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FoxO Transcription Factors and Stem Cell Homeostasis: Insights from the Hematopoietic System

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The forkhead O (FoxO) family of transcription factors participates in diverse physiologic processes, including induction of cell-cycle arrest, stress resistance, differentiation, apoptosis, and metabolism. Several recent studies indicate that FoxO-dependent signaling is required for long-term regenerative potential of the hematopoietic stem cell (HSC) compartment through regulation of HSC response to physiologic oxidative stress, quiescence, and survival. These observations link FoxO function in mammalian systems with the evolutionarily conserved role of FoxO in promotion of stress resistance and longevity in lower phylogenetic systems. Furthermore, these findings have implications for aging in higher organisms and in malignant stem cell biology, and suggest that FoxOs may play an important role in the maintenance and integrity of stem cell compartments in a broad spectrum of tissues.

FoxO Family Members and HSCs

Longevity in higher-level organisms is dependent on the maintenance of tissue homeostasis that is in part determined by the integrity of tissue-specific stem cells. There are several adult tissue compartments in mammalian systems that are highly reliant on stem cells for their maintenance and propagation, including skin, gastrointestinal epithelium, and blood (Blanpain et al., 2004; Radtke and Clevers, 2005; Till and McCulloch, 1961). In addition, there is convincing evidence for the existence of adult tissue stem cells in the central nervous system, and cells with properties of stem cells have been identified in lung in murine models (Kim et al., 2005; Reynolds and Weiss, 1992). In the skin, gut, and hematopoietic systems, stem cells persist for the life of the organism and give rise to committed progenitors that subserve various functions of terminally differentiated cells. For example, in the gut, stem cells that reside in the base of the colonic crypts give rise to progeny that terminally differentiate into colonic epithelial cells. In the hematopoietic system, stem cells give rise to a broad spectrum of terminally differentiated effector cells that are responsible for innate and humoral immune response to infection, hemostatic homeostasis, and oxygen delivery.

Hematopoietic development is regulated by a dynamic balance between HSC self-renewal and differentiation to mature effector cells. The balance between self-renewal and differentiation is of critical importance: too little self-renewal or too much differentiation may jeopardize the ability to sustain hematopoiesis throughout life, whereas excessive self-renewal and/or aberrant differentiation may result in leukemogenesis. The regulation of HSC self-renewal is not fully understood, but recent studies have underscored the importance of cell cycle, apoptosis, and oxidative stress response in HSC homeostasis. Recent data indicate that FoxO family members play a critical role in these physiologic processes in the HSC compartment and thereby regulate maintenance and integrity of HSCs.

Regulation of FoxO Transcriptional Activity

The forkhead box (Fox) family of proteins is a large family of transcription factors with diverse physiological functions. The evolutionary conservation of Fox proteins from yeast to humans, and their diverse biological functions, highlight the importance of these proteins in developmental processes. All members of the Fox family share a conserved 110 amino acid DNA-binding domain that is referred to as the "forkhead box" or "winged helix" domain. Over 100 forkhead genes have been identified to date, and in humans, this family of transcription factors has been subdivided into 19 subgroups (FOXA-FOXS) based on sequence similarity (reviewed in Wijchers et al., 2006).

The FoxO subfamily (FoxO1, FoxO3, FoxO4, and FoxO6) plays an important role as effectors of the PI3K/AKT pathway in diverse cellular processes that include induction of cell-cycle arrest, stress resistance, apoptosis, differentiation, and metabolism (reviewed in Greer and Brunet, 2005). FoxO1, FoxO3, and FoxO4 expression is abundant in most tissues, including those of the hematopoietic system, with highest expression of the different isoforms found in the adipose tissue, brain, and heart, respectively. In contrast, the expression of FoxO6 appears to be restricted to the developing brain and has a variant mechanism for regulation of its transcriptional activity, as described below.

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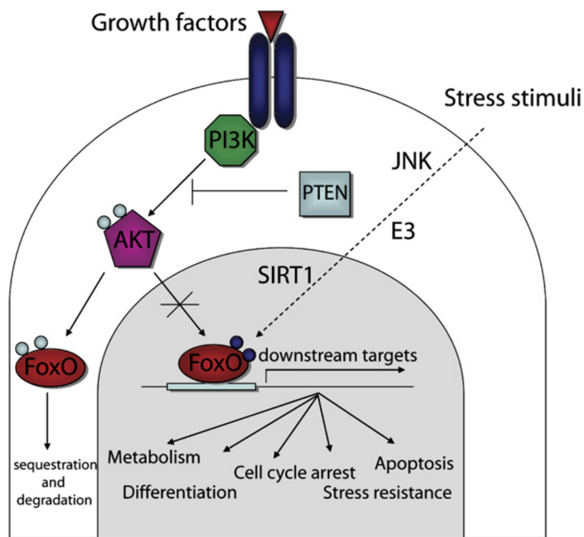

Figure 1. PI3K/AKT and Stress Stimuli-Mediated Pathways Regulate FoxO Function
Activation of the PI3K/AKT pathway in response to insulin or growth factor stimulation results in recruitment and activation of the serine-threonine kinase AKT. Activated AKT inhibits FoxO function by phosphorylation of FoxOs at three conserved residues. The dual specificity lipid and protein phosphatase PTEN antagonizes PI3K/AKT activity. In the presence of stress stimuli, JNK phosphorylates FoxOs at a distinct set of threonine residues and mediates their nuclear import and transcriptional activation of stress resistance-inducing target genes. Similarly, presence of stress stimuli activates deacetylation of FoxOs by SIRT1 and monoubiquitination of FoxOs by E3 ligase, which both promote FoxO-mediated transcription of genes inducing stress resistance.

It is perhaps not surprising, given the diversity of functions enacted by FoxO factors, that there are multiple levels of control of FoxO function in the cellular milieu that include phosphorylation, acetylation, and ubiquitination (reviewed in van der Horst and Burgering, 2007). FoxO phosphorylation can play both inhibitory and activating roles in FoxO function. AKT inactivates FoxO1, FoxO3, and FoxO4 by direct phosphorylation of three conserved serine and threonine residues (Thr32, Ser253, and Ser315 in FoxO3), creating a binding motif for the 14-3-3 chaperone proteins that interfere with the DNA binding domain of FoxOs and facilitate the translocation of FoxOs from the nucleus to the cytoplasm (Brunet et al., 1999, 2002). Cytoplasmic FoxOs are targeted for ubiquitination and proteosomal degradataion. The regulation of FoxO6 activity is not well understood, in that it lacks the C-terminal AKT phosphorylation site and is primarily localized in the nucleus (van der Heide et al., 2005). In addition to AKT, other kinases, such as serum and glucocorticoid inducible kinase (SGK), casein kinase 1 (CK1), dual tyrosine phosphorylated regulated kinase 1 (DYRK1), and I kappa-B kinase β (IKKβ) participate in phosphorylation of specific serine residues in FoxOs and are thought to affect subcellular localization of FoxOs in a manner similar to AKT.

