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FOXO1: A potential target for human diseases

Huarui Lu[1] and **Haojie Huang**[1,2,*]

[1]Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA

[2]Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455, USA

Abstract

The forkhead box O (FoxO) transcription factors are known to be involved in many physiological and pathological processes including apoptosis, cell cycle arrest, stress resistance, glucose metabolism, cellular differentiation and development, and tumor suppression. The environmental cues, such as growth factors, nutrients, oxidative stress and irradiation, can either positively or negatively modulate FoxO proteins' activities, thereby ensuring distinctive transcription programs in the cell. The potent activities of FoxOs are tightly controlled by multiple mechanisms, which include posttranslational modification such as phosphorylation, acetylation, methylation and ubiquination, subcellular localization, and direct protein-protein interaction. Mounting evidence suggests that the human FOXO1 protein, a founding member of the FoxO family is likely involved in carcinogenesis, diabetes and other human diseases. Here we give an overview of most recent findings regarding the regulation and function of FoxO1, its potential role in human diseases and useful animal models for functional studies on FoxO1. Prospective ways in which the discoveries from the basic research of FoxO1 can be utilized for drug targeting and development of novel therapeutics for human diseases are also discussed.

Keywords

FoxO transcription factors; posttranslational modification; apoptosis; the cell cycle; glucose metabolism; cancer; diabetes; muscle atrophy

Introduction

FOXO1 (also known as FKHR - forkhead in rhabdomyosarcoma) belongs to the forkhead box O-class (FoxO) subfamily of the forkhead transcription factors. Other members in this family in humans include FOXO3a, FOXO4 and FOXO6. FOXO1 was initially identified by its fusion with PAX3 (paired box 3) and PAX7 (paired box 7) genes through t(2; 13) (q35; q14) and t(1; 13) (p36; q14) chromosomal translocations, respectively, in human alveolar rhabdomyosarcomas (ARMS) [1, 2]. As shown in Figure 1, the FOXO1 protein consists of four major functional domains that influence the activity of FOXO1: a highly conserved forkhead (FKH) domain (also called DNA binding domain (DBD)), a nuclear

*Correspondence should be addressed to: Haojie Huang, Ph.D., University of Minnesota, MMC 806, 420 Delaware Street S.E., Minneapolis, MN 55455, USA, Phone: +1 612 624 3306; Fax: +1 612 624 9448, huang253@umn.edu.

localization signal (NLS) located in the COOH-terminal basic region of the DBD, a nuclear export sequence (NES) located downstream of the DBD and a COOH-terminal transcription activation domain (TAD). A salient structural feature of forkhead box transcription factors is that proteins in this family inevitably possess a highly conserved 100-residue FKH domain [3]. Structural studies of forkhead proteins began with the solution of the co-crystal structure of the complex of FoxA3 (HNF-3γ) DBD with DNA [4]. The crystallographic analysis reveals that the FKH domain contains three major α-helices and two major wing-like loops [4]. Therefore, proteins in this family are often called winged helix transcription factors. Because of sequence differences in their FKH domains, FoxO proteins form the most divergent subfamily of the forkhead transcription factors. Recently, the three-dimensional structures of the DNA-bound DBDs of FOXO1, FOXO3a and FOXO4 have been solved, which exhibits that FoxO proteins share similar, with some variation, secondary structure content and topological arrangement with FoxA3 [5–7]. Consistent with the *in vitro* finding that conversion of the histidine 215 residue to arginine (H215R) abolishes the binding of FOXO1 to the canonical forkhead response element (FRE) [8, 9], crystal structure analysis of the FOXO1 DBD-DNA complex demonstrates that histidine 215 makes the direct contact with a specific base of DNA [7].

