces FOXO4 transcriptional activity upon growth factor stimulation. However, hyperactivation of the Ras-Ral pathway by introduction of oncogenic Ras results, paradoxically, in the suppression of FOXO4 activity. Currently, both the mechanism by which activated Ral leads to FOXO phosphorylation and the factors that determine the differential effects of Ral signaling on FOXO function remain to be characterized.

Thus, whereas attention has been largely focused on the regulation of FOXO activity by the PI3K-Akt pathway, evidence exists for a role for other signaling pathways in FOXO regulation. Currently, the mechanisms that regulate

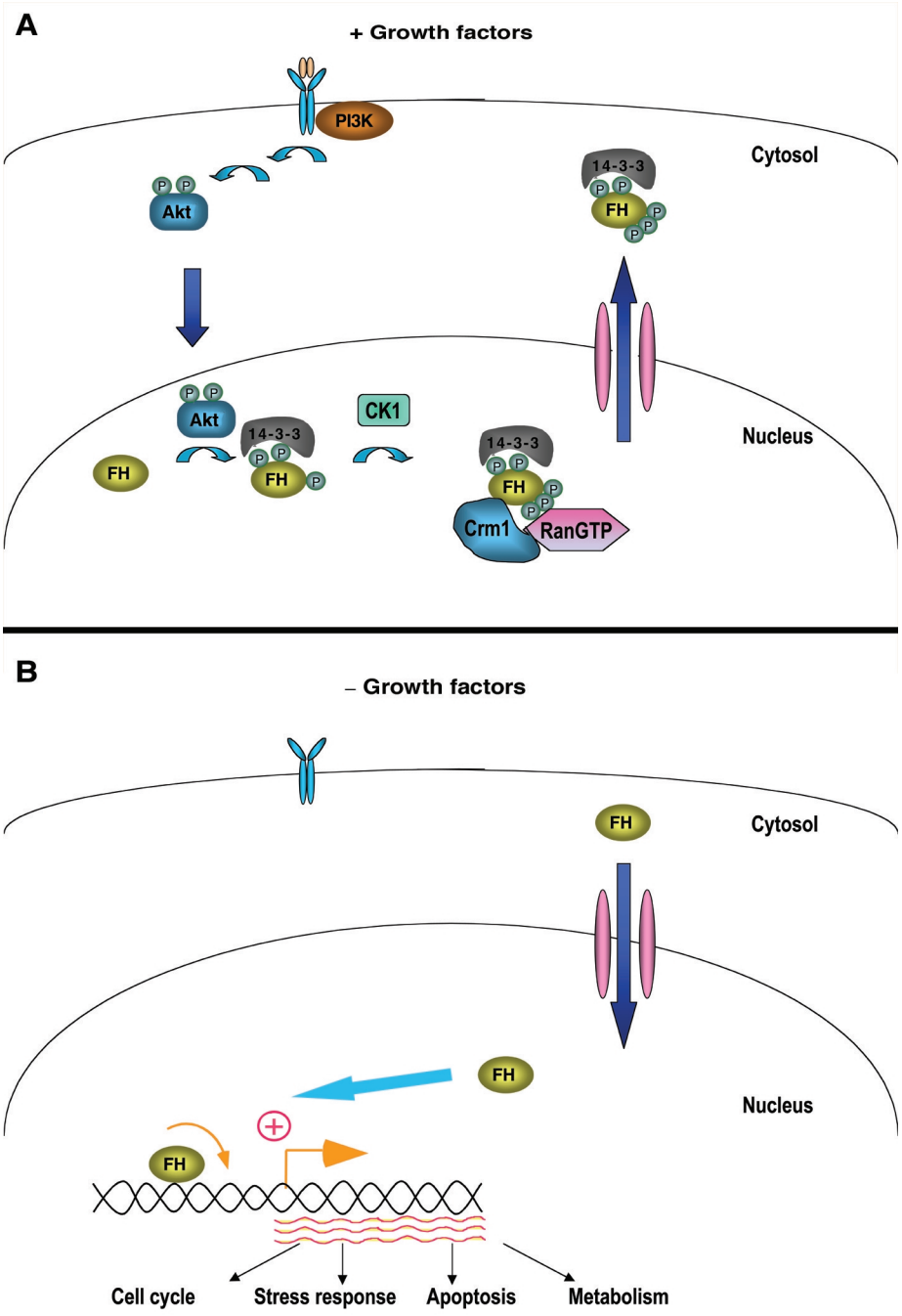


Fig. 2. Regulation of the FOXO transcription factors. (**A**). The addition of growth factor leads to the activation of PI3K, which in turn activates Akt. Akt then translocates into the nucleus where it phosphorylates FOXO transcription factors. This phosphorylation generates sites for 14-3-3 binding and primes FOXO for phosphorylation by CK1. Phosphorylation at these critical residues allows FOXO to interact with the RanGTPase, a critical component of the exportin/Crm-1 export machinery that leads to the export of FOXO from the nucleus. (**B**). In the absence of growth factor, FOXO relocalizes to the nuclear compartment, where it can activate downstream target genes to elicit various cellular responses. FH, FOXO transcription factor; P, phosphates.