Conversely, activation of Jun N-terminal kinase (JNK) or mammalian sterile 20-like kinase-1 (Mst1) in response to stress stimulation results in phosphorylation of FoxOs at a distinct set of threonine residues and results in nuclear import, rather than export, of FoxOs and subsequent transcriptional activation (Essers et al., 2004; Lehtinen et al., 2006). The effects of FoxO phosphorylation by JNK thus appear to be counterregulatory to those mediated by PI3K/AKT phosphorylation. However, it should be noted that stressful stimuli override the negative regulatory effects of growth factors on FoxO activation (Brunet et al., 2004; Wang et al., 2005), suggesting that among the most important functions of these highly evolutionarily conserved proteins is protection of mammalian cells from environmental stress, similar to their role in lower organisms.

There is also complex modulation of FoxO activity by acetylation and deacetylation. FoxOs bind to coactivator and corepressor complexes, such as CREB-binding protein (CBP), p300/CBP-associated factor (PCAF), and SIRT1 deacetylase, and subsequent acetylation or deacetylation affects their transcriptional activity (Brunet et al., 2004; Frescas et al., 2005; Matsuzaki et al., 2005; van der Horst et al., 2004; Vogt et al., 2005; Yang et al., 2005). For example, acetylation of FoxO1 alters DNA binding activity and sensitivity to phosphorylation (Matsuzaki et al., 2005), whereas Sirt1-mediated deacetylation of FoxO1 appears to regulate subnuclear localization and may impact selection of transcriptional programs (Frescas et al., 2005) and has been reported to globally repress FoxO1 transcriptional activity in the context of prostate cancer cells (Yang et al., 2005).

Ubiquitination may also result in either activation or inactivation of FoxO. Polyubiquitination targets FoxOs for proteasomal degradation and requires phosphorylation of FoxOs by AKT, SGK, or IKKβ and cytoplasmic localization (Hu et al., 2004; Huang et al., 2005; Matsuzaki et al., 2003; Plas and Thompson, 2003). Conversely, oxidative stress can induce monoubiquitination of FoxOs in the cytoplasm or nucleus and thereby mediate FoxO activation (van der Horst et al., 2006).

Thus, unique combinations of phosphorylation, acetylation, and ubiquitination of FoxOs provide mechanisms to "fine tune" FoxO function (Vogt et al., 2005). Taken together, these posttranslational layers of control of FoxO activity provide insight into the seeming paradox that FoxOs can regulate both a protective response to stressful stimuli as well as regulation of cell death, and mechanistic explanations for FoxOs' ability to orchestrate different transcriptional programs depending on the nature of the environmental stimulus.

Diverse Physiological Roles of FoxOs In Vitro and In Vivo

Whereas FoxO inactivation by PI3K/AKT pathway favors enhanced cell survival, cell proliferation, and stress sensitivity, activation of FoxOs leads to apoptosis, cell-cycle arrest, and stress resistance in most tissue contexts. In the absence of growth factors or insulin, or in the presence of stress stimuli, FoxO members reside in the nucleus and are active as transcription factors (Figure 1). Their

activation engages several transcriptional programs that include proapoptotic signaling via induction of *TRAIL*, *FasL*, and *Bim* (Brunet et al., 1999; Dijkers et al., 2000; Modur et al., 2002). In addition, FoxOs alter the expression of a spectrum of genes that cumulatively result in cell-cycle arrest. These include increased expression of *p27*, *p130*, and *p21* and repression of *Cyclin D* expression that contributes to G1/S arrest (Kops et al., 2002b; Medema et al., 2000; Seoane et al., 2004), activation of *Cyclin G2* that contributes to G0/G1 arrest (Martinez-Gac et al., 2004), and activation of *Cyclin B* and *Polo-like kinase* associated with G2/M arrest (Alvarez et al., 2001; Seoane et al., 2004).

Furthermore, the oxidative stress response is regulated in part by FoxO induction of *MnSOD* and *catalase* (Kops et al., 2002a; Nemoto and Finkel, 2002; Tran et al., 2002). In concert with mediation of stress resistance, FoxOs also facilitate DNA damage repair by upregulating the expression of genes such as *GADD45* and *DDB1* (Ramaswamy et al., 2002; Tran et al., 2002). In differentiating cells, FoxOs can either promote or inhibit differentiation, depending on the tissue context and FoxO isoform. For example, expression of FoxO1 inhibits differentiation of adipocytes and myoblasts (Hribal et al., 2003; Nakae et al., 2003), whereas FoxO3 potentiates differentiation of erythroid cells (Bakker et al., 2004). In addition, activation of FoxOs causes atrophy of fully differentiated skeletal and cardiac muscle cells by decreasing protein synthesis and cell size (Sandri et al., 2004; Stitt et al., 2004). Loss of function of FoxOs in conditional knockout models as a consequence of excision mediated by interferon-inducible promoters also results in tumorigenesis, but in a highly tissue dependent and selective manner (Paik et al., 2007). Lastly, FoxOs are important regulators of glucose metabolism by upregulating the expression of genes involved in gluconeogenesis (reviewed in Barthel et al., 2005). The basis for the highly context-dependent effects of FoxO gain or loss of function is not well understood. However, there are several potential explanations for these differences that include varying levels of expression or redundancy among different family members in different tissues or unique environmental stresses encountered by various tissue compartments. In addition, it is not clear why mammalian systems have four closely related FoxO family members, whereas *Drosophila* or nematodes have a single FoxO ortholog. However, it is tempting to speculate that there is a degree of functional redundancy that reflects the importance of these transcription factors in maintaining integrity of mammalian systems and that there are also distinctive and nonoverlapping functions that subserve specific physiologic needs within a specific tissue compartment.

Experimental support for functional redundancy comes from loss-of-function studies in the murine system, in which there may be minimal phenotypes associated with loss of a single FoxO family member. For example, *FoxO4*-deficient animals are viable and do not show any overt phenotype (Hosaka et al., 2004), and *FoxO3*-deficient animals are born with normal Mendelian frequencies, although females become infertile due to global primordial follicle activation with subsequent oocyte exhaustion that indicates a central role for FoxO3 in this germ cell compartment. *FoxO3*-deficient mice also exhibit defects in glucose uptake and autoinflammation (Castrillon et al., 2003; Lin et al., 2004). *FoxO1* deficiency results in embryonic lethality at day E10.5 due to a defect in angiogenesis (Furuyama et al., 2004; Hosaka et al., 2004), indicating a nonredundant role for FoxO1 in vasculogenesis. The phenotype of *FoxO6*-deficient animals is yet to be reported, but its restricted expression pattern suggests that it may play a role in embryologic development of the central nervous system (Hoekman et al., 2006).