All FoxO proteins contain a nuclear localization signal (NLS). The NLS consists of three arginine residues present in the COOH-terminal end of the FKH domain, and three lysine residues located 19 residues (in case of FOXO1) downstream of the described arginine residues [10, 11]. Since the arginine residues in the NLS motif overlap with the RXRXXS/T Akt phosphorylation motif, phosphorylation of the serine residue in the Akt phosphorylation consensus site influences the function of NLS. Recently, a second functional NLS has been described in the N-terminus of the FOXO1 protein [12]. FoxO transcription factors also contain a leucine-rich region that can function as a nuclear exportation signal (NES) [10, 13, 14]. The NES motif was originally identified by subcellular localization experiments when FOXO1 deletion mutants were fused to green fluorescent protein [13]. These experiments reveal that loss of the putative NES results in impaired nuclear export of FOXO1. Also, a functional LXLL motif ($L_{461}KEL_{464}L_{465}$), which is required for the interaction of transcription factors with transcription co-regulators, has been defined in the FOXO1 protein [15]. Taken together, mounting evidence indicates that these domain structures of FoxO proteins not only provide a molecular basis for their functions in modulation of gene transcription, but also are frequently targeted for regulation by various signaling pathways.

Regulation and function of FOXO1

Regulation by upstream signaling pathways

The FoxO1 transcription factor plays important roles in various cellular functions including proliferation, differentiation, cell survival, glucose metabolism, longevity and oxidative stress resistance [16]. Human FOXO1, as well as the other members of the FOXO family are the downstream substrates of the PI3K-Akt pathway [8, 13, 17–20]. Studies in *C. elegans* demonstrate that mutations in the insulin receptor gene DAF-2 result in increased life span, which is dampened by mutations in the DAF-16 gene, a *C. elegans* ortholog of FOXO factors [21, 22]. Further studies show that all mammalian FoxO proteins except FoxO6

contain three highly conserved putative Akt phosphorylation consensus sites [8, 13, 17–19]. Akt phosphorylates FoxO proteins *in vitro* and *in vivo*, resulting in their nuclear exclusion and abrogation of their cellular functions. Other than the PI3K-Akt pathway, human FOXO proteins can also be phosphorylated and inactivated by serum and glucocorticoid-inducible kinase (SGK) [23], the dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK) [24], IκB kinase (IKK) [25], cyclin-dependent kinases 1 (CDK1) and 2 (CDK2) [26, 27], extracellular signal-regulated kinase (ERK) [28], and p38 mitogen-activated protein kinase [29]. Thus, phosphorylation stands at the central stage for regulation of mammalian FoxO1 functions. Recent studies reveal that other regulatory mechanisms exist in addition to phosphorylation. For example, acetylation/deacetylation of human and mouse FoxO proteins provides another layer of regulation. The transcription coactivators CREB-binding protein (CBP), p300 and their associated proteins, such as p300- and CBP-associated factor (PCAF), possess intrinsic histone acetyl-transferase (HAT) activity [30, 31]. These proteins directly acetylate transcription factors through their transcription factor acetyl-transferase (FAT) activities [32]. CBP interacts directly and acetylates mouse FoxO1 *in vitro* and *in vivo*, thereby dampening the transcription activity of FoxO1 [33]. This effect is reversed by SIRT1, a human ortholog of the NAD-dependent protein deacetylase silent information regulator 2 (Sir2) in yeast. SIRT1 binds to and deacetylates the mouse FoxO1 at lysine residues K242, K245, and K262 under oxidative stress conditions [33, 34]. These modifications antagonize CBP-induced acetylation and inhibition of mouse FoxO1 [33, 34]. A most recent finding shows that reactive oxygen species induce the formation of cysteine-thiol disulfide-dependent complexes of FoxO and CBP/p300 and that modulation of FoxO activity by CBP/p300-mediated acetylation is dependent on the formation of the redox-dependent complex [35]. FOXO proteins are also regulated by the ubiquitination proteasome system. Insulin treatment decreases the protein level of FOXO1 in human HepG2 cells through polyubiquitination [36]. Significantly, mutations in the three Akt recognition sites prevent polyubiquitination of human and mouse FoxO1 proteins *in vivo* and *in vitro* [37]. Similarly, treatment of chicken [36] embryonic fibroblasts with platelet-derived growth factor (PDGF) resulted in a decrease in the level of the FoxO1 protein and this effect was inhibited by the proteasome inhibitor lactacystin or PI3K inhibitor LY294002 [38]. This suggests that Akt-mediated phosphorylation is required for the ubiquitination and subsequent proteasome degradation of mammalian FoxO1 [36]. Moreover, the F-box protein Skp2, a substrate-binding component of the Skp1/Cullin1/F-box protein (SCF) complex E3 ligase, interacts with and promotes the polyubiquitination of FOXO1. Of the three Akt phosphorylation sites, phosphorylation of serine 256 is required for Skp2-mediated ubiquitination of human FOXO1 [37]. More recently, Yamagata et al. reported that protein arginine methyltransferase-1 (PRMT1) methylates FOXO1 at conserved arginine residues within a Akt phosphorylation consensus motif and blocks Akt-mediated phosphorylation and inhibition of FOXO1 *in vitro* and *in vivo*, thereby representing another regulatory mechanism for FOXOs [39].

