FoxOs at the Crossroads of Cellular Metabolism, Differentiation, and Transformation

Review

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Forkhead transcription factors of the FoxO subfamily are emerging as a shared component among pathways regulating diverse cellular functions, such as differentiation, metabolism, proliferation, and survival. Their transcriptional output is controlled via a two-tiered mechanism of phosphorylation and acetylation. Modest alterations of this balance can result in profound effects. The gamut of phenotypes runs from protection against diabetes and predisposition to neoplasia, conferred by FoxO loss of function, to increased cellular survival and a marked catabolic response associated with gain of function.

In the roundworm *C.elegans*, insulin/insulin-like growth factor (IGF) signaling regulates metabolism, reproduction, and life span. Mutations of the insulin/IGF receptor ortholog *Daf2* cause *dauer*, a characteristic arrest in diapause that results in increased life span. The effect of *Daf2* mutations can be rescued with complete penetrance by null alleles of *Daf16*, the ortholog of mammalian FoxO (*F*orkhead b*OX*-containing protein, *O* subfamily) transcription factors. Moreover, gain-of-function mutant alleles of the gene encoding the NAD-dependent deacetylase *Silent Information Regulator* (*Sir*) *2.1* prolong life span in a *Daf16*-dependent fashion (Finch and Ruvkun, 2001). These twin observations provide the underpinning for investigations of the role of FoxO proteins in mammalian metabolism and life span.

Three of the four FoxOs (1, -3a, and –4) are substrates of the serine/threonine kinase Akt, while FoxO6 lacks some phosphorylation sites and exhibits a unique pattern of subcellular localization. Genetic epistasis in *C. elegans* and biochemical evidence in mammalian cells indicates that Akt regulates FoxO activity by inducing a prompt and sustained nuclear exclusion. This is an oversimplification, since inhibition of FoxO-dependent transcription can be achieved with constitutively nuclear FoxO mutants, indicating that multiple mechanisms regulate FoxO activity.

There is broad consensus on the fact that Akt-dependent phosphorylation is crucial to the regulation of FoxO function. However, the observation that worms with extra copies of the *Sir2.1* deacetylase have increased longevity and that *Daf16* is epistatic to *Sir2.1* gain-of-function alleles raised the intriguing possibility that FoxO is regulated by the seven mammalian *Sir* orthologs, or *Sirtuins* (Sirt1–7). Nuclear Sirtuins can deacetylate transcription factors, such as p53 (Luo et al., 2001; Vaziri et

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al., 2001) and MyoD (Fulco et al., 2003), while cytoplasmic Sirtuins deacetylate enzymes (Starai et al., 2002) and structural proteins like tubulin (North et al., 2003). Sirt1-dependent deacetylation dampens p53 transcriptional effects, thus enabling cells to survive under conditions that would otherwise cause p53-dependent death. Two studies now expand the repertoire of Sirt1 targets by including FoxO. These papers show that FoxO is acetylated in response to cellular stress by the acetyltransferase activity of the nuclear hormone receptor coactivators Cbp and p300. Expression of Sirt1 causes FoxO deacetylation. These data are consistent with the genetic evidence in *C. elegans*, indicating that *Daf16* is downstream of *Sir2.1*. While these studies provide proof of principle that FoxO is modified through acetylation, the functional consequences thereof are less clear. Motta et al. report that FoxO deacetylation decreases expression of *p27*[kip], a prominent FoxO target in the regulation of cell cycle progression (Motta et al., 2004). Contrariwise, Brunet et al. propose that the effects of deacetylation are target gene specific, such that expression of proapoptotic genes is inhibited, while expression of genes that regulate cell cycle arrest (*p27*[kip]) and resistance to oxidative stress (*MnSOD*) is increased (Brunet et al., 2004). Both papers propose that the net effect of deacetylation is to promote cellular survival under stress and protect against apoptosis by fine-tuning FoxO transcription. As discussed below, the discrepancy between the two reports underscores that more work will be required to understand the impact of deacetylation on FoxO function.