Evolutionary Conservation of FoxO Function in Stress Resistance and Longevity

FoxO family members were first identified in *C. elegans* as the ortholog DAF-16 (Kenyon et al., 1993). Loss of function of DAF-16 reverts a longevity phenotype in nematodes mutant in the DAF-2 insulin/IGF-1 receptor ortholog. Furthermore, DAF-16 prolongs lifespan in part by induction of the dauer phenotype, a developmentally arrested larval stage that is observed during times of environmental stress. DAF-16 mediates its effects on longevity by activating stress response genes, such as *MnSOD* (Honda and Honda, 1999), as well as a number of other targets, including *cki-1*, *egl-10*, and *lin-2*, among others (Baugh and Sternberg, 2006; Oh et al., 2006), and this process is mediated by a number of DAF-16 regulators, such as *SMK1* and *kri-1* (Berdichevsky et al., 2006; Berman and Kenyon, 2006; Lehtinen et al., 2006; Wolff et al., 2006). Similarly, the *Drosophila* ortholog dFOXO acts as an effector of the PI3K/AKT pathway, and its activation results in increased resistance to stress and enhanced longevity (Giannakou et al., 2004; Hwangbo et al., 2004). Thus, evolutionary and functional conservation of FoxO as an effector of PI3K/AKT is indicative of the importance of this group of transcription factors in maintaining and regulating cellular homeostasis (Figure 2).

The Role of FoxOs in Regulating the Size of the HSC Compartment

Recent data from several groups indicate that FoxOs play essential regulatory roles in a number of physiologic processes that influence hematopoietic stem cell number and function. For example, young adult mice engineered with conditional knockout alleles of *FoxO1*, *FoxO3*, and *FoxO4* and excised in the hematopoietic system with an Mx-driven Cre recombinase show a marked reduction of HSC (LT-LSK) numbers, which correlates with a functional deficiency of long-term repopulation in both the competitive and noncompetitive reconstitution assays (Tothova et al., 2007). Furthermore, aged germline *FoxO3* knockout animals also show a reduction of the HSC pool and a deficient repopulating capacity in secondary and tertiary competitive transplantation assays (Miyamoto et al., 2007). The relative subtlety of the HSC phenotype of *FoxO3* germline knockout animals, and the need for excision of all three *FoxOs* to fully manifest defects in apoptosis and cell-cycle aberrations, indicates that there is a

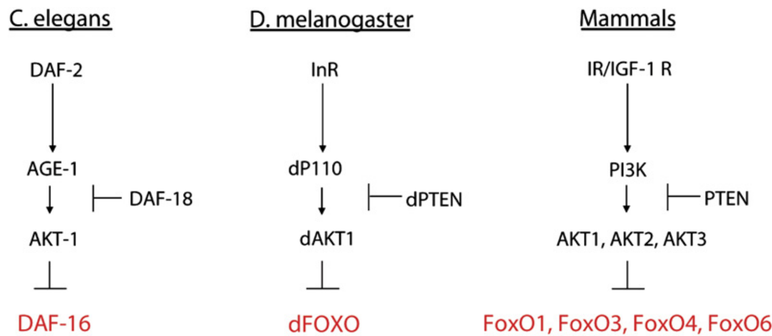

Figure 2. PI3K/AKT/FoxO Signaling Is Highly Conserved across Species
The *C. elegans*, *D. melanogaster*, and mammalian orthologs of FoxOs are highly conserved in their ability to mediate stress resistance and increase in lifespan as downstream effectors of the PI3K/AKT pathway.

significant degree of functional redundancy among the different FoxO members.

FoxOs and HSC Quiescence

FoxO members play an important role at the G0-G1, G1-S, and G2-M checkpoints by direct transcriptional modulation of proteins that regulate these transitions. Loss of FoxO1, FoxO3, and FoxO4 in the adult hematopoietic system of animals results in a striking increase in the proportion of HSCs (LSKs) in the active phases of cell cycle (S/G2/M). This finding is restricted to the HSC compartment and not present in the myeloid progenitor compartment and correlates with an HSC-restricted modulation of FoxO target genes, including *Rb/p130*, *Cyclin G2*, *p27*, *p21*, and *Cyclin D2* (Tothova et al., 2007). Similarly, germline loss of FoxO3 results in increased exit of $CD34^{-}$LSKs, but not $CD34^{+}$LSKs, into cycle and is accompanied by decreased *p27* and *p57* expression. Furthermore, germline loss of FoxO3 renders mice more susceptible to cell-cycle-dependent myelotoxic agents such as 5-FU (Miyamoto et al., 2007).

These effects of FoxOs on cell cycle and quiescence in the HSC compartment can be understood in part from studies of mouse models deficient in components of the cell-cycle machinery, including direct targets of FoxO, such as *p21*, *p27*, and *D Cyclins*. Mice deficient for $p21^{cip1/waf1}$ show increased numbers of HSCs and increased HSC cycling, implicating p21 as a negative regulator of HSC proliferation (Cheng et al., 2000b). Similarly, $p27^{kip1}$ is a negative regulator of progenitor ($Sca1^{+}Lin^{+}$) proliferation in studies of *p27*-deficient mice that show normal numbers and proliferation of HSCs but increased number and proliferation of progenitors (Cheng et al., 2000a). Embryos triply deficient for Cyclin D_1, Cyclin D_2, and Cyclin D_3 show reduced numbers of fetal liver HSCs and progenitors and impaired proliferative ability of both cell types, indicating a positive role of D Cyclins in HSC proliferation (Kozar et al., 2004). Finally, p16 has been recently shown to drive age-associated changes in the HSC compartment, such as decreased self-renewal and increased apoptosis with stress (Janzen et al., 2006). These studies collectively indicate that proper regulation of the cell-cycle machinery is essential for the maintenance of the HSC and progenitor pools and provide mechanistic insights into the effects of loss of function of FoxOs on HSC cell-cycle abnormalities.

The abnormalities in the HSC cell-cycle profile in the context of FoxO deficiency are likely to have important consequences on stem cell fate. The decrease in the G0 population and significantly reduced numbers of HSCs in young conditional *FoxO1/3/4* knockout animals or aged *FoxO3* knockout animals suggest that *FoxO*-deficient stem cells may not be able to re-enter cell cycle properly and are thereby deficient in their ability to self-renew. In addition, there is a proportional increase in the number of cycling cells, suggesting that some HSCs are unable to exit the cell cycle in the absence of FoxOs and thus accumulate in S/G2/M. *FoxO*-deficient HSCs are therefore driven out of quiescence into cell cycle, resulting in depletion of the stem cell pool.