Over recent years it has become evident that FoxO factors are insulin-sensitive transcription factors with an array of downstream targets and interacting partners identified. Central to insulin-mediated inhibition of FoxOs is a shuttling mechanism that regulates subcellular localization of FoxO proteins, thereby regulating their transcriptional function. The transport

of FoxO proteins through the nuclear pore is dependent on active-transport mechanisms, which usually requires adaptor proteins called importin or exportin receptors that mediate either import or export, respectively. Importins and exportins recognize specific NLSs and NESs present in the transported proteins. As discussed above, FoxO proteins possess both NLS and NES motifs. The functions of NLS and NES are affected by posttranscriptional modifications, particularly phosphorylation and monoubiquitination [10, 11, 40, 41]. For instance, phosphorylation of FoxO proteins by various protein kinases, including Akt, SGK, DYRK1A, and CDK2 promotes export of these proteins to the cytoplasm, where FoxO proteins are sequestered by chaperone proteins 14-3-3. Interestingly, other protein kinases, such as JNK, can phosphorylate FOXO proteins, thereby triggering the relocalization of FoxO proteins from the cytoplasm to the nucleus in response to oxidative stress stimuli [42, 43].

Apoptosis

The observation that Akt phosphorylates and inhibits the transcriptional activity of FoxO proteins suggests that these proteins may play roles in apoptosis. Forced expression of a constitutively active form of FOXO1 triggers apoptosis in certain types of cancer cells, such as human LNCaP prostate cancer cells [44–47]. FOXO1-induced apoptosis in LNCaP cells can be inhibited by treatment of cells with the synthetic androgen R1881 [44]. An unbiased gene profiling study using adenovirus-mediated overexpression of FOXO1 revealed that tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL), a death receptor ligand, is regulated by FOXO1 in the human prostate cancer cell line LAPC4, which is responsible for FOXO1-induced apoptosis [46]. FoxOs also promote apoptotic cell death by transcriptionally upregulating Fas ligand (FasL) [17]. In addition to the death receptor ligands, mammalian FoxO1 is involved in the transactivation of Bim, a proapoptotic member of the Bcl-2 family, which functions in the intrinsic mitochondrial apoptotic pathway [48, 49]. It has been shown that CDK-mediated phosphorylation of FOXO1 at serine 249 plays an important role in regulation of genotoxic stress-induced cell death [26]. Silencing of human FOXO1 by small interfering RNA (siRNA) diminished DNA damage-induced cell death in both p53-deficient and -proficient cells [26]. Following treatment of cells with the CDK inhibitor roscovitine, expression of Bim was increased, accompanied by the increased cleavage of Poly (ADP-ribose) polymerase (PPAR), a marker of apoptosis [26]. These findings indicate that mammalian FoxO1 plays an important role in apoptosis through up-regulation of proapoptotic genes.

Cell cycle regulation

While in some types of cells FOXO1 plays a role in apoptosis, FOXO1 activation also regulates cell cycle progression. Activation of FOXO1 increases the transcription and half-life of cyclin-dependent kinase inhibitor $p27^{KIP1}$ [47, 50]. Another study reports that FoxO1 affects cell cycle progression via regulation of the nuclear localization of $p27^{KIP1}$ in porcine granulosa cells [51]. Suppression of the D-type cyclins (cyclin D1 and D2) has also been linked to mammalian FoxO1-mediated cell cycle arrest [9, 52]. Transcriptional profiling experiments reveal that expression of a constitutive active form of human FOXO1 suppresses expression of cyclin D1 and D2 [9]. Chromatin immunoprecipitation assays show that human FOXO1 is associated with the cyclin D1 promoter [9]. A mutant form of

human FOXO1 (H215R), which cannot bind to the canonical FRE to induce expression of $p27^{KIP1}$, still suppresses the promoter activity of cyclin D1 and D2 and induces cell cycle arrest at G1 [9], although the underlying mechanism is not fully understood. Nonetheless, activation of mammalian FoxO1 arrests the cell-division cycle at G1 through either stimulating or repressing gene transcription.