dephosphorylation of FOXO family members remain uncharacterized. Given the life-and-death decisions that are regulated by FOXO, it is likely that FOXO factors have evolved to integrate multiple signaling pathways to determine cell fate. Further work will be required to unravel the apparent complexity of signaling cascades that impinge on this family of transcriptional regulators.

Subcellular Localization of FOXO Transcription Factors. With the discovery that Akt activity blocks FOXO-dependent transcription in mammalian cells, a mechanism to account for this inhibition was immediately suggested by the observation that Akt promoted export of FOXO factors from the nucleus (*22*). FOXO family members are phosphorylated by Akt within the nucleus before they are exported (*37*, *38*), and we now have a detailed picture of the mechanisms by which phosphorylation controls FOXO subcellular localization.

The first clues to the mechanism of phosphorylation-dependent FOXO relocalization came from the realization that phosphorylation of the FOXO transcription factors by Akt generates two consensus binding sites for 14-3-3 proteins (RSXpSXP) (*39*). 14-3-3 proteins are members of a family of cellular chaperones that interact with their protein ligands in a phosphorylation-dependent manner (*40*). 14-3-3 family members interact with FOXO, and this interaction is dependent on phosphorylation of the two consensus sites by Akt (*22*). The importance of the 14-3-3 interaction has been demonstrated in in vitro binding assays done with DAF-16, the FOXO ortholog in *Caenorhabditis elegans* (*41*). DAF-16 is efficiently phosphorylated by Akt in vitro, which promotes DAF-16's association with 14-3-3. Whereas Akt phosphorylation alone has no effect on DAF-16 binding to DNA, the phosphorylation-dependent interaction with 14-3-3 is sufficient to displace DAF-16 from DNA. Furthermore, mutation of the two canonical 14-3-3 binding sites within FOXO3a blocks FOXO export in response to growth factor treatment (*38*). Thus, binding of 14-3-3 proteins to FOXO that has been phosphorylated by Akt is required for the release of FOXO factors from DNA and their subsequent translocation to the cytoplasm.

Although 14-3-3 binding appears to be sufficient to displace FOXO factors from the DNA, the localization of FOXO proteins within the nucleus is regulated by the presence of nuclear localization signal (NLS) and nuclear export signal (NES) motifs in these transcription factors. The sequences required for efficient nuclear localization of FOXO proteins have been mapped to a series of basic amino acid residues that constitute a nonclassical NLS located COOH-terminal to the DNA binding domain (*26*, *37*, *38*). This region was both necessary and sufficient to drive nuclear localization when fused to green fluorescent protein (GFP), and mutation of basic residues within this motif impairs nuclear localization of FOXOs (*37*, *38*). A conserved site of phosphorylation by Akt is contained within this motif, and a FOXO4 mutant in which this site was converted to a glutamic acid to mimic phosphorylation exhibited reduced nuclear localization (*37*), suggesting that Akt phosphorylation may promote export in part by disrupting NLS function. Additionally, subsequent binding by 14-3-3 may also act to mask NLS function by blocking the interaction of FOXO with the nuclear import machinery.

FOXO transcription factors also contain leucine-rich regions (Fig. 1A) that can function as NES (*26*, *37*, *38*). One NES was identified in subcellular localization experiments with FOXO1 deletion mutants fused to GFP. These experiments revealed that the loss of the putative NES resulted in impaired nuclear export of FOXO1 (*26*). Mutation of NES motifs in FOXO3a blocks FOXO export out of the nuclear compartment, even when the sites of Akt phosphorylation and 14-3-3 interaction are intact (*38*). However, an intact NES is not sufficient for export, because a FOXO3a mutant that can no longer bind 14-3-3 remains localized to the nuclear compartment, even though this mutant retains intact NES sequences. These results suggest that effective nuclear export requires both 14-3-3 binding and an intact NES on FOXO.