Metabolic Functions of FoxO Proteins

The physiologic functions of FoxO proteins, as deduced from loss- and gain-of-function experiments in transgenic and knockout mice, are complex. Mice lacking the three main FoxO isoforms display remarkably different phenotypes (Hosaka et al., 2004): FoxO1 ablation is embryonic lethal due to defective angiogenesis. On the other hand, haploinsufficiency of *FoxO1* protects against insulin resistance caused by defective insulin signaling (Nakae et al., 2002) and rescues diabetes due to pancreatic β cell failure caused by ablation of *Insulin receptor substrate 2* (*Irs2*) (Kitamura et al., 2002). Moreover, transgenic mice overexpressing a constitutively active FoxO1 mutant in liver develop diabetes, presumably as a result of increased glucose production (Nakae et al., 2002). FoxO3a has a selective effect on ovarian function. Ablation of FoxO3a causes premature ovarian failure as a result of accelerated differentiation and consequent depletion of primary ovarian follicles (Castrillon et al., 2003; Hosaka et al., 2004). The latter observation suggests an additional mechanism to account for the well-known association among metabolism, life span, and reproduction.

Metabolic studies point to FoxO1 as the long-sought insulin-regulated transcription factor responsible for insulin action on gene expression (Figure 1). In liver, FoxO1 straddles two key pathways of insulin signaling, metabo-
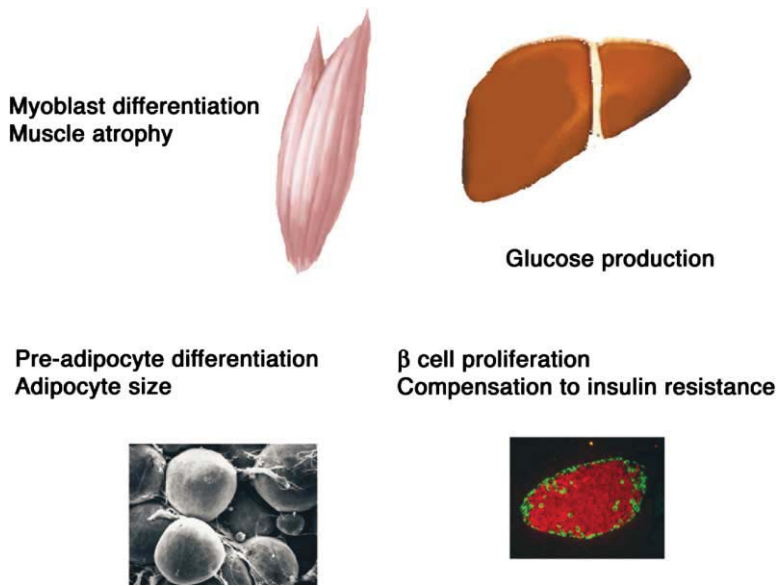
Figure 1. Synopsis of the Metabolic Actions of FoxO1 in Different Target Tissues

lism and cellular proliferation. It promotes transcription of genes that increase glucose production, acting in concert with the Pparγ coactivator Pgc1α (Puigserver et al., 2003). Since insulin control of hepatic glucose production is key to the development and treatment of diabetes, this makes FoxO1 a premier target for therapeutic intervention in this increasingly common disease. Moreover, since diabetes increases oxidative stress through the generation of reactive oxygen species, it is possible that an increase in FoxO-dependent transcription mediates the deleterious effects of hyperglycemia (so-called "glucose toxicity") as well. The potential importance of FoxO1 in metabolic disease is underscored by the effect of FoxO1 haploinsufficiency to prevent genetic (Kitamura et al., 2002; Nakae et al., 2002) as well as environmental forms of diabetes (Nakae et al., 2003). In addition, FoxO1 regulates organismal growth by controlling IGF binding protein-1 expression, which in turn modulates IGFs bioavailability. FoxO can also affect growth through its actions on the cell cycle, an effect that is conserved from *Drosophila* to mammals (Junger et al., 2003; Puig et al., 2003).

FoxOs and Cellular Differentiation
While the metabolic functions of FoxO proteins were to some extent predictable, based on work in *C. elegans*, their role in cellular differentiation was not. Evidence from cultured cells and genetically modified mice indicates that FoxOs integrate extracellular cues with the transcriptional cascade that controls differentiation of pre-adipocytes (Nakae et al., 2003), myoblasts (Hribal et al., 2003), pancreatic β cells (Kitamura et al., 2002), and thymocytes (Leenders et al., 2000). These observations dovetail with the antidifferentiative effects FoxO3a in the female gonad, as described above (Castrillon et al., 2003; Hosaka et al., 2004).

