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REVIEW

FOXO transcription factors in cancer development and therapy

Alexandra Coomans de Brachène[1] · Jean-Baptiste Demoulin[1]

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Abstract The forkhead box O (FOXO) transcription factors are considered as tumor suppressors that limit cell proliferation and induce apoptosis. FOXO gene alterations have been described in a limited number of human cancers, such as rhabdomyosarcoma, leukemia and lymphoma. In addition, FOXO proteins are inactivated by major oncogenic signals such as the phosphatidylinositol-3 kinase pathway and MAP kinases. Their expression is also repressed by micro-RNAs in multiple cancer types. FOXOs are mediators of the tumor response to various therapies. However, paradoxical roles of FOXOs in cancer progression were recently described. FOXOs contribute to the maintenance of leukemia-initiating cells in acute and chronic myeloid leukemia. These factors may also promote invasion and metastasis of subsets of colon and breast cancers. Resistance to treatment was also ascribed to FOXO activation in multiple cases, including targeted therapies. In this review, we discuss the complex role of FOXOs in cancer development and response to therapy.

Keywords FOXO1 · FOXO3 · FOXO4 · Cancer stem cells · Tumor-initiating cells · Cell cycle · Cell invasion · Metastasis

✉ Jean-Baptiste Demoulin
JB.Demoulin@uclouvain.be

[1] de Duve Institute, MEXP-UCL 74.30, Université catholique de Louvain, Avenue Hippocrate 75, B1.74.05, 1200 Brussels, Belgium

Introduction

The forkhead box (FOX) family of proteins consists of 19 sub-families of transcription factors that share a highly conserved DNA-binding domain of approximately 110 amino acids, the forkhead box domain (also known as the winged-helix domain). Within this family, the O subgroup contains four members: FOXO1 (FKHR), FOXO3 (FKHRL1), FOXO4 (AFX) and FOXO6 [1]. The first three are ubiquitously expressed, at different levels depending on the tissue [2, 3]. On the contrary, FOXO6 is expressed only in the central nervous system [4]. To determine whether they play distinct or redundant functions, knock-out (KO) mice were produced for the different FOXO family members. *Foxo1*$^{-/-}$ embryo die because of incomplete vascular development; *Foxo3*$^{-/-}$ female mice are infertile due to abnormal ovarian follicle development; whereas *Foxo4*$^{-/-}$ mice do not present any obvious abnormalities [5]. These phenotype differences may be related to functional differences between FOXO isoforms as well as distinct patterns of expression.

The expression and activity of FOXO factors are strongly controlled by post-translational modifications such as phosphorylation, acetylation, methylation and ubiquitination (reviewed in [6]). A major mechanism of regulation of FOXOs consists of phosphorylation by AKT (also called Protein Kinase B, PKB) on three residues (T32, S253 and S315 of FOXO3) following growth factor stimulation [7], leading to FOXO inactivation. Indeed, these phosphorylations allow the binding of 14-3-3 proteins to FOXOs and their export from the nucleus to the cytoplasm (reviewed in [8]). The sequestration of FOXOs in the cytoplasm maintains them in an inactive state, which can be rapidly reversed. In addition, following their nuclear exclusion, FOXOs can also be ubiquitinated and degraded by

proteasomes. The mechanisms that direct FOXOs to degradation rather than sequestration might be related to the intensity of the signal that triggers nuclear export [6]. Unlike FOXO1, 3 and 4, FOXO6 is phosphorylated by AKT on two residues only, which inactivates FOXO6 without inducing its export from the nucleus to the cytosol [4]. Because FOXO6 has a restricted pattern of expression and is regulated differently than the three other isoforms, we will use the general denomination "FOXOs" to refer to FOXO1, 3 and 4.

In addition to AKT, other kinases have been described as negative regulators of FOXOs, such as SGK (serum and glucocorticoid-regulated kinase), CK1 (casein kinase 1), DYRK1A (dual-specificity tyrosine-phosphorylation-regulated kinase 1A) and, more recently, ERK (extracellular signal-regulated kinase) and IKK (IκB kinase) (reviewed in [8]). In contrast, FOXOs can be activated by JNK (c-Jun N-terminal kinase), MST1 (Mammalian Ste20-like kinase) and AMPK (AMP-activated protein kinase). Under oxidative stress conditions, MST1 and JNK phosphorylate FOXOs, in particular FOXO4, and induce its translocation from the cytoplasm to the nucleus. Likewise, in response to nutrient stress, AMPK also phosphorylates and activates FOXOs to induce the expression of genes involved in energy metabolism and stress resistance [9]. In addition to the regulation of FOXOs by post-translational modifications, our laboratory showed that the PI3K-AKT pathway also represses the expression of these factors at the mRNA level [10].