The nuclear export of proteins can occur through several different pathways, but pharmacological studies have demonstrated that FOXO export from the nucleus is mediated by the exportin or Crm-1 export receptor (*26*, *37*, *38*). Crm-1 associates with components of the nuclear pore and controls the nuclear exit of various proteins, and Crm-1 function is potently inhibited by the small molecule leptomycin B (*42*). Leptomycin B treatment effectively blocks FOXO export from the nucleus. Furthermore, Crm-1 associates with FOXOs. This association requires that the FOXO NES sequences be intact, but surprisingly, the association of FOXO with Crm-1 occurs independently of FOXO phosphorylation or 14-3-3 binding (*37*, *38*). These findings suggest that although the three Akt regulatory sites are important for the proper subcellular localization of FOXOs, their phosphorylation does not affect the interaction of FOXOs with Crm-1. However, the CK1-mediated phosphorylation of FOXO1 at Ser^{322} and Ser^{325} may modulate the association of FOXOs with the nuclear export machinery because mutations at these sites abrogate the interaction of FOXO1 with the Ran GTPase, a component of the Crm-1 nuclear export complex (*33*).

The ability of Crm-1 to associate with FOXO in the absence of growth factor signaling raised the possibility that FOXO factors might be dynamically shuttled into and out of the nucleus even in the absence of extracellular stimuli. This hypothesis has been confirmed by heterokaryon fusion experiments (*37*). In this assay, nucleocytoplasmic shuttling can be observed when donor and acceptor nuclei are readily distinguishable following cell fusion. Accumulation of the tagged protein in the acceptor nucleus can only occur upon protein export from the donor nucleus and reimport from the common cytoplasm into the acceptor nucleus. A constitutively nuclear FOXO4 mutant in which all three Akt regulatory sites were converted to alanines accumulates over time in the acceptor nucleus. Thus, FOXO subcellular localization appears to be a dynamic process that is determined by the relative rate of FOXO nuclear import and export.

Taken together, the current data suggest a model for FOXO nuclear export in which FOXO phosphorylation in response to growth factor stimulation leads to 14-3-3 binding and the displacement of FOXO from the DNA. FOXO export then proceeds in a manner that requires both 14-3-3 binding and intact FOXO NES sequences. Although 14-3-3 proteins themselves have a NES-like sequence that has been reported to bind Crm-1, the putative 14-3-3 NES appears to have no role in the nuclear export of FOXO factors (*38*). Phosphorylation of Ser^{322} and Ser^{325}, putative CK1 phosphorylation sites, may enhance the rate of FOXO export by increasing the interaction of FOXO with the Ran-Crm-1 nuclear export complex. In addition to its effects on FOXO export from the nucleus, phosphorylation of FOXO also interferes with the function of the FOXO NLS, slowing the rate of nuclear import. In the presence of growth

factors, the combination of these events shifts the balance of nuclear import and export, leading to the rapid exclusion of FOXO transcription factors from the nucleus.

Cellular and Organismal Effects of FOXO Proteins

In contrast to the detailed study of FOXO regulation at the molecular level, much still remains to be determined regarding the role of FOXO family members at the cellular and organismal levels. The transcriptional targets that mediate the effects of FOXO activity on cell survival, cell cycle control, and DNA damage repair are just beginning to be identified.

Regulation of Apoptosis by FOXO. The first activity to be ascribed to FOXO factors in vertebrates was the induction of programmed cell death. The growth factor-PI3K-Akt signaling pathway acts in a variety of cell types to promote survival (*9*). The observation that Akt phosphorylation inhibited FOXO-dependent transcription by inducing FOXO translocation to the cytoplasm immediately suggested that FOXO transcriptional activity might play a role in apoptosis (*22*). Indeed, overexpression of a constitutively active form of FOXO in which all three Akt regulatory sites are converted to alanines promotes apoptosis in a number of cell types in culture (*22*, *24*, *43-46*). Importantly, the pro-apoptotic activity of this mutant is dependent on DNA binding, suggesting that these effects are due to FOXO transcriptional activity (*24*). Subsequent studies have shown that FOXO-induced cell death appears to be mediated by transcriptional regulation of a number of target genes. Initially, the death cytokine Fas ligand (FasL) was identified as one mediator of FOXO-induced cell death (*22*). DNA mobility-shift experiments revealed that putative FOXO binding elements in the FasL promoter bound FOXO3a, a result subsequently confirmed by chromatin immunoprecipitation experiments in HeLa cells (*47*). FOXO3a was also shown to activate a FasL promoter-driven reporter in fibroblasts, and this activation was inhibited by both constitutively active Akt and growth factor treatment. Once induced by FOXO, secreted FasL binds and activates the cell surface death receptor Fas in a paracrine or autocrine manner, which in turn activates the Fas-dependent cell death pathway (*48*). Support for a role for FasL in FOXO-mediated cell death comes from studies using cerebellar granule neurons. In this cell type, the ability of FOXO3a to induce cell death was diminished by addition of soluble Fas to block FasL binding to the cells (*22*). Overexpression of a constitutively active FOXO3a mutant in Jurkat cells also led to cell death, and this effect was substantially attenuated in cells deficient in components of the Fas signaling pathway. Taken together, these findings indicate that FOXO-induced apoptosis is mediated at least in part by the transcriptional up-regulation of FasL.