FoxOs and Their Effects on HSC Survival

The role of apoptosis in HSC homeostasis has been tested in H2K-BCL-2 transgenic mice engineered to overexpress the antiapoptotic Bcl2 protein, a member of the Bcl2 family of proteins, in all hematopoietic cells. These mice show increased numbers of HSCs, accompanied by decreased cycling and a competitive advantage in reconstitution assays. This observation underscores the importance of the role that apoptosis plays in regulating and limiting stem cell numbers (Domen et al., 2000), and as noted above, FoxOs play an important role in regulation of this process. Germline loss of FoxO3 resulted in no apparent changes in apoptosis of hematopoietic stem and progenitor populations (Miyamoto et al., 2007); however, loss of FoxO1, FoxO3, and FoxO4 in the adult hematopoietic system results in significantly increased levels of apoptosis in both HSC and myeloid progenitor compartments (Tothova et al., 2007). At face value, this finding is paradoxical, because FoxOs are known to promote, rather than impair, apoptosis. However, there is recent evidence from a murine model of induced arthritis that *FoxO3* deficiency causes an increase in apoptosis that is correlated with increased levels of FasL production (Jonsson et al., 2005). It has therefore been suggested that FoxOs may act as either a repressor or an activator of FasL production, depending on the specific external stimulus and/or tissue context. Thus, enhanced apoptosis and increased exit out of quiescence due to *FoxO* deficiency may act in concert to decrease the pool size of HSCs available for self-renewal.

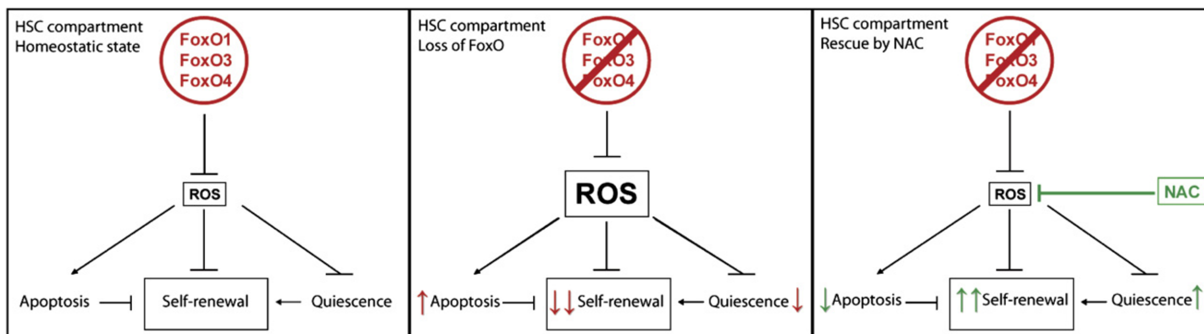

Figure 3. Antioxidant Treatment of *FoxO*-Deficient Mice Reverses Increased ROS Levels in the HSC Compartment and Restores Proper HSC Function
Treatment of *FoxO1/3/4*-deficient animals with the antioxidant NAC restores levels of ROS, as well as proper functions in the HSC compartment, including HSC number, cell cycling, and apoptosis, and implicates ROS as the causal agent in the *FoxO*-deficient HSC phenotype.

FoxOs Mediate HSC Resistance to Physiologic Oxidative Stress

Effect of Oxidative Stress on HSC Self-Renewal

Regulation of oxidative stress in the HSC compartment is critical for the maintenance of HSC self-renewal. For example, increased levels of reactive oxygen species (ROS) impair HSC self-renewal in *Atm*-deficient mice via a p38 MAPK-p16/p19-Rb-dependent mechanism (Ito et al., 2004; Ito et al., 2006). In brief, *Atm*-deficient mice develop early-onset bone marrow failure that correlates with increased levels of ROS, activation of the p38 MAPK pathway, and increased expression of p16 and p19 in the HSC compartment. Treatment of *Atm*-deficient animals with antioxidant N-acetyl-L-cysteine (NAC), as well as a p38 MAPK-specific inhibitor, rescues these mice from bone marrow failure. In addition, changes similar to those observed in *Atm*-deficient mice were also present in HSCs isolated from mice that were treated with the oxidant buthionine sulfoximine (BSO), as well as in HSCs that underwent multiple rounds of serial transplantation (Ito et al., 2006). Collectively, analysis of the *Atm*-deficient HSCs shows that maintenance of the proper oxidative environment is essential for normal HSC function, although the exact mechanisms of ATM deficiency-induced increase in ROS and subsequent p38 MAPK activation remain to be elucidated.

FoxOs Affect HSC Integrity by Regulating ROS

FoxO family members protect quiescent cells from oxidative stress by upregulation of genes involved in their detoxification, such as *MnSOD*, *catalase*, and *GADD45*. HSCs (LSKs), but not myeloid progenitor cells isolated from *FoxO1/3/4* conditional knockout animals, show increased ROS, which correlates with enrichment of genes that regulate ROS in wild-type versus *FoxO*-deficient HSCs (Tothova et al., 2007). Similarly, germline loss of FoxO3 results in increased levels of ROS in the HSC (LSK) compartment and correlates with decreased level of SOD2 and catalase expression and increased activation of p38 MAPK in CD34$^-$LSK cells (Miyamoto et al., 2007).

As observed in *Atm*-deficient mice, the HSC defect in *FoxO*-deficient mice is reverted with the antioxidant NAC (Figure 3). NAC treatment of the triple *FoxO* conditional knockout mice restores the size of the HSC compartment, and the rescue of the HSC compartment size correlates with reversion of the cell cycle and apoptosis defects, as well as the hematopoietic colony forming unit ability, long-term cobblestone area forming ability, short-term repopulating ability in vivo, and restoration of a FoxO transcriptional program (Tothova et al., 2007).

Treatment of *FoxO3* germline knockout animals with NAC reveals a partial inhibition of p38 MAPK activation in CD34$^-$LSK cells, and treatment with the p38 MAPK inhibitor rescues the defect in LTC-IC formation (Miyamoto et al., 2007). These experiments collectively imply that ROS accumulation and p38 MAPK activation impair HSC function in *FoxO*-deficient animals.