FOXOs control diverse cellular functions, such as cell growth, survival, metabolism and anti-oxidant state, by regulating the expression of many genes (for a comprehensive list of FOXO targets, see [11]). Because of their anti-proliferative and pro-apoptotic functions, FOXO factors have been considered as tumor suppressors. Indeed, their expression and activity are altered in many cancers. However, recent studies have described new and unexpected functions of FOXOs in the resistance to cancer treatment and in the promotion of cancer, suggesting a complex role of FOXO factors in this disease. This will be the topic of the present review.

FOXOs as tumor suppressors

FOXO factors are often considered as tumor suppressors. This makes sense given their cellular functions as cell cycle and apoptosis regulators. This is also supported by the phenotype of FOXO knock-out mice. *Foxo1*$^{+/-}$ *Foxo3*$^{-/-}$ *Foxo4*$^{-/-}$ germline mutant mice as well as compound mutant mice with four or five deleted FOXO alleles present a modest tumor phenotype that emerges very late in life [12]. The conditional deletion of all *Foxo1/3/4* alleles in adult tissues leads to the appearance of lymphoblastic thymic lymphomas and hemangiomas. The restricted tumor spectrum in triple FOXO KO mice (particularly the absence of carcinoma) was surprising and contrasted with the devastating results of PI3K signaling deregulation. The authors suggested that other arms of the PI3K-AKT signaling, such as mTOR, may play more crucial roles in epithelia tumorigenesis [12]. Nevertheless, this should not overshadow the importance of FOXOs in other cell lineages.

In mice, the tumor suppressor activity of FOXOs is visible only after inactivation of four to six alleles. This is unlikely to occur frequently in human tumors, perhaps explaining the rarity of genetic alterations inactivating FOXO loci in human cancers. However, as discussed below, cancer cells use a more efficient way of inactivating FOXOs at the protein and mRNA levels via different oncogenic signaling pathways and micro-RNAs.

An oncogenic signaling network controls FOXO activity

Some major signaling pathways, such as those involving PI3K, Ras or IKK, have been linked to FOXOs in the context of cancer (Fig. 1).

The PI3K-AKT pathway

As already mentioned above, FOXOs are targeted and inactivated by the PI3K-AKT pathway [7]. Yet, this signaling pathway is often constitutively active in cancers due to gain-of-function mutations in genes encoding tyrosine kinases, RAS or PI3K itself, or due to loss-of-function mutations of PTEN, for instance (reviewed in [13]). In these cancers, FOXOs are expected to be in the inactive cytosolic phosphorylated state, thus promoting cell survival and proliferation. This was indeed demonstrated in a number of studies. For instance, transformation of pre-B lymphocytes with BCR-ABL requires the PI3K-AKT pathway and, in particular, the suppression of FOXO3-induced apoptosis [14]. Likewise, the expression of FLT3-ITD, a mutant receptor that is commonly found in acute myeloid leukemia, leads to the activation of the PI3K-AKT pathway with subsequent FOXO3 phosphorylation and inactivation in transfected Ba/F3 cells. By doing so, FLT3-ITD represses FOXO3-induced expression of p27^{KIP1} (CDKN1B) and Bim (BCL2L11) and maintains cell proliferation and survival [15]. The PI3K pathway is also often deregulated and activated in thyroid, cervical and breast cancers. In breast cancer cell lines, the targeted depletion of PI3K using small-interfering RNA (siRNA) reactivates FOXO1, 3 and 4, which induce a cell cycle arrest and apoptosis [16]. The same anti-proliferative and pro-