Glucose metabolism

Studies in *Drosophila* have shown that the dFOXO protein is involved in the regulation of metabolism [53]. Cell culture studies in mammals confirm that FoxO1-induced transcription of gluconeogenic genes, such as G6Pase [54, 55] and PEPCK [56], contributes to insulin-regulated metabolism. Transactivation of these genes by FoxO1 depends on its interaction with the transcription coactivator PGC-1α. PGC-1α interacts with mouse Foxo1 within the DNA binding domain and functions in concert with FoxO1 in stimulating gluconeogenesis in the liver [57]. Interaction with coactivators including CBP is also important for the ability of *C. elegans* DAF-16 and its human FOXO homologues to function cooperatively with other transcription factors, including glucocorticoid receptor [58]. Disruption of this cooperation may contribute to the inhibitory effect of insulin on glucocorticoid-induced expression of genes including IGFBP-1, G6Pase and PEPECK. Moreover, the interaction of mammalian FoxO1 with peroxisome proliferator-activated receptor-γ (PPARγ) [59] and hepatocyte nuclear factor-4 (HNF-4) [60] is of great interest in the context of insulin-regulated gene expression. HNF-4 plays an important role in the regulation of glucose and lipid metabolism in the human liver and these effects are mediated, at least in part, by the interaction of HNF-4 with FOXO1 [60].

Muscle growth and differentiation

FoxO1 has also been implicated in regulating differentiation in muscle cells [61]. Studies with transgenic mice supported the concept that mouse FoxO1 regulates muscle mass and indicated that FoxO1 is important in determining the formation of type 1 versus type 2 muscle fibers [62]. Interestingly, mouse FoxO1 and FoxO3a protein levels increase in muscle during fasting and insulin deficiency [63], and it would be interesting to determine whether the increased levels of these proteins are related to Akt-mediated degradation [64]. FoxO1 stimulates the expression of both pyruvate dehydrogenase kinase 4 (PDK4) [63, 65], which limits the flux of pyruvate through the Krebs cycle. FoxO1 also regulates the expression of lipoprotein lipase (LPL) [62], promoting the ability of muscle cells to utilize fatty acids for metabolism. Cell culture studies indicate that mammalian FoxO1 proteins can stimulate fatty acid uptake and oxidation in skeletal muscle cells, by promoting the translocation of CD36 (a fatty acid transfer protein) to the cytoplasmic membrane and affecting the expression of genes involved in the regulation of microsomal and mitochondrial oxidative pathways [66]. Thus, mammalian FoxO1 proteins play an important role in muscle growth and differentiation by regulating glucose and lipid metabolisms in muscle cells.

In addition to the function of FoxO1 as a transcriptional factor, increasing evidence suggests that FoxO1 also possess transcription-independent functions by directly interacting with and regulating the activities of many other transcription factors, which include the androgen

receptor, estrogen receptor, signaling transducer and activator of transcription-3 (STAT3), among others [45, 67–73].

Implication of FOXO1 in diseases

As outlined above, the FoxO1 protein acts as a multifunctional protein in very diversified settings. It is not surprising that this molecule has been implicated in many diseases (Table 1).