Regulation of ROS during Hematopoietic Development

The HSC-specific increase in levels of ROS that are causally implicated in the *FoxO*-deficient stem cell phenotype is also accompanied by a marked increase in ROS levels with the transition from HSCs to myeloid progenitors that is unaffected by *FoxO* deficiency. This observation suggests that there might be a FoxO-independent developmental program that regulates ROS levels in myeloid progenitors. Gene set enrichment analysis (GSEA) of HSCs and myeloid progenitors of *FoxO1/3/4* conditional knockout animals supports the existence of a developmentally regulated program that is engaged with the transition from HSCs to myeloid progenitors. Of note, *FoxO*-deficient and wild-type myeloid progenitors show enrichment for the same subset of ROS genes, and this gene set is different from that observed in the HSC compartment. These data suggest that there is a subset of ROS genes that is regulated by FoxOs in the HSC compartment and serves to decrease levels of ROS and, with this, their deleterious effects on HSC survival and function. In the context of HSCs, the $\sim$10-fold increase in ROS results in a striking decrease in the longevity of these cells that are normally capable of self-renewal over the lifetime of the organism in which they reside. However, with the transition of HSCs to myeloid progenitors, GSEA identifies a developmentally regulated transcriptional program that is FoxO independent and that is

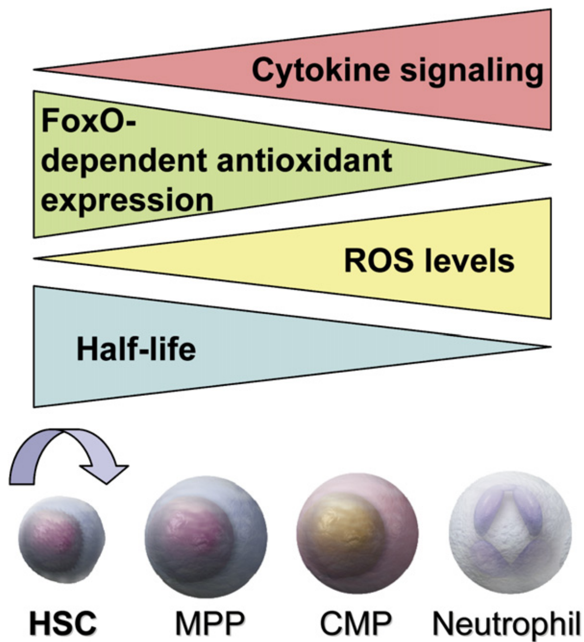

Figure 4. FoxO Regulation of ROS during Hematopoietic Development and Its Proposed Effects on Fate Determination and Longevity
FoxO transcription factors may contribute to fate determination by providing compartmentalization of antioxidant enzymes. Under normal hematopoietic homeostasis, HSCs are some of the longest-lived cells as a result of their ability to maintain low levels of ROS. Decreased FoxO activity correlates with decreased FoxO-dependent antioxidant expression and increased ROS levels, which may act as the required signal directing the transition from HSCs to more mature fates. Loss of FoxOs results in a significant increase in ROS in the HSC compartment with a subsequent decrease in lifespan of these cells. (Artwork courtesy of Eric Smith.)

accompanied by a ~100-fold increase in ROS (Tothova et al., 2007).

It is perhaps not surprising that ROS levels would be dramatically elevated with this developmental transition of HSCs to myeloid progenitors. A spectrum of terminally differentiated myeloid lineage cells, including neutrophils, monocytes, eosinophils, and mast cells, are a first line of host defense to infectious agents, such as bacteria. These cells employ a variety of strategies to kill their prey, including ROS. Thus, HSCs and myeloid progenitors have opposing relationships to ROS. HSCs must be protected from the effect of ROS to maintain quiescence, self-renewal, and longevity. In contrast, commitment by the HSC compartment toward myeloid differentiation requires marked upregulation of ROS to subserve the role of terminally differentiated myeloid lineage cells as professional generators of ROS with bactericidal intent. Current experimental evidence supports the notion that this dichotomous relationship to ROS between these intimately related hematopoietic compartments is regulated, perhaps not unexpectedly, by two different transcriptional programs. The ROS protective program in HSCs is enacted by FoxO transcription factors that are highly redundant in this role, whereas an ROS generating program is engaged by an as yet unknown, FoxO-independent mechanism to facilitate normal terminal differentiation of myeloid cells (Tothova et al., 2007). It will be of considerable interest to gain mechanistic insights into this transition.

It is also of interest that increased ROS dramatically shortens the lifetime of HSCs, yet myeloid progenitors have ~10-fold higher levels of ROS than *FoxO*-deficient HSCs. It seems likely that there are mechanisms that serve to protect myeloid progenitors from ROS exposure during development. Nonetheless, terminally differentiated myeloid cells are among the shortest-lived cells in mammals. It is tempting to speculate that they must generate high levels of ROS for their normal function but have an ephemeral existence as a consequence.

FoxOs are expressed with the transition from HSCs to myeloid progenitors, which raises the question of how ROS levels can override the inherent activity of FoxOs in repressing levels of ROS. Myeloid progenitors differentiate under the influence of a spectrum of exogenous cytokines, thus it is possible that there is an inherently higher level of activation of the PI3K/AKT pathway in this compartment compared with HSCs, with resultant inactivation of FoxO. Indeed, wild-type $CD34^{-}$LSKs show greater accumulation of nuclear FoxO3 than $CD34^{+}$LSKs, which have increased levels of Akt activation and lipid raft formation (Yamazaki et al., 2006). Even with negation of the countervailing influence of FoxOs on ROS, the observation that increased ROS in myeloid progenitors is not dependent on FoxOs strongly supports the hypothesis that there is a developmentally regulated program that increases ROS in myeloid progenitors.

The deleterious effects of high levels of ROS are well known—indeed these are in part responsible for cytocidal activity in terminally differentiated myeloid cells. However, there is evidence that certain ROS, such as hydrogen peroxide (H_2O_2), can not only act as oxidative stressors, causing cellular damage, but also as important and highly selective signaling molecules that regulate a variety of biological functions (reviewed in Veal et al., 2007). Building on the premise that ROS can act as signaling molecules, and the observation that levels of ROS are significantly higher in myeloid progenitors than HSCs, it could be speculated that increased levels of ROS act as the required intracellular signal directing the transition from HSCs to myeloid progenitor fates by triggering exit out of quiescence and skewing the balance away from self-renewal toward differentiation (Figure 4). In vivo, this transition could be enabled by FoxO-driven compartmentalization of antioxidant expression within the relatively hypoxic bone marrow niche, and/or modulation of oxidizing proteins with catalytic site cysteines that are exquisitely sensitive to redox potential or hypoxia-inducible factor (HIF) expression.