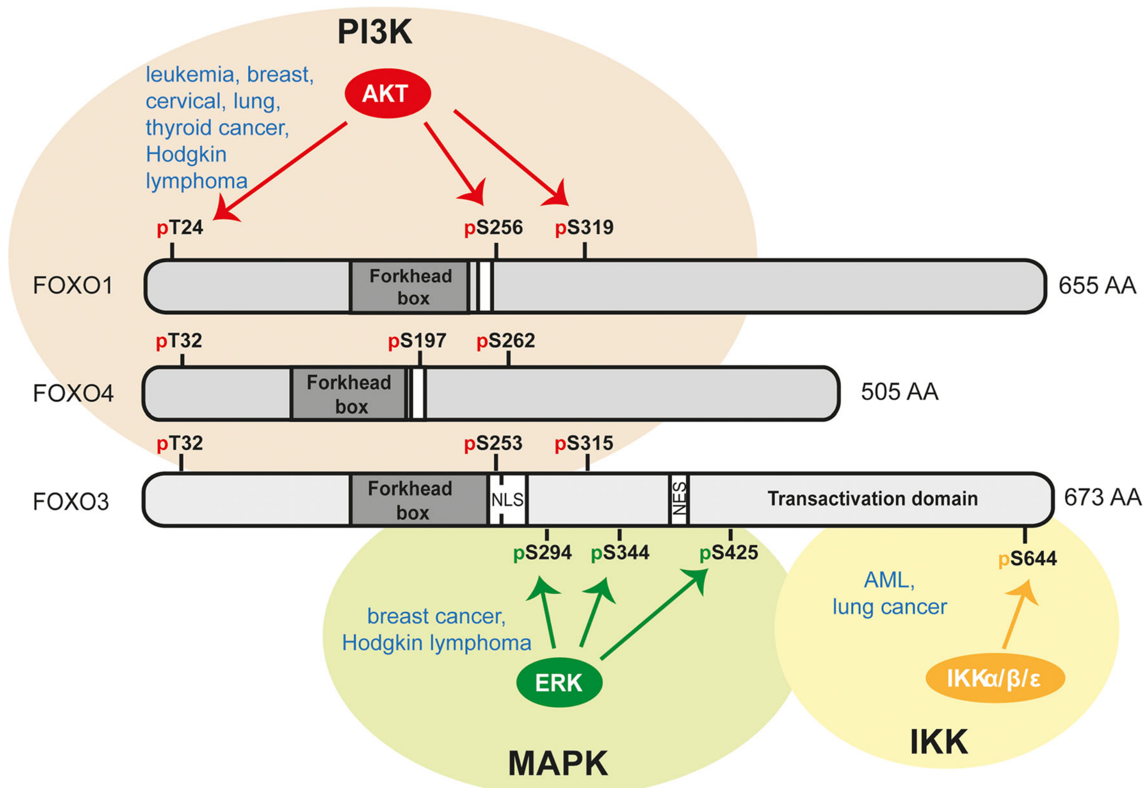

Fig. 1 FOXO phosphorylation downstream oncogenic signaling pathways. *Schematic* representation of the major signaling pathways related to cancer and their link with FOXOs. Three kinases, AKT, ERK and IKK, can phosphorylate FOXOs leading to their inactivation and degradation. These regulations have been demonstrated in several cancer types, as indicated. The phosphorylation site positions correspond to human FOXOs. FOXO1 phosphorylation by ERK has also been suggested [99]. *AA* amino acids

apoptotic effects were observed when FOXO1 was reactivated in cervical cancer cell lines treated with LY294002, a PI3K inhibitor [17]. PI3K inhibition also activates FOXOs and restores $p27^{KIP1}$ expression in a mouse model of lung cancer [18]. Other drugs that inhibit this pathway restore FOXO activity. For instance, in thyroid cancer cells, a chemopreventive non-steroidal anti-inflammatory drug, sulindac sulfide, blocks the PI3K-AKT pathway and leads to the activation of FOXO3, which increases the expression of Bim, GADD45A and $p27^{KIP1}$ to promote cell cycle arrest and apoptosis [19]. The expression of FOXO1 is often reduced in Hodgkin lymphomas compared to B lymphocytes, which normally express it at a high level. The repression of FOXO1 in these cells can be attributed to diverse mechanisms including constitutive activation of AKT and ERK. In these cells, the reintroduction of FOXO1 was also shown to reduce cell proliferation and increase apoptosis [20].

The Ras-MEK-ERK pathway

ERK can also phosphorylate FOXO3 on three serine residues (S294, S344 and S425), which are distinct from AKT substrates, allowing its interaction with the E3-ubiquitin ligase MDM2 [21]. FOXO3 is polyubiquitinated by MDM2 and subsequently degraded by proteasomes. In human breast cancer tissues, a reverse correlation between MDM2 and FOXO3 expression was observed and a higher tumor grade was associated with MDM2-positive and FOXO3-negative cancer tissues, highlighting the pathological relevance of this relationship [21]. In glioblastoma, both ERK and AKT were shown to control FOXO3 [22]. FOXO3 degradation could thus partially account for ERK-mediated tumorigenesis.