In some cases, however, FOXO-induced cell death appears to be independent of Fas signaling. Up-regulation of the pro-apoptotic Bcl-2 family member Bim plays a role in FOXO-dependent cell death upon cytokine withdrawal in a number of hematopoietic cell types (*44-46*). FOXO3a activation is sufficient for induction of Bim expression in Ba/F3 cells, and Bim appears to be a direct target of FOXO, as FOXO-induced up-regulation of Bim mRNA is independent of new protein synthesis (*44*). Bim expression appears to represent a major mediator of FOXO-induced cell death because Bim-deficient hematopoietic stem cells are largely resistant to cell death induced by growth factor withdrawal or inhibition of the PI3K signaling pathway (*46*). Bim functions at the mitochondria, where it acts to disrupt mitochondrial integrity and promote release of cytochrome c, initiating a cascade of proteolytic events that are hallmarks of programmed cell death.

In addition to inducing the expression of pro-apoptotic genes, FOXO transcription factors may indirectly down-regulate the expression of genes involved in cell survival. FOXO4 suppresses the expression of Bcl-XL, a pro-survival member of the Bcl-2 family (*49*). The suppression of Bcl-X_L by FOXO4 appears to be mediated through the transcriptional repressor Bcl-6. Analysis of transcripts up-regulated upon FOXO4 activation identified Bcl-6 as a FOXO-induced gene. FOXO4 can bind and transactivate the Bcl-6 promoter, leading to an increase in the expression of this transcriptional repressor. Bcl-6 in turn appears to bind to the promoter of the pro-survival gene Bcl-X_L, repressing its expression. Consistent with this idea, FOXO activation was found to repress transcription of Bcl-X_L. Thus, by indirectly suppressing the expression of pro-survival genes and inducing cell death genes, FOXO activation contributes to the initiation of programmed cell death.

FOXO Factors and Cell Cycle Regulation. Although in some cell types FOXOs play a role in intiating apoptosis, FOXO activation can also regulate cell cycle progression under other circumstances. FOXO activity is regulated by PI3K-Akt and Ras activity, and both of these signaling pathways are important for cell cycle progression through G_1 phase. Activated PI3K signaling is sufficient to induce new DNA synthesis in serum-starved fibroblasts (*50*), and experiments with blocking antibodies show that DNA synthesis induced by treatment with a number of growth factors requires endogenous PI3K (*51*). Several studies have also demonstrated the ability of PTEN, a negative regulator of the PI3K-Akt pathway, to induce cell cycle arrest (*52*, *53*). The importance of Ras signaling in cell cycle progression has been extensively characterized (*54*). Thus, signaling pathways that regulate FOXO activity also have a clear role in regulating cell proliferation, suggesting the possible involvement of FOXO family members.

Overexpression of FOXO4 in several cell lines, including A14 cells, the PTEN-deficient glioblastoma cell line U87MG, and the Ras-transformed cell line RR7, halts cell cycle progression in the G_1 phase, and this effect is dependent on FOXO transcriptional activity (*55*). Furthermore, expression of a dominant negative form of FOXO4 resulted in increased cell proliferation, suggesting that endogenous FOXO activity acts to restrict the proliferative capacity of cells. Subsequent studies in a variety of cell types have confirmed that overexpression of a constitutively active form of FOXO induces arrest in the G_1 phase of the cell cycle (*36*, *45*, *56*, *57*). FOXO activity mediates G_1 cell cycle arrest at least in part by inducing expression of the cell cycle inhibitor p27kip1 (*55*, *56*). p27kip1 acts to block the activity of the cyclin E-cyclin-dependent kinase 2 (CDK2) complex, thereby preventing entry into S phase (*58*). p27kip1 likely represents a direct target of FOXO activation because multiple FOXO binding sites are present in the p27 promoter, and FOXO activity can induce expression of a p27 promoter-driven reporter gene. p27kip1 appears to be a major mediator of FOXO-induced G_1 arrest because p27kip1-deficient fibroblasts are largely resistant to FOXO4-induced G_1 arrest (*55*). The precise mechanism by which FOXOs regulate the abundance of p27kip1 still remains an issue of debate. In one study, a FOXO-induced increase in p27kip1 protein level was attributed entirely to increased transcription (*55*). However, another study reported

that FOXO-induced accumulation of p27kip1 also involves stabilization of the p27kip1 protein (*56*).