Nowhere has the gamut of FoxO functions been better illustrated than in muscle development and function. During myoblast differentiation, phosphoinositol 3-kinase (PI3K) signaling leads to myoblast fusion and activation of the terminal differentiation program. This requires transient exclusion of FoxO from the nucleus. Accordingly, a constitutively nuclear FoxO1 inhibits C2C12 myoblast differentiation, while a transcriptionally inactive mutant is capable of partially rescuing the inhibition of differentiation mediated by PI3K inhibition (Hribal et al., 2003). Interestingly, the same effect is seen in response to activation of Sirt1 (Fulco et al., 2003), raising the possibility that the effects of FoxO and Sirt1 on differentiation are epistatic. However, once the differentiation process has commenced, a constitutively nuclear FoxO mutant can increase myotube formation in primary mouse myoblast cultures (Bois and Grosveld, 2003). This study is seemingly in contrast with the findings in C2C12 cells, but in primary myoblasts, PI3K signaling via Akt appears to have a paradoxical antidifferentiative effect, so the two systems are hardly comparable. Nonetheless, these data emphasize that FoxO activity is tightly regulated during cellular differentiation. The importance of this regulation is underscored by the fact that unchecked FoxO activity, as found in some chromosomal translocations, results in alveolar rhabdomyosarcomas (ARMS) (Xia et al., 2002), an aggressive pediatric cancer thought to arise from the same progenitor cells as striated muscle (see below).

The other end of the spectrum of FoxO functions is now explored in two papers addressing the relationship between FoxO and muscular atrophy. Alterations of muscle mass in wasting syndromes, including muscular atrophy, are invariably accompanied by the induction of two ubiquin ligases, MAFbx (also known as Atrogin-1) and MuRF1. Two studies now demonstrate that these ubiquitin ligases are FoxO targets, and that FoxO inhibition prevents their induction, opening a new front in the struggle to treat muscular atrophy (Sandri et al., 2004; Stitt et al., 2004). Thus, multiple pathways converge onto a single transcription factor to control different functions at different stages of differentiation. In differentiating cells, a transient inhibition of FoxO activity appears to be a *sine qua non* to coordinate exit from the cell cycle
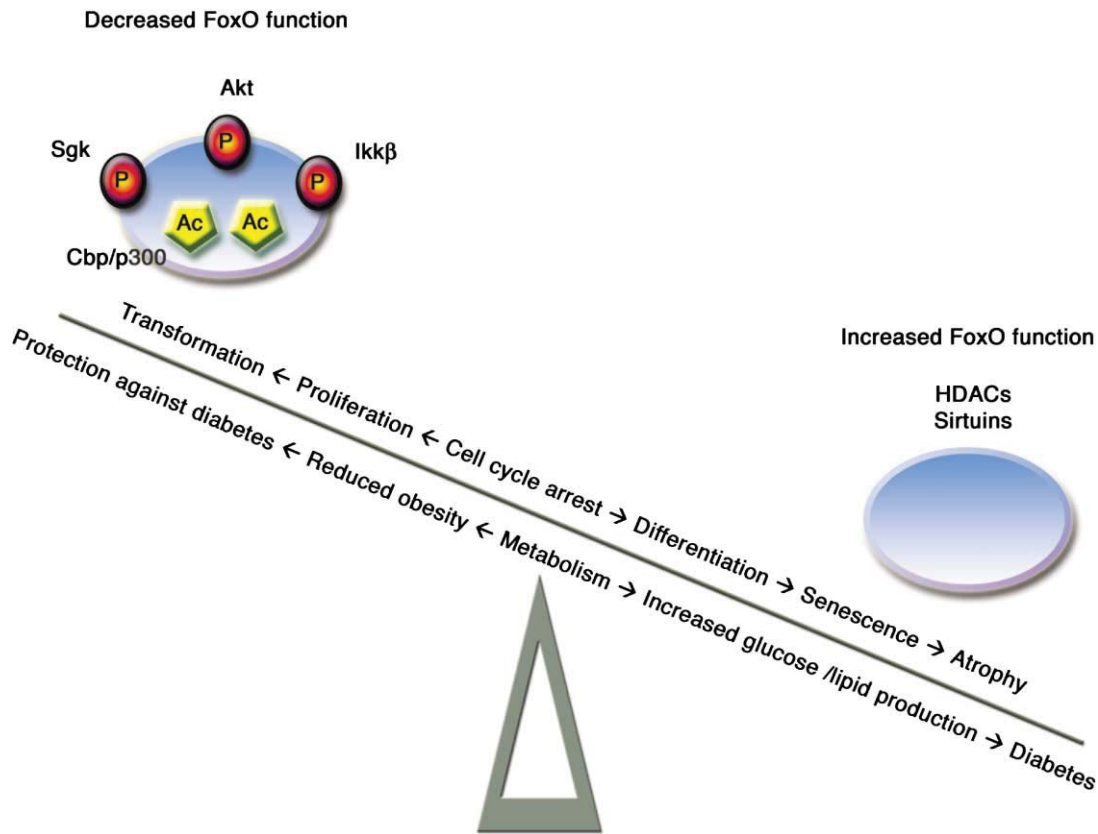
Figure 2. Pathophysiologic Consequences of Altered FoxO Transcription