The IKK pathway

First described for its role in innate immune response and inflammation, the IKK-NF-κB pathway now emerges as an important signaling pathway in cancer development. FOXO3 is a direct target of IKKα and β, which phosphorylate residue S644 and induce its nuclear exclusion and degradation by proteasomes [23]. In AML, FOXO3 is often localized in the cytoplasm as a result of its phosphorylation by constitutively active IKK, rather than AKT. By doing so, IKK stimulates cell survival and proliferation and

favors tumorigenesis [24]. IKKε, another member of the IKK family, is also able to phosphorylate FOXO3 on residue S644, thereby blocking apoptosis. In human lung cancer, the phosphorylation of FOXO3 on S644 is correlated to IKKε expression [25]. These data suggest that the regulation of FOXO3 by different members of the IKK family could be a key mechanism driving tumorigenesis.

FOXO regulation by micro-RNAs in cancer

Several micro-RNAs (miRs), including miR-96, miR-182 and miR-183, have been identified as regulators of FOXO expression in different cancer types (Table 1). Overexpressed miR-96 was reported to promote tumor cell proliferation by targeting FOXO3 in breast cancer and FOXO1 in transitional cell carcinoma (a type of bladder cancer) [26, 27]. In melanoma cells, miR-182 is up-regulated and targets FOXO3 and MITF, enhancing invasiveness [28]. Furthermore, in the breast cancer cell line MCF7, miR-27a acts together with miR-96 and miR-182 to target FOXO1 and promote tumoral cell growth [29]; while in classical Hodgkin lymphoma, FOXO1 is frequently down-regulated, in part via the combined action of miR-96, miR-182 and miR-183 [20]. In endometrial cancer, FOXO1 is also down-regulated by micro-RNAs, including miR-96, miR-182 and miR-183, which play a role in cancer cell proliferation and survival [30]. FOXO1 is also down-regulated by miR-370 in prostate cancer [31], by miR-135b in osteosarcoma cells [32], by miR-1269 in hepatocellular carcinoma [33] and by miR-411 in lung cancer [34]. Based on cell line studies, these miRs were shown to favor cancer cell proliferation and survival.

FOXO anti-tumoral functions

Hundreds of studies have linked the tumor suppressor activity of FOXOs to the regulation of genes involved in cell cycle arrest (e.g., $p27^{KIP1}$, CDKN1A/p21) and cell death (e.g., FasL, Trail, Bim). These two functions have been extensively reviewed and, by consequence, will not be further detailed [1, 35]. In addition, FOXO factors play important anti-tumoral activities by interfering with senescence induced by an oncogene, angiogenesis, resistance to oxidative stress and the control of cell invasion (Fig. 2). These functions will be detailed below. Finally, whether other physiological roles of FOXOs are relevant to cancer development remains to be investigated. FOXOs may alter cancer cell metabolism, for instance.

Table 1 Micro-RNAs targeting FOXOs in cancer

Cancer	Micro-RNA	FOXO	Function of the interaction	References
Breast	miR-27a miR-96 miR-182	FOXO1	Favors cell proliferation and viability	[29]
Endometrium	miR-9 miR-27 miR-96 miR-153 miR-182 miR-183 miR-186	FOXO1	Induces deregulated cell cycle control and impaired apoptotic response in endometrial cancer cells	[30]
Melanoma	miR-182	FOXO3	Promotes migration and survival of melanoma cells	[28]
Breast	miR-96	FOXO3	Mediates breast cancer cell proliferation	[26]
Hodgkin lymphoma	miR-96 miR-182 miR-183	FOXO1	Involved in autonomous growth and survival of cHL cells	[20]
Prostate	miR-370	FOXO1	Favors proliferation of human prostate cancer cells	[31]
Bladder	miR-96	FOXO1	Regulates FOXO1-mediated apoptosis	[27]
Osteosarcoma	miR-135b	FOXO1	Promotes proliferation and invasion of osteosarcoma cells	[32]
Hepatocellular carcinoma	miR-1269	FOXO1	Promotes cell proliferation	[33]
Lung cancer	miR-411	FOXO1	Promotes cell growth	[34]