FOXO1 in carcinogenesis

Since FOXO1 regulates a number of cellular processes such as apoptosis, cell cycle arrest and DNA repair that are highly relevant to cancer, it suggests that FOXO1 functions as a tumor suppressor. Indeed, the human FOXO1 gene was initially identified in the studies of the chromosomal translocations found in pediatric malignant rhabdomyosarcomas [1]. Although the presence of chromosomal translocation involving the FOXO1 gene in human tumors does not establish a causative role of FOXO1 in tumorigenesis, these genetic alterations of FOXO1 suggest a potential function of FOXO1 in cancer development. Because the human PAX3-FOXO1 fusion protein possesses much more potent transcription activity than PAX3, a gain-of-function model has been proposed [74]. However, tumorigenesis is not observed in PAX3-FOXO1 knock-in mice [75, 76]. This has been confirmed by transgenic expression of the chimeric human PAX3-FOXO1 gene [77]. These findings suggest that PAX3-FOXO1 alone is not sufficient to initiate tumor formation. Alternatively, loss of one allele of the FOXO1 gene due to chromosomal rearrangement may predispose cells to deregulated cell cycle control and impaired apoptosis. Surprisingly, there are no wild type FOXO1 proteins detected in human alveolar rhabdomyosarcoma (ARM) cell lines although the mRNA of the wild-type FOXO1 gene is readily detected [1, 78]. Moreover, levels of the F-box protein Skp2 are much higher in human ARM cell lines that carry the t(2; 13) chromosomal translocation than in normal skeletal muscle cells [79]. Forced expression of the PAX3-FKHR fusion protein results in an increase in the levels of Skp2 protein, which has been shown to work in concert with Akt in inducing the proteasome degradation of human FOXO1 protein [37]. Thus, it can be speculated that the chimeric protein from the chromosomal translocation could lead to a complete loss of function of FOXO1 via proteasome-mediated degradation and therefore trigger cell transformation and tumor formation.

The role of FOXO1 in tumorigenesis is further supported by the findings in prostate cancer. The PTEN tumor suppressor gene is commonly mutated or deleted in human prostate cancer. Loss of PTEN results in constitutive activation of Akt, which in turn leads to the phosphorylation and inhibition of human FOXO1. Indeed, expression of cytoplasmic, phosphorylated FOXO1 proteins is correlated with prostate cancer progression [80]. Moreover, hemizygous deletion at the FOXO1 gene locus was detected in approximately 30% of prostate cancer cell lines, xenografts, and primary prostate tumors [72]. These findings suggest that FOXO1 may function as a tumor suppressor in the prostate. In line with this notion, forced expression of a constitutively active form of human FOXO1 that cannot be phosphorylated by Akt induces apoptosis in human prostate cancer cell lines [37, 47]. In contrast, expression of this mutant induces G1 arrest rather than apoptosis in human

PTEN-null renal carcinoma and glioma cells [47], suggesting that FOXO1 may function as a tumor suppressor via different mechanisms in different tissue origins.

FOXO1 in diabetes

The FOXO1 protein regulates cellular metabolism in the liver, muscle, adipose tissue and pancreas. Alterations in FOXO1 function might result in disorders of metabolism, including diabetes. Transgenic approaches have been used to study the function of FoxO1 *in vivo*. Haploinsufficiency of the FoxO1 gene restores insulin sensitivity and rescues the diabetic phenotype in insulin resistant mice by reducing hepatic expression of gluconeogenetic genes and increasing the expression of insulin-sensitive genes in adipocytes [81]. In contrast, transgenic expression of a gain-of-function mutant of mouse FoxO1 in liver and pancreatic β cells results in diabetes, which is accompanied by increased hepatic glucose production and impaired β cell compensation due to decreased expression of the pancreas/duodenum homeobox gene-1 (Pdx1) [82]. The importance of FoxO1 in the regulation of gluconeogenesis is further supported by adenovirus-mediated ectopic expression of a dominant negative form of FoxO1. Mice expressing dominant negative FoxO1 exhibit decreased blood glucose levels and reduced expression of both G6Pase and PEPECK genes [83]. FoxO1 also appears to exert an important role in pancreatic β cells. Haploinsufficiency of FoxO1 reverses β cell failure in insulin receptor substrate-2-null mice [82]. This effect appears to be mediated through partial restoration of β cell proliferation and increased expression of the pancreatic transcription factor Pdx1, a key regulator of β cell development. Moreover, FoxO1-heterozygous mice are resistant to diet-induced diabetes [84]. Although increased FoxO1 activity contributes to the phenotype of insulin-resistance and diabetes in animal models, it is important to note that FoxO1 does not function solely as an insulin antagonist with a pro-diabetic function. For instance, mouse FoxO1 has been shown to stimulate the expression of the adiponectin receptor, which is thought to promote insulin sensitivity [85].

FOXO1 in atrophy

Forced expression of a constitutively active form of mouse FoxO3a causes atrophy of fully differentiated skeletal and cardiac muscle cells by inducing the expression of atrogin-1, an F-box protein component of a muscle-specific ubiquitin E3 ligase complex, subsequent protein degradation, and eventually muscle atrophy [86–88]. Consistent with these observations, transgenic mice with skeletal muscle-specific overexpression of FoxO1 display a decrease in the size of type I and II muscle fibers and a marked reduction in gene expression in type I fibers [62]. Taken together, these data reveal a link between FoxO proteins and muscle atrophy.