There is a consensus that phosphorylation inactivates FoxO, thus removing a constraint to cellular proliferation and potentially to tumorigenesis. On the other hand, the functional consequences of acetylation are not clear, so this part of the diagram should be viewed as a working model, and not as an established fact. Red circles represent phosphorylation sites, and yellow pentagons acetylation sites.

with activation of the terminal differentiation program. A sustained loss of FoxO function at this stage results in unchecked proliferation and, potentially, in neoplastic transformation. On the other hand, unrestrained FoxO activity in terminally differentiated cells appears to promote cellular senescence, but—when taken to extremes—can result in cellular atrophy and promote a catabolic state (Figure 2).

FoxO and Cancer

A role for FoxO family members in tumorigenesis was first suggested by their involvement in two chromosomal translocations associated with human cancer. In leukemia, *FOXO2* (previously known as Af6q21) and *FOXO4* (previously known as AFX) have both been shown to participate in chromosomal translocations with the Trithorax-related transcription factor *MLL* (So and Cleary, 2003). In solid tumors, the t(2;13) and t(1;13) translocations that uniquely associate with ARMS have been shown to result in the fusion of the coding regions of either *PAX3* at 2q35 or *PAX7* at 1q31 to *FOXO1* at 13q14 (Xia et al., 2002). Two models have been proposed to explain the role of *FOXO1* in tumorigenesis. One model is predicated on the assumption that tumors arise from a FoxO gain of function. The main evidence supporting this view is that the FoxO1/Pax3 fusion protein is a much more potent transcriptional activator than Pax3

to increase the expression of Pax3 target genes (Xia et al., 2002). However, mice bearing *PAX3-FOXO1* transgenes or different versions of the *Pax3*-targeted *PAX3-FOXO1* mutant allele fail to develop tumors (Anderson et al., 2001; Lagutina et al., 2002; Relaix et al., 2003). Thus, an alternative model has been proposed, suggesting that FoxO loss of function is a pivotal event in tumorigenesis. Since cancer can be thought of as a perturbation of the critical balance between cell proliferation and cell death, disrupting a gene with roles in both cell cycle arrest and apoptosis could contribute to cellular survival and confer an advantage to transformed cells (Burgering and Kops, 2002). It is possible that the disruption of one *FOXO* allele in ARMS results in a partial loss of FoxO protein and promotes cell survival and/or transformation in cancer by altering its normal function to restrain cell cycle progression.

In addition to the chromosomal translocations described above, there is growing evidence that FoxOs play a role in other forms of cancer as well. The tumor suppressor gene PTEN has been shown to negatively regulate FoxO through the PI3K/Akt pathway. In PTEN-deficient cells, FoxO1 and FoxO3a are inactive, and restoration of functional PTEN induces either apoptosis or cell cycle arrest via upregulation of $p27^{kip}$. This effect can be mimicked by FoxOs, indicating that they act downstream of PTEN, potentially on cyclins D1 and

D2 (Birkenkamp and Coffer, 2003). In prostate cancer, where 60%–80% of tumors have mutations in PTEN, overexpression of FoxOs resulted in apoptosis through direct induction of TRAIL, a proapoptotic member of the tumor necrosis factor family (Modur et al., 2002). Therefore, any decrease in PTEN decreases FoxO activity and in this manner negatively affects direct targets of FoxO, such as TRAIL and other indirect targets, ultimately promoting tumor cell survival. It has also been shown in prostate cancer that androgen receptor and FoxO1 are capable of forming a complex, which impairs the DNA binding activity of FoxO1, thereby reducing its ability to induce cell cycle arrest and apoptosis. The same pattern of response to FoxO proteins can be demonstrated in a variety of cell types that are not necessarily PTEN deficient. Whether cells demonstrate cell cycle arrest or the apoptotic phenotype is likely to reflect differences in cell types and/or physiological conditions. In this respect, it should be noted that FoxOs are able to induce apoptosis via FasL and BIM.