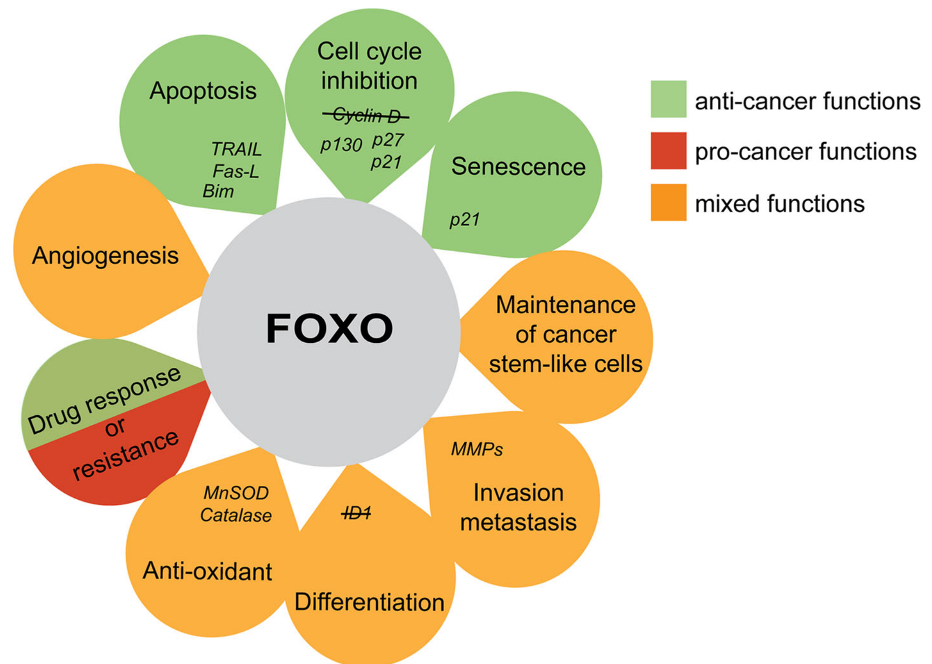

Fig. 2 FOXO functions in cancer. FOXOs are involved in diverse physiological processes, such as cell cycle arrest, apoptosis, and oncogene-induced senescence, which prevent tumor development and contribute to cancer cell killing by various drugs (*green*). By contrast, FOXOs also play pro-tumoral roles, in the resistance to certain treatments, for instance (*red*). Ambiguous functions of FOXOs have been described in angiogenesis, oxidative stress resistance, differentiation, cancer stem cell maintenance and the control of cell invasion and metastasis (*orange*). Key target genes are indicated in *smaller letters*. Repressed genes are *crossed*

FOXOs control invasiveness

The role of FOXOs in the control of cell migration and invasion was investigated in different cellular models. In prostate cancer, AEG1 (astrocyte-elevated gene-1, metadherin or MTDH) is often over-expressed and plays a role in cell invasion. AEG1 knock-down reduces cell viability and invasiveness and increases FOXO3 expression and its nuclear localization. The pro-invasive effect of AEG1 could partially be caused by FOXO3 repression [36]. FOXO3 expression is also decreased in invasive urothelial cancer and is correlated with patient survival. In urothelial cancer cells, FOXO3 downregulation increased the expression of Twist1 and cell motility [37]. In prostate cancer, FOXO4 down-regulation by the PI3K-AKT pathway correlates with metastasis. FOXO4 limits prostate cancer cell migration and invasion in vitro, at least in part by antagonizing the transcription factor RUNX2 [38]. Similarly, FOXO1 has also been shown to negatively regulate RUNX2 transcriptional activity and RUNX2-mediated migration and invasion of prostate cancer cells [39].

FOXOs in oncogene-induced senescence

Oncogene-induced senescence protects organisms from tumor formation by limiting the development of benign lesions. In an attempt to clarify the mechanisms involved in this process, Courtois-Cox et al. showed that aberrant activation of Ras triggers senescence through a negative feedback loop that suppresses Ras and PI3K signaling, leading to activation of FOXO1 and 3 [40]. Remarkably, expression of an activated FOXO mutant was enough to induce senescence of human fibroblasts. The oncogene $BRAF^{V600E}$ can also promote senescence through a MEK-ROS-JNK pathway. Indeed, $BRAF^{V600E}$ signaling through MEK induces increased ROS levels and JNK activation. FOXO4 is then activated via its phosphorylation by JNK leading to FOXO4-induced CDKN1A/p21 expression and senescence [41]. These studies have expanded the role of FOXOs as tumor suppressors capable of promoting senescence in response to an oncogene.