Linking the Role of FoxOs in HSCs to Other Components of the PI3K/AKT Pathway

The essential role of the PI3K/AKT pathway in hematopoietic homeostasis and maintenance of HSC integrity is supported by recent reports of hematopoietic defects in mice with conditional loss of PTEN in the hematopoietic

compartment. PTEN (phosphatase and tensin homolog) is a dual specificity lipid and protein phosphatase that converts PtdIns(3,4,5)P_3 to PtdIns(3,4,5)P_2 and antagonizes PI3K/AKT activity. Deletion of PTEN results in hyperactivation of the PI3K/AKT pathway, and activation or inactivation of its multiple downstream targets, including GSK3β, FoxO, mTOR, IKK, Bad, p27, and ASK1. Mice deficient for *Pten* in the hematopoietic system show reduction in the number of HSCs and develop myeloproliferative disease that rapidly evolves to acute leukemia (Yilmaz et al., 2006; Zhang et al., 2006).

Pten-deficient mice have many phenotypic similarities to *FoxO*-deficient mice. There are similar perturbations of the myeloid and lymphoid compartments, associated with development of a myeloproliferative phenotype and T cell lymphomas, although *FoxO*-deficient mice do not develop acute leukemia. Perhaps the most striking similarity, however, is the HSC phenotype in the two models. Deficiency of Pten or FoxO results in enhanced cycling of HSCs and impaired multilineage reconstitution, with consequent depletion of the stem cell pool. In *FoxO*-deficient mice, the HSC phenotype is mechanistically linked to increased levels of ROS. However, it is not known to what extent ROS contributes to the HSC phenotype in *Pten*-deficient mice.

Pten inactivation results in AKT activation, and activated AKT phosphorylates and inactivates FoxO. Thus, one possible explanation for the similarity of the *Pten*- and *FoxO*-deficient HSC phenotypes is that the Pten deficiency is attributable to loss of function of FoxOs in this context. However, treatment of *Pten*-deficient animals with rapamycin, a specific inhibitor of mTOR, restores normal HSC function. This finding taken at face value would suggest that mTOR activation in *Pten*-deficient mice is the primary basis for the observed HSC phenotype. These data are also puzzling in light of literature suggesting that rapamycin inhibition of mTOR signaling results in feedback activation of AKT that would be predicted to inactivate FoxO function (Harrington et al., 2004). These data thus present another interesting paradox that might be explained by off-target effects of rapamycin or by as yet unidentified pathways activated by mTOR that influence FoxO function.

mTOR (mammalian target of rapamycin) is a serine/threonine kinase that acts as the catalytic subunit of two structurally and functionally distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (reviewed in Bhaskar and Hay, 2007). mTORC1 contains the core components mTOR and mLST8/GβL in a complex with raptor, regulates ribosomal biogenesis and protein synthesis, and is negatively regulated by the TSC1/TSC2 (*t*uberous *sc*lerosis) complex. AKT activates mTORC1 in part by direct phosphorylation and by phosphorylation and inactivation of TSC1/TSC2. mTORC2 has the same core components complexed with rictor and SIN1, but in contrast, mTORC2 activates AKT by phosphorylation of residue Ser473. The mechanism(s) of regulation of mTORC2 activity is not well understood, although insulin and growth factor stimulation have been shown to result in mTORC2 activation.

An attractive explanation that unites the phenotypes of *FoxO*- and *Pten*-deficient HSCs derives from a recent report (Sarbassov et al., 2006) demonstrating a previously unrecognized mechanism of action of rapamycin. Rapamycin, in addition to inhibition of the mTORC1 complex, may also inhibit the assembly and subsequently the function of the mTORC2 complex in a tissue-specific manner, including hematopoietic cells. In contrast to inhibition of mTORC1 that results in feedback activation of AKT, inhibition of mTORC2 results in loss of phosphorylation and inactivation of AKT and is correlated with increased FoxO activity. Genetic strategies that result in loss of function of the mTORC2 complex provide further support for this observation. For example, genetic deletion of the rictor component of mTORC2 results in hemiphosphorylation of AKT in which Ser473 is not phosphorylated. Hemiphosphorylated AKT lacks the ability to phosphorylate and thereby inactivate FoxO, but there is no apparent effect on other AKT targets (Guertin et al., 2006). These data provide further evidence that functional mTORC2 complex is essential for AKT-mediated inactivation of FoxO. In addition, these findings are in consonance with the observation that inactivation of the mTORC2 by prolonged rapamycin treatment results in a context-dependent reactivation of FoxO, including hematopoietic and AML cell lines (Sarbassov et al., 2006; Zeng et al., 2007). Therefore, it is possible that restoration of the *Pten*-deficient HSC phenotype with rapamycin may be attributed to reactivation of FoxO, and this hypothesis warrants further investigation.

Role of FoxOs in Other Embryonic and Somatic Stem Cell Compartments

It is of interest to relate the effects of *FoxO* deficiency in the hematopoietic system to other tissue contexts. Studies of *FoxO3* germline knockout animals have shown that isolated *FoxO3* deficiency results in oocyte exhaustion and infertility due to global activation of the primordial ovarian follicle (Castrillon et al., 2003). These phenotypic attributes in germ cells are similar to those observed in HSCs and suggest that FoxOs may play an important role in the maintenance and integrity of stem cell compartments in a broad spectrum of tissues. Studies of the role of FoxOs in the maintenance of other tissue stem cell compartments will be facilitated by the availability of the triple *FoxO* conditional knockout mouse model that can be crossed to different tissue-specific Cre recombinase mouse strains. In addition, the similarities between the role of FoxOs in regulation of quiescence and longevity of HSCs in vertebrates, and regulation of dauer diapause and life span determination in *C. elegans* by the FoxO ortholog DAF-16, further highlight the evolutionarily conserved role of FoxOs in regulation of longevity.

FoxO and Cancer

FoxOs as Tumor Suppressors

FoxO inactivation is a frequent event in cancer development. Constitutive activation of the PI3K/AKT pathway with concomitant inactivation of FoxO is a hallmark of

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many human cancers, including leukemia, breast cancer, glioblastoma and prostate cancer (reviewed in Altomare and Testa, 2005). FoxOs are thus attractive candidates as tumor suppressors in this context, given their role in regulation of cell-cycle arrest, apoptosis, DNA repair, and stress resistance.

FoxO transcription factors were initially identified in humans by virtue of their involvement in chromosomal translocation breakpoints in human tumors. These include t(2;13)(q35;q14) or t(1;13)(p36;q14) that result in expression of a *Pax3-FoxO1* or *Pax7-FoxO1* fusion gene, respectively, in alveolar rhabdomyosarcoma (Anderson et al., 1998; Davis et al., 1994; Galili et al., 1993). In addition, acute myeloid leukemias (AMLs) are associated with t(6;11)(q21;q23) or t(X;11)(q13;q23) that result in expression of *MLL-FoxO3* and *MLL-FoxO4* fusion genes, respectively (Borkhardt et al., 1997; Hillion et al., 1997; Parry et al., 1994).