Animal models for functional studies on FoxO1

The potent functions of FoxO1 under physiological and pathological conditions are further supported by the findings in studies with animal models (Table 2). FoxO1-null ($FoxO1^{-/-}$) mice die at embryonic day 10.5 due to several developmental defects, including incomplete vascular development in embryos and yolk sacs [89, 90]. $FoxO1^{-/-}$ embryos are approximately 50% the size of their wild-type ($FoxO1^{+/+}$) littermates. Cardiac looping of

FoxO1$^{-/-}$ embryos is retarded and the pericardium is distended compared to FoxO1 wild-type and heterozygous (FoxO1$^{+/-}$) embryos. Furthermore, significant defects in the formation of the vascular system are observed in FoxO1$^{-/-}$ embryos. The dorsal aorta appears thin and disorganized and the intersomitic vessels are irregularly developed in FoxO1$^{-/-}$ embryos. The head vasculature of FoxO1$^{-/-}$ embryos appears to lack properly formed branches of the internal carotid artery. Developed vasculatures are not present in FoxO1$^{-/-}$ yolk sacs either [11]. The observation of highly expressed FoxO1 in a variety of developing embryonic vasculature supports a critical role of this transcription factor in vascular formation [10, 11]. An *in vitro* differentiation system composed of embryonic stem (ES) cells demonstrated that the endothelial cells derived from FoxO1$^{-/-}$ ES cells displayed a remarkably different morphological response to exogenous vascular endothelial growth factor (VEGF) compared with their wild-type counterparts, suggesting that FoxO1 is important in endothelial cell response to angiogenic stimuli including VEGF [10].

As mentioned above, germline homozygous deletion of FoxO1 (FoxO1$^{-/-}$) results in an embryonic lethal phenotype. However, FoxO1 heterozygous mice (FoxO1$^{+/-}$) are viable and exhibit no obvious abnormalities. Heterozygous knockout of the FoxO1 gene partially rescues insulin resistance in animals with haploinsufficiency of the insulin receptor gene and protects against the development of diabetes [81]. This result was achieved through downregulation of glucogenetic gene expression in the liver and concomitant upregulation of insulin-sensitizing genes. FoxO1 haploinsufficiency reversed the characteristic pancreatic β-cell failure, which is thought to be highly related to type 2 Diabetes. These data suggest that FoxO1 is involved in the regulation of β cell proliferation and functions as a negative regulator of insulin sensitivity [82]. Consistent with the finding that FoxO1 becomes constitutively active when the Akt phosphorylation residue serine 253 is converted to the nonphosphorylatable alanine, β cell-specific transgenic expression of this mutant of FoxO1 resulted in mice that developed glucose intolerance, β cell failure and diabetes in an age-dependent manner. These observations provide further support to the notion that FoxO1 is an important downstream regulator of the insulin pathway and therefore FoxO1 could be a premier target for therapeutic intervention of diabetes.

Transgenic mice that specifically overexpress FoxO1 in skeletal muscle have been created [62]. These mice had a reduced skeletal muscle mass and an impaired glycemic control capability after oral glucose and intraperitoneal insulin administration. These results suggest that FoxO1 is a negative regulator of both skeletal muscle mass and function. Activation of FoxO1 may also be involved in the pathogenesis of sarcopenia, the age-related decline in muscle mass in humans, which is often associated with obesity and diabetes. Moreover, mice expressing the Pax3-FoxO1 transgene or various versions of Pax3-FoxO1 mutant alleles are developmentally abnormal but fail to develop tumors [75–77]. In contrast, mice with a complete deletion of FoxO1 by homozygous knock-in of Pax3-FoxO1 develop alveolar rhabdomyosarcoma (ARM) in p53- and ink4a-deficient background [91]. This is the first mouse model of ARM that was generated by conditional expression of Pax3-FoxO1 fusion gene and conditional deletion of Ink4a/Arf and p53 genes in terminally differentiated, Myf6-expressing skeletal muscle. These tumors recapitulate the histological features of human ARMs. More recently, mouse models with conditional deletion of FoxO1, FoxO3a