In chronic myeloid leukemia, cytokine- and BCR/ABL-mediated inhibition of TRAIL occurs through FoxO3a phosphorylation. Moreover, FoxO3a has been implicated as a downstream effector of STI571-induced cell cycle arrest in BCR/ABL-expressing cells. Finally, FoxO1 phosphorylation is associated with significantly shorter survival of patients with AML, leading to the suggestion that phospho-FoxO1 may be a useful marker for identifying AML patients likely to have unfavorable clinical outcomes.

FoxO3a activity has been shown to elevate p27^{kip} expression and induce cell cycle arrest. FoxO3a and FoxO4 have also been shown to inhibit the cell cycle through downregulation of cyclin D by a p27^{kip}-independent mechanism. Thus, p27^{kip} is not required for FoxO-induced cell cycle arrest, but appears to buttress the antiproliferative effect of FoxO.

In breast cancer, FoxO3a has been shown to upregulate BIM, a BH3 domain protein very effective at inducing apoptosis. In paclitaxel-sensitive breast cancer, paclitaxel appears to upregulate FoxO3a, which subsequently increases BIM expression, ultimately decreasing cell survival and contributing to the tumor response to paclitaxel. On the other hand, stimulation of p21-activated kinase-1 (Pak-1) and estrogen receptor-α by estrogen treatment in mammary cancer cells promotes cell survival by inducing phosphorylation and nuclear exclusion of FoxO1 in a Pak1-dependent manner (Birkenkamp and Coffer, 2003).

In their investigation of the relationship between Akt phosphorylation (pAkt) and FoxO3a expression in primary breast tumors, Hu et al. found that in some pAkt-negative tumors, FoxO3a was excluded from the nucleus. These same tumors exhibited high expression of IκB kinase β, a key modulator of the NF-κB proinflammatory pathway, and had a poor survival rate. The study goes on to show that FoxO3a is a direct target of the IκB kinase. Phosphorylation by IκB causes cellular relocalization of FoxO3a to the cytoplasm, accompanied by ubiquitination and degradation by the proteasome pathway. Furthermore, overexpression of FoxO3a can override IκB stimulation of cell cycle progression, proliferation, and tumorigenesis in mice, indicating that—in this setting—FoxO3a acts as a tumor suppressor (Hu et

al., 2004). IκB is important to regulate several cytokine-dependent pathways and is a prominent target of the anti-inflammatory actions of salicylates (Yin et al., 1998). FoxO phosphorylation by IκB may thus provide a mechanistic link between cancer and inflammation.

In *C.elegans*, *Daf16* straddles *Daf2* (insulin/IGF receptor) and *Daf4/Daf7* (TGFβ) signaling pathways. A study by Seoane and colleagues now demonstrates that a similar interaction occurs in mammalian cells, where FoxO acts at the intersection of three critical pathways—the Smad, PI3K, and FoxG1 pathways (Seoane et al., 2004). The growth inhibitory gene *p21^{Cip1}* is activated when Smad proteins, mediators of TGFβ signaling, form a complex with FoxO proteins. This process is negatively regulated by PI3K and positively regulated by FoxG1, a member of a different subfamily of Fox genes. FoxG1 binds to the FoxO-Smad complexes to block *p21^{Cip1}* expression. The implication for cancer is clear. While mutations of TGFβ have been described in some cancers, more often tumor cells cease to maintain the cytostatic response to TGFβ. In the absence of the cytostatic response, TGFβ becomes a potent inducer of cell proliferation, invasion, and metastasis. Seoane et al. show that glioblastoma cells have increased levels of FoxG1 and PI3K/Akt activity, resulting in the suppression of *p21^{Cip1}* and cytostasis and promoting cell survival and proliferation, precisely the desired strategy for successful tumor growth and spread (Seoane et al., 2004). Interestingly, *p21^{Cip1}* is also a prominent FoxO target during pre-adipocyte differentiation (Nakae et al., 2003).