The suppression of the D-type cyclins has also been implicated in FOXO-mediated growth arrest (*59*, *60*). As cells progress through G_1, D-type cyclins are synthesized and form a complex with their corresponding catalytic partners CDK4 and CDK6. These cyclin D-CDK complexes function to inactivate the retinoblastoma tumor suppressor protein, permitting progression into S phase (*61*). Transcriptional profiling experiments revealed that expression of activated FOXO1 suppressed expression of cyclin D1 and D2 (*59*). FOXO1 was also found to associate with the cyclin D1 promoter by chromatin immunoprecipitation. Furthermore, a mutant form of FOXO1 that was unable to recognize the canonical FOXO binding site and could not induce expression of p27kip1 still suppressed the expression of cyclin D1 through binding to its promoter and retained the ability to induce G_1 arrest in a tumor cell line. Thus, as they do in the control of cell survival, FOXO factors likely block G_1 progression by both stimulating and repressing gene transcription. FOXOs appear to increase expression of cell cycle inhibitors and repress expression of factors required for cell proliferation. These effects appear to be mediated in part by FOXO acting through a noncanonical DNA binding site.

FOXO activity has also been observed to induce cells to exit the cell cycle and enter a nondividing, quiescent state (G_0) (*62*). This switch is characterized by reduced protein synthesis and increased abundance of the retinoblastoma family protein p130. p130 itself appears to represent a direct target of FOXO, and although the FOXO-induced increase in p130 does not affect cyclin E-dependent kinase activity and is therefore not believed to have a role in the FOXO-induced G_1 arrest, p130 does appear to be required for the FOXO-induced decrease in protein synthesis that has been correlated with cellular quiescence. FOXO thus acts to block cell cycle progression prior to DNA synthesis and to induce cellular quiescence via a variety of target genes.

FOXO activity is also important during later phases of the cell cycle. The introduction of a constitutively active mutant of FOXO in cells that were synchronized in S phase results in a delay in the G_2-M transition, and similar effects were observed upon treatment of cells with an inhibitor of PI3K-Akt signaling (*63*, *64*). Transcriptional profiling experiments have identified the growth arrest and DNA-damage response gene *gadd45a* as a FOXO target that functions in the G_2 arrest. Gadd45a is up-regulated under conditions of stress or growth factor deprivation, and gadd45 contributes to G_2 arrest by preventing the interaction of the cyclin-dependent kinase cdc2 with cyclin B (*65-67*). Ectopic expression of FOXO3a increases expression of gadd45a mRNA and protein in a manner that is dependent on FOXO3a DNA binding. Furthermore, the up-regulation of gadd45 appears to be important for the FOXO-induced G_2 delay because this effect was partially compromised in *gadd45a*-deficient fibroblasts.

A role for the PI3K signaling pathway has also been established in the execution of the mitotic program in mammalian cells. Although expression of a constitutively active mutant of the PI3K catalytic subunit accelerated cell cycle reentry, the transfected cells were delayed in their transition from M phase into the following G_1 phase; the cells accumulated in mitotic telophase and displayed incomplete cytokinesis (*47*). Inhibition of endogenous FOXO activity through the use of a dominant interfering form of FOXO also resulted in defective M phase progression. Conversely, activation of FOXO in the G_2 phase of the cell cycle resulted in an accelerated completion of M phase. Chromatin immunoprecipitation and reporter gene experiments demonstrated that FOXO3a could bind and transactivate the promoters of the human cyclin B and polo-like kinase (PLK) genes, two genes whose products have critical roles in M phase progression. Cyclin B levels rise in G_2 phase and allow for entry into and progression through M phase, whereas exit from mitosis is promoted by cyclin B ubiquitination and degradation. PLK controls this ubiquitination process by regulating the anaphase-promoting complex, a ubiquitin ligase that causes cyclin B degradation and M phase completion (*68-70*). These results suggest that FOXO family members play a key role in controlling mitosis by regulating the expression of a set of genes that act as crucial mediators of M phase progression.