and FoxO4 individually or together have been developed by using the inducible Mx-Cre transgene [92]. By 60 weeks of age, 100% of FoxO1$^{-/-}$ females displayed mild hemangiomas in the uterus and occasionally in perirenal fat with no abnormalities in other tissues. FoxO1/4$^{-/-}$ mice also displayed mild hemangiomas in the uterus and occasionally in other tissues, and all FoxO1/3a$^{-/-}$ mice displayed vascular abnormalities with similar tissue distribution as FoxO1/3a/4$^{-/-}$ mice. In contrast, vascular abnormalities were not detected in FoxO3a$^{-/-}$ mice up to 21 weeks, germline FoxO3a/4$^{-/-}$ mice up to 2 years, and FoxO1$^{+/-}$, FoxO3a$^{-/-}$ and FoxO4$^{-/-}$ mice up to 40 weeks of age. These genotype-phenotype correlations indicate FoxO1 is the most potent regulator of adult vascular homeostasis, with less, but physiologically important contributions from the other FoxOs. Deletion of all three FoxO genes results in lineage-restricted tumor phenotypes. When Cre-mediated gene disruption of all three FoxO genes was induced at 4~5 weeks of age, the mice developed lymphoblastic thymic lymphomas between 19 and 30 weeks of age with spread to spleen, liver, and lymph nodes. These studies provided evidence that the FoxO genes are tumor suppressors *in vivo*. They also addressed the issue of functional redundancy of FoxO genes, as loss of all three FoxO alleles was required for the malignant phenotype. Disruption of any combination of two of the three FoxO genes resulted in a mild to moderate phenotype. Using the same system of Cre-mediated disruption of all three FoxO genes, but focusing on the hematopoietic system revealed a significant decrease in the long-term hematopoietic stem cell (HSC) population and the common lymphoid progenitor [93]. A significant increase in the number of HSCs exiting G0/G1 and entering G2/M was observed in FoxO-deficient mice, suggesting that FoxOs are important in maintaining the quiescent state of HSCs and preserving their replication and renewable capacity. FoxO-deficient HSCs also exhibit increased levels of apoptosis. Similar to the tumor phenotype described above, deficiency in any one or two FoxO genes in combination does not produce the apoptotic or cell-cycle arrest phenotype, which corroborates the functional redundancy of FoxO proteins.

Thus, the animal models developed and studied to date have revealed much about the multiple roles of FoxO factors at the complex organismal level. However, many questions are unanswered and further investigation is warranted. Uncovering the multifaceted aspects of FoxO regulation and function will provide important insights into the processes involving FoxO proteins including development, differentiation, immunity, proliferation, metabolism and aging.

FOXO1 as a drug targeting

To date, several drug candidates that directly target FOXO1 are known to be in development and some of them has been patented (Table 3). As inactivation of FOXO1 appears to be a crucial step in tumorigenesis, restoring activity of this factor represents a potential effective therapeutic strategy. Increasing evidence demonstrates that many protein kinases like Akt, SGK, DYRK1A, CK1 and CDK1/2 can phosphorylate FOXO1 and exclude FOXO1 from the nucleus. Accordingly, developing compounds that disrupt the phosphorylation of FOXO1 by these kinases could be a potential choice for cancer-related drug design. Moreover, modulation of subcellular localization of FOXO1 could be another possibility. As the FOXO1 protein is constantly exported from the nucleus in a CRM1-dependent manner, nuclear export inhibitors, including CRM1 inhibitors such as leptomycin B and

semisynthesized derivatives of leptomycin B [94], could be used as potential anti-cancer agents. A high throughput, chemical genetic screen for inhibitors of FOXO1 nuclear export has been reported and some compounds specific to the PI3K/Akt/FOXO1 signaling pathway are under further investigation [95]. Recently, a bromotyrosine derivative, psammaplysene A, was identified to compensate for PTEN loss by inducing relocalization of FOXO1 to the nucleus in PTEN-deficient cells [96]. Moreover, a cell-based imaging screen that monitored the translocation of FOXOs as the AKT effector proteins identified pyrazolopyrimidine derivatives as potent inhibitors of PI3K as well as FOXOs relocators. Other small molecule compounds such as D4476 [97] and ETP-45658 [98], have also been shown to be able to influence the intracellular localization and function of FOXO proteins.