A more general role of FoxOs in tumorigenicity stems from their function as negative regulators of cell proliferation and cell survival. Thus, inactivation of FoxOs, either by means of nuclear exclusion or posttranslational modifications, is a frequent event in multiple cancers, such as breast or prostate cancer (Hu et al., 2004; Modur et al., 2002). Moreover, FoxOs are thought to interact with a number of tumor suppressors and oncogenes, such as p53, SMAD, and β-catenin (Essers et al., 2005; Nemoto et al., 2004; Seoane et al., 2004; You and Mak, 2005). Despite these data providing strong support for a role for loss of function of FoxO family members in cancer, analysis of single *FoxO* knockout models has identified a subtle cancer proclivity only in *FoxO3* germline knockout animals (Paik et al., 2007).

Recent development of a mouse model engineered with conditional knockout alleles of *FoxO1*, *FoxO3*, and *FoxO4* provides direct evidence that FoxO transcription factors are bona fide, functionally redundant tumor suppressors in that the loss of these FoxO family members results in development of thymic lymphomas and widespread hemangiomas (Paik et al., 2007). The tumor spectrum observed in these mice is of a narrower range than might have been anticipated based on widespread expression and physiologic importance of FoxOs in various tissues. This may be due in part to the observation that use of Mx-Cre restricts excision to certain tissue compartments, and it will be of interest to assess loss of FoxO function on tumorigenesis in other contexts. Nonetheless, excision was documented in a number of tissues in this model system in which tumors did not develop, indicating that there is context-dependent tumor suppression mediated by FoxO (Paik et al., 2007).

FoxOs and Malignant Stem Cells

Cancer is a disorder of self-renewal, and yet the stem cell defects in *FoxO*-deficient HSCs are somewhat at odds with the documented role of FoxOs as a tumor suppressor. However, in both the myeloproliferative phenotype and in the T cell lymphoma of *FoxO1/3/4* knockout animals, the available evidence suggests that self-renewal is restored to the *FoxO*-deficient cells that would be expected to lack such potential. For example, the T cell lymphoma is readily transplantable to secondary recipients and in both the T cell and myeloproliferative phenotypes there is complete excision of all three *FoxO* alleles (Paik et al., 2007; Tothova et al., 2007). The apparent restoration of self-renewal potential in the context of FoxO deficiency is likely related to the acquisition of secondary mutations. Although the mechanism for potentiation of second mutations is not known, it is possible that oxidative damage in the absence of FoxOs is a contributing factor.

Many cancers, including leukemia, seem to depend on a small population of cancer stem cells for their continued growth and propagation (reviewed in Huntly and Gilliland, 2004). The existence of leukemia stem cells (LSCs) was first demonstrated in the context of acute myeloid leukemias by Dick and colleagues (Bonnet and Dick, 1997). LSCs comprise only a small fraction of leukemia cells that have limitless self-renewal capacity and provide long-term growth potential of the tumor. In light of the cancer stem cell theory, it is possible that the second hit mutations in *FoxO*-deficient mice may serve a role to compensate for the self-renewal defects that are present in the HSC compartment as a result of FoxO loss. This could be accomplished by mutations that result in restoration of normal levels of ROS in the leukemic stem cell compartment and/or activation of physiologic mechanisms that render leukemic stem cells less susceptible to oxidative damage. Examples could include mutations that result in increased expression of ROS scavenging enzymes or those that result in downregulation of p16 that would be predicted to dissociate the detrimental effects of ROS on self-renewal of the HSC compartment.

The finding that FoxOs play a key role in the maintenance of normal ROS levels in HSCs could have significant therapeutic implications in the realm of malignant hematopoiesis. Observations in the context of normal hematopoietic stem cells suggest the possibility that the self-renewal potential of leukemic stem cells might be sensitive to high levels of reactive oxygen. If so, this liability may be an Achilles heel for a cancer stem cell and might be exploited therapeutically (Huang et al., 2000). Indeed, there is evidence suggesting that treatment of human AML stem and progenitor cells with parthenolide, a naturally occurring molecule that can induce ROS, preferentially targets AML cells and induces robust apoptosis (Guzman et al., 2005). Most, if not all, myeloid leukemias, and indeed most solid tumors, are characterized by constitutive activation of the PI3K/AKT pathway with resultant FoxO deficiency. Based on recent findings related to *FoxO*-deficient and normal HSCs, it is tempting to speculate that leukemia stem cells—or other cancer stem cells—exert significant cellular homeostatic mechanisms to manage the obligate increase in ROS associated with loss of function of FoxO family members. It is thus plausible that oxidative agents that would have minimal effects on normal hematopoietic constituents might be lethal for leukemia stem cells that must manage the increase in ROS that is associated with constitutive activation of the PI3K/AKT/FoxO axis

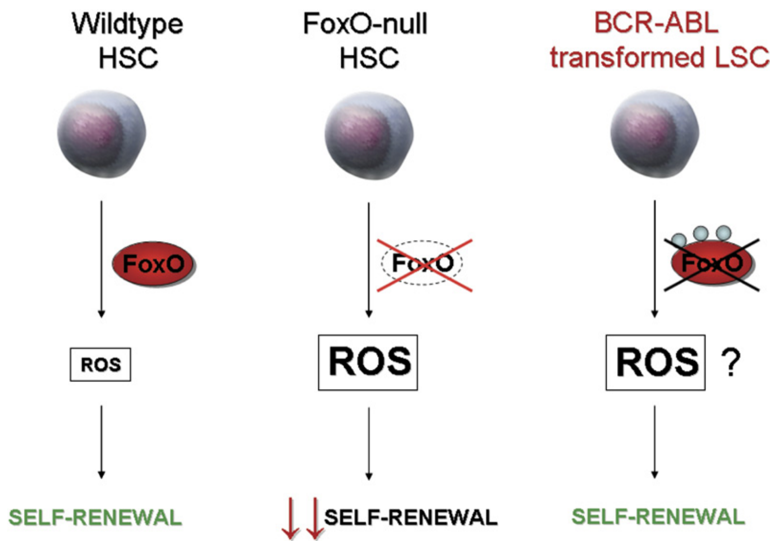

Figure 5. FoxOs in Normal versus Malignant HSCs
Genetic loss of FoxOs in the HSC compartment results in increased levels of ROS and loss of self-renewal of HSCs. Most, if not all, myeloid leukemias, including those mediated by BCR/ABL, are characterized by constitutive activation of the PI3K/AKT pathway with resultant posttranslational FoxO inactivation. It remains to be determined whether leukemic stem cells have increased levels of ROS and what mechanisms render their unlimited self-renewal potential in spite of predicted high levels of ROS.