Thus, in addition to their roles as mediators of apoptosis, FOXO factors regulate cell proliferation at multiple phases of the cell cycle. Surprisingly, FOXO activity appears to exert opposing effects at different points in the cell cycle; under normal physiological conditions, FOXO family members regulate the expression of genes such as cyclin B and PLK that promote progression through M phase, whereas ectopic FOXO expression can act to arrest cells in G_1 and G_2. The G_1-S and G_2-M transitions represent important sites of checkpoint regulation by the cell to ensure replicative fidelity, raising the possibility that at these critical checkpoints, FOXO factors may sense and act to coordinate cellular responses to DNA damage or other stress stimuli.

Roles of FOXO in Stress Responses and DNA Repair. The first evidence that FOXOs might regulate the cellular response to environmental stress derives from studies in the nematode *C. elegans* in which the FOXO ortholog DAF-16 was found to be activated by stress stimuli. Agents such as heat and juglone, a reactive oxygen species generator, trigger the translocation of DAF-16 from the cytoplasm to the nucleus (*71*, *72*). In addition, inactivating mutations in the *C. elegans* PI3K signaling pathway confer increased resistance to a variety of environmental insults, including ultraviolet irradiation and oxidative stress (*73-75*). This increased resistance is dependent on DAF-16 and may be explained by the transcriptional up-regulation of superoxide dismutase (SOD) and catalase, two enzymes involved in the cellular detoxification of reactive oxygen species (ROS).

A role for FOXO family members in conferring cellular resistance to stress has been conserved in mammals. Ectopic expression of a transcriptionally active FOXO3a increases both ROS scavenging and cellular survival in response to oxidative stress (*76*, *77*). Again, transcriptional profiling experiments in mammalian cells have greatly aided in the identification of numerous FOXO-regulated stress-responsive genes. These studies have isolated FOXO-regulated genes involved in a variety of stress responses ranging from antioxidant defense to DNA repair (*59*, *63*). In particular, FOXO3a, like its nematode counterpart, appears to enhance expression of the antioxidant enzymes mitochondrial manganese SOD2 and catalase (*59*, *76*, *77*).

The ability of FOXO family members to induce cell cycle arrest at the G_1-S and G_2-M checkpoints also suggests a possible involvement of FOXO factors in DNA-damage repair. Indeed, expression of activated FOXO3a induces repair of a transfected ultraviolet (UV)-damaged reporter plasmid (*63*). This effect appears to be dependent on FOXO3a transcriptional activity, because it required the presence of the FOXO DNA binding

domain. Although a complete mechanism to account for FOXO-induced DNA repair is still lacking, up-regulation of GADD45a appears to play a role. Mice lacking the Gadd45a gene show defects in DNA nucleotide excision repair (*78*, *79*), and FOXO3a-induced DNA repair was found to be partially impaired in GADD45a-null fibroblasts (*63*). These findings suggest that in addition to ROS detoxification, FOXO3a also induces a transcriptional program resulting in DNA-damage repair in response to certain types of stress.

More work needs to be done to fully elucidate how FOXO factors mediate such diverse cellular processes as apoptosis, cell cycle progression, and resistance to stress stimuli. Additional transcriptional profiling experiments should continue to uncover FOXO target genes involved in each of these processes. It is still unclear whether different FOXO family members control different biological processes and regulate distinct downstream targets. Expression analyses have revealed that FOXO family members are present in all tissues examined, as might be expected given the role of FOXOs in apoptosis and the stress response (*5*, *7*, *80*). Yet the enrichment of the different FOXO factors in different tissues, such as FOXO1 in adipose tissue and liver versus FOXO3a in the brain, suggests that FOXO1 may play a more dominant role in insulin signaling and metabolism (see below), whereas FOXO3a may be more involved in the central regulation of processes that affect the organism as a whole such as aging. Because current studies have largely used tissue culture systems to explore the cellular function of FOXO factors, FOXO function will need to be analyzed at the organismal level through the use of knockout and knock-in mice.

A Role for FOXO in Carcinogenesis? Given the involvement of FOXO transcription factors in cellular responses such as cell cycle control, DNA repair, and apoptosis, it is not surprising that these factors may play a role in carcinogenesis. The signaling pathways that modulate FOXO activity have been clearly implicated in tumorigenesis. Ras, PI3K, and Akt all function as oncogenes (*81-84*). PTEN, the lipid phosphatase that opposes PI3K signaling in the cell, has also been implicated as a tumor suppressor in mice (*85*). In addition, p27kip1, the product of a FOXO target gene, also appears to act to suppress tumor formation because p27kip1-deficient mice are predisposed to tumorigenesis (*86*). Thus, FOXO family members function within a pathway whose dysregulation can lead to tumorigenesis.