In the studies described above, inactivation of FoxO appears to be an important step in carcinogenic transformation and would argue that *FoxO* genes be classified as tumor suppressors. Cancer-related drug discovery has been focused on inhibitors of oncoproteins that are activated in tumor cells. One of the best-known examples is Gleevac, the inhibitor of the leukemia-associated BRC/ABL fusion gene product. Developing chemical molecules that act to restore the function of a defective tumor suppressor gene would seem to pose a more difficult problem. However, recently Kau et al. identified several small molecules that act as inhibitors of FoxO1 nuclear export in PTEN-deficient cells (Kau et al., 2003). These molecules fall into two classes: general inhibitors of the nuclear export receptor CRM1 and specific inhibitors of the PI3K/Akt-dependent export pathway (Figure 2). Whether any of these compounds will make their way to the clinic remains to be seen. However, given the variety of cancers in which FoxO proteins are likely to play a role, the prospect of potential treatment modalities aimed at restoring FoxO activity is an exciting one.

At the Crossroads of Metabolism and Neoplasia
The biology of FoxO transcription factors provides a glimpse into the complex relationship among cellular proliferation, transformation, and metabolism. In humans, epidemiological studies indicate that rates of cancer prevalence increase with increasing body mass (Calle et al., 2003). Does this association reflect a common etiology? The transcriptional thread activated by FoxO represents a potential unifying mechanism to regulate both cellular differentiation and metabolism. Thus, the FoxO target *p21^{cip}* is critical for both TGFβ-depen-

dent transformation and insulin-dependent adipogenesis, providing a mechanism by which alterations of FoxO function could tip the balance between cellular differentiation and neoplastic transformation. Another mechanism could be envisioned to involve FoxO inactivation by Iκβ, as the latter also mediates insulin resistance (Yuan et al., 2001).

From a therapeutic standpoint, the identification of acetylation-dependent mechanisms of FoxO regulation expands the potential repertoire of drugs by which FoxO activity could be modulated to include HDAC inhibitors. Since these compounds are coming to the fore as antineoplastic agents, the present findings raise the question of whether they could be pressed into service against the raising tide of metabolic diseases. In addition, as Sirtuin agonists have been shown to control metabolism in yeast, it is worth exploring the idea that antagonists may counteract FoxO-dependent transcription, thus breaking the vicious cycle in which altered metabolism contributes to oxidative stress via increased glucose oxidation.

A Shared Pathway to Control Metabolism, Longevity, and Tumorigenesis under FoxO Control?

The seemingly contradictory effects of deacetylation on FoxO function cannot be easily reconciled at this point (Motta et al., 2004; Brunet et al., 2004). The crux of the matter is that, while deacetylation is generally thought to decrease gene expression, in *C. elegans* increased deacetylation appears to result in a FoxO gain-of-function. On the other hand, although FoxO tends to be viewed by a majority of investigators working in the field as a transcriptional activator, there are examples of FoxO-dependent transcriptional suppression (Kitamura et al., 2002). Thus, increased FoxO activity would not necessarily be predicted to increase gene expression across the board.

As we have seen, inactivation of FoxO proteins seems to play an important role in cancer progression. While in principle it would be theleologically attractive to marshal the gamut of cellular functions related to longevity and neoplastic transformation under FoxO control (Figure 2), the reader should view this as a working model and not as a consensus. In mammalian cells, it may prove impossible to tease out the effects of FoxO deacetylation from those caused by deacetylation of histones and other Sirt1 targets, like p53. In this regard, it should be noted that both studies show that FoxO-dependent transcription is regulated by both NAD-dependent and -independent deacetylases (Motta et al., 2004; Brunet et al., 2004). Additionally, both studies focused on FoxO targets that regulate cell cycle and removal of reactive oxygen species. They did not delve into a panoply of additional FoxO targets that have profound consequences on cellular survival and transformation, such as those identified through genetic screens for *Daf16* targets in *C. elegans* (Lee et al., 2003; Murphy et al., 2003). Moreover, some effects widely ascribed to FoxO in cellular systems, such as induction of apoptosis, do not occur in transgenic mice overexpressing constitutively active FoxO (Nakae et al., 2002). Finally, while neither study addressed in detail the stoichiometry of

FoxO acetylation, the report by Brunet and colleagues indicates that only a fraction of endogenous FoxO is bound to Sirt1 at any given time. If so, this would suggest that small changes in the amount of deacetylated FoxO have major consequences on cellular functions and indicate that deacetylation is paramount in regulating FoxO activity. The studies on the FoxO/Sirt1 interaction represent an important stepping stone toward the demonstration that control of vertebrate life span, cell differentiation, metabolism, and reproduction can be ascribed to FoxO target genes. The challenge is now to address how seemingly contrasting in vivo functions of FoxO can yield a coordinated organismal response when modulated through acetylation.

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