(Figure 5). This concept is now emerging within other pathways, such as the RAS-RAF-MEK pathway as well, where tumor cells harboring activating mutations in the RAS-RAF-MEK pathway can be selectively killed with ligands binding directly to mitochondrial voltage-dependent anion channels (Yagoda et al., 2007).

It is also interesting to speculate that agents that directly or indirectly inhibit the PI3K/AKT pathway in cancer might paradoxically protect the leukemic stem cell population. For example, imatinib treatment of BCR-ABL-positive CML may result in molecular remission but does not cure the disease due to lack of eradication of a quiescent BCR-ABL-positive stem cell population. It is possible that, in the leukemia stem cell compartment, inhibition of PI3K/AKT signaling by inhibiting BCR-ABL activity would result in reactivation of FoxOs and induction of quiescence and stress resistance in the leukemia stem cell population. This could potentially explain the persistence of this population of cells in the presence of therapeutic and pharmacologically active concentrations of imatinib.

Therapeutic Targeting of FoxOs in Regenerative and Cancer Medicine

FoxOs are key regulators of HSC integrity, and depletion of the HSC compartment upon loss of FoxOs shares certain phenotypic attributes of aging. These observations suggest the intriguing possibility that therapeutic activation of FoxOs could result in lifespan prolongation of HSC or other tissue stem cells, which in turn could be beneficial in the context of bone marrow transplantation or bone marrow failure syndromes. There are several potential ways by which FoxOs could be targeted for activation. These strategies might include development of specific inhibitors of mTORC2 complex formation or function,

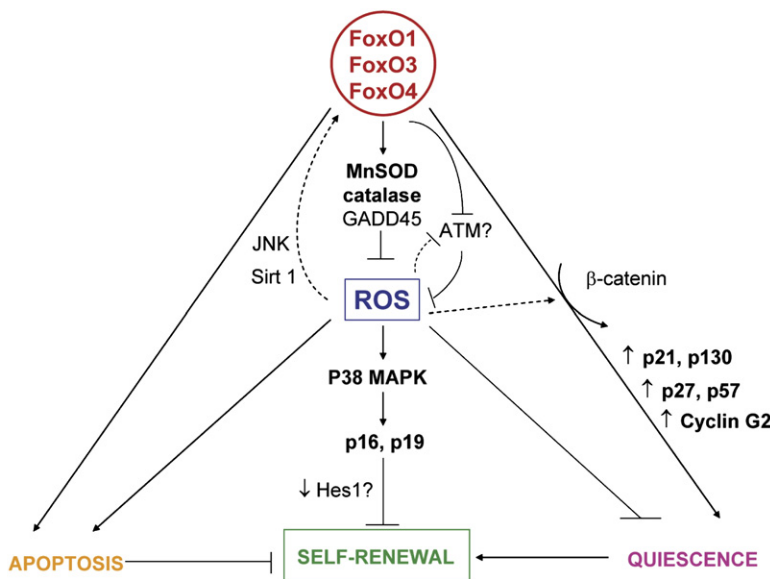

Figure 6. Proposed Model of FoxO Function in the Maintenance of the Integrity of HSC Compartment
FoxOs maintain quiescence, enhance survival, and mediate resistance to physiologic oxidative stress and thereby maintain self-renewal of the HSC compartment. (All of the target genes in bold have been directly investigated in studies of FoxOs in HSCs to date, all of the target genes in regular font have been linked to FoxO function in previous studies, and all of the target genes with a question mark remain to be investigated as downstream targets of FoxOs that contribute to the maintenance of HSC self-renewal.)

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therapeutic agents that prevent shuttling of FoxOs out of the nucleus (Kau et al., 2003), or agents affecting the phosphorylation, acetylation, and ubiquitination status of these proteins.

More selective inhibition of the PI3K/AKT pathway targeting FoxO reactivation could potentially be accomplished by use of selective inhibitors of Akt phosphorylation on Ser473. Recent data demonstrated that mouse embryo fibroblasts (MEFs) derived from rictor null mice that lack a functional mTORC2 complex do not phosphorylate AKT on Ser473, and the sole functional consequence of rictor deficiency in the context of PI3K/AKT signaling is the inability of hemiphosphorylated AKT to phosphorylate and inactivate FoxO (Guertin et al., 2006). Therefore, inhibitors specifically targeting the mTORC2 complex could be of potential clinical use by enabling selective reactivation of FoxO.

Putting It All Together: A Model of FoxO Function in the HSC Compartment

FoxO transcription factors play a critical role in hematopoietic homeostasis by regulating the HSC compartment. Experimental evidence thus far suggests that, under homeostatic conditions, FoxO transcription factors maintain self-renewal of hematopoietic stem cells, and is consistent with the hypothesis that FoxOs cooperate to affect quiescence of HSCs by regulation of mediators of the G0-G1 and G1-S arrest in this compartment, including *Rb/p130*, *Cyclin G2*, *p27*, *p57*, *p21*, and *Cyclin D2*, and prevent aberrant entry into cycle. Second, FoxOs collectively act to inhibit apoptosis in the HSC compartment and therefore contribute to the maintenance of the stem cell pool size. Third, current evidence is consistent with a critical role of FoxOs in mediating resistance to physiologic oxidative stress in the HSC compartment through known downstream targets of FoxO, including *MnSOD*, *catalase*, *ATM*, and *p16*, and thereby potentiate properties of self-renewal. A proposed model includes a positive feedback loop between ROS and FoxO activation, because it has been well established that phosphorylation of FoxOs by JNK or deacetylation of FoxOs by SIRT1 results in nuclear localization and activation of FoxO. Furthermore, increased levels of ROS result in activation of p38 MAPK, at least in the context of *FoxO3* germline deficiency, with a subsequent increase in p16 expression, which may inhibit self-renewal by downregulation of Hes 1. Finally, oxidative stress-enhanced binding of FoxOs to β-catenin may counteract the deleterious actions of ROS on HSC self-renewal. A speculative but attractive hypothesis is that FoxOs could also potentiate β-catenin's ability to engage self-renewal programs (Figure 6).

Thus, studies of FoxOs in mammals have several important implications. They suggest a central role for management of reactive oxygen in hematopoietic stem cell homeostasis. These studies further present the interesting conundrum of the requirement for low ROS for HSC integrity but an absolute requirement for a marked increase in ROS during hematopoietic development. Furthermore, these studies suggest a role for targeting ROS in treatment of cancer and suggest that FoxOs may be useful targets for enhancing stem cell longevity and perhaps in tissue regeneration. Thus, cumulatively, analysis of FoxO function across species has informed not only our understanding of stem cell function and integrity but also has suggested important potential therapeutic applications.

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