In fact, the individual members of the FOXO family were initially isolated as the sites of chromosomal breakpoints present in human tumors such as rhabdomyosarcomas and leukemias (*3*, *4*, *87-89*). Chromosomal rearrangement that occurred during the oncogenic process results in the fusion of the FOXO transactivation domain to the DNA binding domains of other unrelated transcription factors. In rhabdomyosarcomas, the transactivation domain of FOXO1 was found fused to the DNA binding domains of the developmentally regulated transcription factors PAX3 or PAX7, which are normally involved in early neuromuscular differentiation (*3*, *90*). Similarly, in acute leukemias the transactivation domains of FOXO3a and FOXO4 are fused to the DNA binding domain of the mixed-lineage leukemia protein MLL, which normally functions as a positive regulator of Hox genes during embryonic development (*4*, *6*).

Although the presence of translocation events involving FOXO genes in human tumors does not establish a causative role for these mutations in tumorigenesis, these events provide further hints of a possible role for FOXO factors in cancer development. Assuming an oncogenic role for these translocations, these fusion products may result in a gain-of-function, dysregulating cell physiology due to excessive transcription of PAX and MLL target genes. Alternatively, loss of an allele of the FOXO gene due to chromosomal rearrangement may predispose cells to dysregulated cell cycle control and impaired apoptosis. To examine the first possibility, transcriptional profiling experiments were done to identify changes in gene expression induced by the PAX3-FOXO1 chimera (*91*). This fusion protein aberrantly induced developmental programs of transcription, leading to dysregulated cell growth and differentiation. However, despite the ability of PAX3-FOXO1 to transform cultured fibroblasts (*92*, *93*), PAX3-FOXO1 knock-in mice showed no sign of malignancy (*94*). Similar results were also observed upon transgenic expression of the chimeric PAX3-FOXO1 protein (*95*), suggesting that PAX3-FOXO1 activity alone is not sufficient to trigger tumor formation.

In contrast to the PAX3-FOXO1 animal models, human patients with rhabdomyosarcoma often possess a chromosomal translocation event that results in the expression of chimeric FOXO proteins that dominantly interfere with the function of the endogenous forkhead factors (*96*). Thus, the chromosomal translocation event would lead to the loss of one allele of the FOXO gene, whereas the chimeric protein would dominantly interfere with the function of the remaining intact allele. In combination, these events may result in a nearly complete loss of FOXO function, which may lead to dysregulation of cell proliferative and apoptotic pathways that result in neoplasia.

Although the true role of the FOXO family in tumorigenesis awaits the development and analysis of appropriate animal models, these transcriptional regulators clearly function within a signaling pathway that appears to play a critical role in tumor formation. The involvement of FOXO factors in cellular processes such as apoptosis, cell proliferation, and DNA repair is reminiscent of the tumor suppressor protein p53 (*97*). Furthermore, several FOXO target genes such as GADD45, Wip1, and PA26 are also targets of transcriptional activation by p53 (*98*, *99*, *100*), suggesting that under some circumstances these factors may cooperate in target gene activation. The functional relation between FOXO family members and p53 remains to be explored.

FOXO and Aging. Recent evidence in several organisms suggests that organismal aging is under genetic control and involves the growth factor-PI3K-Akt-FOXO pathway. Genetic studies in several model organisms have demonstrated a role for the PI3K pathway in aging (*101-103*). In *C. elegans*, mutations of the insulin receptor or PI3K can lead to a two- to threefold extension in life-span (*104*, *105*). Several large-scale screens for mutations that suppress the longevity phenotype of PI3K-Akt pathway mutants identified null mutations in the FOXO ortholog DAF-16 (*20*, *21*). The ability of DAF-16 mutants to suppress the longevity phenotype strongly suggests that the FOXO transcription factor DAF-16 is a primary target of the PI3K-Akt signaling pathway and promotes life-span extension in the nematode. In mammals, studies of aging have been limited because of the relatively long lifetimes of the experimental organisms. Nevertheless, experiments that recapitulate the worm's longevity phenotype have recently been performed in mice. Mice that are haploinsufficient for the insulin-like growth factor-1 (IGF-1) receptor (*Igf1r*) or have the insulin receptor (*Insr*) knocked out in fat-specific tissues exhibit an extended life-span of 26 and 18%, respectively (*102*, *103*). In the long-lived