In summary, because of its broad spectrum of cellular functions implicated malignant and metabolic diseases, the FOXO1 protein could be targeted for the treatment of these diseases. However, it is worth noting that the precise mechanism as to how FOXO1 controls gene expression in response to a specific condition is largely unknown. In addition, most studies to date are performed *in vitro*. More *in vivo* investigations into whether the FOXO1 protein could be a therapeutic target are warranted. The disease-oriented novel mouse models, in which the FoxO1 gene can be deleted in a tissue-specific manner [91–93], should provide keen insights into the role of FOXO1 in the development of diseases including cancer and diabetes. Small molecule inhibitors that can reactivate FOXO1 activity by specifically blocking the targeting of FOXO1 by the upstream regulators such as Akt, IKK, CDK1, CDK2, and Skp2 may hold the promise for drug targeting.

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Fig. 1.

The schematic diagram of FOXO1 protein domain structure. FKH: conserved forkhead domain; NLS: nuclear localization signal; NES: nuclear export sequence; TAD: transactivation domain.

Table 1

FOXO1-implicated diseases

Target Protein	Disease	Validation Level	References
FOXO1	Rhabdomyosarcoma, Alveolar	Candidate	[1, 2]
FOXO1	Prostatic neoplasm	Candidate	[3, 4]
FOXO1	Diabetes mellitus	Candidate	[5]
FOXO1	Carcinoma, Renal cell	Candidate	[6]
FOXO1	Glioma	Candidate	[7]
FOXO1	Muscular Atrophy	Candidate	[8, 9]
FOXO1	Endometrial neoplasm	Candidate	[10]
FOXO1	Endometriosis	Candidate	[11]
FOXO1	Stomach neoplasm	Candidate	[12]

Table 2

FoxO-targeted mouse models

Target	Model	Disease/phenotype/assay	References
FOXO1	FoxO1-null mice	Embryonic lethal at day 10.5, abnormal angiogenesis in yolk sacs and embryos	[13, 14]
FOXO1	FoxO1 heterozygous mice	Viable and fertile, no obvious abnormalities	[5, 15]
FOXO1	floxed allele of FoxO1	Mild hemangiomas in the uterus and perirenal fat by 60 weeks of age; premature death due to the development of hemangiomas since ~50 weeks of age	[16]
FOXO1	PAX3-FoxO1 transgenic mice	Pigmentry disturbance of abdomen, hindpaws, tail and neurological alteration	[17]
FOXO1	heterozygous knock-in of PAX3-FoxO1	Developmental abnormalities including intraventricular septum defects, tricuspid valve insufficiency and diaphragm defects; Malformation of some hypaxial muscles; died around the time of birth	[18]
FOXO1	Conditional homozygous knock-in of PAX3-FoxO1	Lower frequency of tumor occurrence, but increased frequency of tumors when disruption of ink4a/arf and p53	[19]
FOXO1	Homogenous deletion of FoxO1 in pro-opiomelanocortin (Pomc)-expression neurons	Normal appearance with reduced body weight until ~ 6 weeks, which is associated with reduced food intake	[20]

Table 3

Patent publications related to FOXO1

Number	Assignee name	Brief description
US2008260700	Accili, Domenico; Kitamura, Tadahiro	Established an immortalized pancreatic duct cell derived from a primary adult pancreatic duct epithelial cell culture, wherein the immortalized cell expresses wild type FoxO1 or mutated FoxO1; A method for treating beta cell failure with a subject spontaneously immortalized pancreatic ductal cell line expressing mutated FoxO1
US2008248995	Karnieli, Eddy; Armoni, Michal	A method for detecting a modulator of transcription of a human PPAR gene promoter, wherein said FoxO1 protein binds directly and specifically through its DNA binding domain to a DNA region within the PPAR-gamma 2 promoter. A compound wherein the peptide is derived from the FoxO1 DNA-binding domain and some analog of said peptide.
EP1907016	Cho, Charles; Chanda, Sumit	A method for identifying modulating compounds that affect FoxO1 localization.
US2008153767/WO2004031350	Dobie, Kenneth; Bhanot, Sanjay	A series of antisense compounds, compositions are claimed for modulating the expression of FoxO1, a method for treatment of diseases associated with expression of FoxO1, in particular for treating diabetes