Igf1r$^{+/-}$ mice, no defect was observed in food intake, growth, energy metabolism, physical activity, or fertility. However, these mice, like their long-lived counterparts in the worm, were discovered to have a greater resistance to oxidative stress (*102*). The long-lived, fat-specific *Insr*$^{-/-}$ (FIRKO) mice exhibited no abnormality in food intake, but these mice did display a reduced fat mass and were protected against age-related deterioration in glucose tolerance (*103*). Because the insulin-PI3K-Akt-FOXO signaling pathway is very well conserved across species, it is tempting to speculate that the double knockout of the *Insr/Igf1r*$^{-/-}$ and *FOXO*$^{-/-}$ will rescue the longevity phenotype in mammals as it does in the worm. At present, *Insr*$^{+/-}$ *FOXO1*$^{+/-}$ mice have been generated, but no data have been presented with respect to their life-span (*106*). These mice, however, do recapitulate the epistatic relation between FOXO1 and the insulin receptor, analogous to that of DAF-16 and DAF-2 in the worm.

Accumulating evidence suggests a link between resistance to oxidative stress and longevity. It has been proposed that the physiological generation of ROS during cellular metabolism may result in cumulative damage, limiting life expectancy (*107*). Genetic links have been found between stress resistance and increased life-span in *C. elegans*, *Drosophila*, and mice (*108*). Various life-extending mutations in the nematode are also correlated with increased resistance to environmental stress, and both processes are dependent on DAF-16 function (*109*). Thus, in the worm, FOXO activity may promote longevity via the activation of a transcriptional program of protection against and detoxification of ROS, as discussed previously.

In mammals, the adaptor protein p66shc has been found to play a role in longevity (*110*). p66shc is a cytoplasmic signal transducer that couples mitogenic signals from activated receptors to intracellular signaling pathways such as the Ras GTPase. Exposure of cells to oxidative stress leads to the phosphorylation and activation of p66shc, and targeted deletion of p66shc in the mouse results in increased resistance to oxidative stress and extended life-span. p66shc appears to functionally interact with mammalian FOXO factors; FOXO-dependent transcription is increased in p66shc-deficient cells, and p66shc signaling is required for the phosphorylation and inactivation of FOXO3a (*76*). Although much work remains to be done, these findings suggest that endogenous p66shc may inhibit the ability of FOXO factors to induce expression of stress response genes that contribute to the life-span extension in mammals.

FOXO family members also regulate cellular metabolism, and metabolic changes appear to modulate life-span, because caloric restriction increases longevity in a number of species (*101*). In the nematode, DAF-16 plays a role in dauer formation, a developmentally arrested state characterized by reduced metabolic activity (*20*, *21*). In mammals, FOXO family members act to shift glucose metabolism from glycolysis to gluconeogenesis by inducing the expression of a number of metabolic genes, including glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (PEPCK) (*106*, *111-113*); however, whether these genes are direct targets of FOXOs is still uncertain. In addition to increasing hepatic glucose production, which may lead to glucose intolerance and possibly diabetes (*114*), the shift toward glucose production may decrease the number of substrates for mitochondrial respiration, the major source of endogenous ROS. Furthermore, as the *Insr*$^{-/-}$ FIRKO mice indicate, the loss of insulin signaling in adipose tissue, which is a major site of energy metabolism, lends to an increase in longevity. This finding suggests that FOXO's ability to regulate cellular metabolism may be contributing to the overall increase in longevity that is observed. Thus, activating FOXO activity might lead to a decreased production of damaging ROS and contribute to an extended life-span in part through the alteration of cellular metabolism.

Conclusions and Future Directions

Over the last several years remarkable progress has been made in understanding the signaling pathways and mechanisms that regulate FOXO function. The PI3K-Akt-FOXO pathway is an evolutionarily conserved signaling pathway that has been adapted to modulate a variety of diverse biological processes, including cell survival, proliferation, and stress responses. Analysis of transcriptional profiling experiments has begun to shed light on the transcriptional targets that mediate these varied outcomes. The factors that determine the specific biological output of FOXO signaling still remain uncharacterized, and much work remains to be done to determine how FOXO factors initiate alternative transcriptional programs upon activation. In addition, the importance of FOXO family members in organismal processes such as tumorigenesis and aging await the further development and analysis of animal models with mutations in or deletions of the FOXO factors. These studies may reveal new insights into the molecular basis of cancer and diabetes and shed light on the mechanism of organismal aging.

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