FOXOs regulate angiogenesis

Angiogenesis is a physiological process through which new capillaries grow from pre-existing blood vessels and which is required for tumor growth. The mechanism of angiogenesis involves stimulation of endothelial cells by angiogenic factors (e.g., VEGF) to promote their proliferation, migration and the formation of tubes [42]. FOXOs have been involved in this process as both pro- and anti-angiogenic factors. Their role in tumoral angiogenesis is not clearly defined yet.

Major evidence for FOXO1 pro-angiogenic function stems from embryonic development studies. Indeed, *Foxo1*$^{-/-}$ mice die at E11.5 due to severely impaired vascular development of embryos and yolk sacs. The analysis of endothelial cells isolated from these *Foxo1*$^{-/-}$ embryos showed an abnormal morphological response to angiogenic stimuli such as VEGF-A [43]. In accordance with these data, in adult endothelial cells, some VEGF-regulated pro-angiogenic genes, such as the vascular cell adhesion

molecule-1 (VCAM-1), are expressed via FOXO [44]. By contrast, FOXO1 is inactivated by angiopoietin-1, another key angiogenic factor. In endothelial cells, FOXOs induce the expression of genes involved in blood vessel destabilization and remodeling (e.g. angiopoietin-2) and apoptosis (TRAIL and BCL-6) [45]. In addition, in a murine model of hind limb ischemia, *Foxo3*$^{-/-}$ mice exhibit increased capillary density and limb perfusion 14 days after the induction of ischemia compared to wild-type mice, which suggests that FOXO3 regulates postnatal vessel formation and maturation in vivo [46].

In line with these effects, the role of FOXOs was investigated in tumor angiogenesis. The analysis of 272 tissue samples from gastric cancer patients showed that FOXO1 is constitutively phosphorylated and inactivated in 85 % of tumor cells. FOXO1 phosphorylation correlates with a higher expression of the angiogenic regulators VEGF and HIF-1α and with larger microvessel areas, which is an accepted measure of neoangiogenesis in cancer. This suggests that the inactivation of FOXO1 in gastric tumors is part of a mechanism to promote angiogenesis, but further in vivo experiments need to be performed to confirm these relationships [47]. Finally, as previously mentioned, triple *Foxo1/3/4* conditional knock-out mice develop hemangiomas, suggesting that these factors are tumor suppressors for endothelial cells [12].

FOXOs in oxidative stress responses

It is now well known that FOXO factors are involved in the response to oxidative stress by promoting cellular detoxification via the induction of superoxide dismutase (SOD2) and catalase expression. By protecting cells from excessive ROS accumulation, FOXOs may prevent cancer development. This is well illustrated by studies in hematopoietic stem cells, in which FOXOs are essential to maintain ROS homeostasis. In *Foxo3*$^{-/-}$ mice, the accumulation of ROS leads to a myeloproliferative syndrome. This is mediated by ROS-enhanced cytokine signaling and could be prevented by addition of antioxidant such as *N*-acetylcysteine [48, 49].

Several reports have linked FOXOs to autophagy. In particular, FOXO1 was shown to promote autophagy in response to oxidative stress, which may contribute to its tumor suppressor activity [50]. Interestingly, the induction of autophagy is independent from FOXO transcriptional activity.

FOXO crosstalk with p53

Different studies reported that FOXO3 interacts with the tumor suppressor p53 at different levels. Indeed, they can physically interact [51], FOXO3 can stabilize p53 [52] or activate it indirectly via the up-regulation of p19^{ARF} (CDKN2A), an upstream regulator of p53 [53]. Furthermore, in fibroblasts, p53 binds on a site in the second intron of FOXO3 to induce its expression during DNA damage. In these cells, FOXO3 is dispensable for p53-mediated cell cycle arrest, possibly because of compensation by other factors or other FOXO isoforms. Nevertheless, FOXO3 is required, at least in part, for p53-induced apoptosis. Moreover, FOXO3 loss does not increase the rate of tumor development in p53-deficient mice but influences the tumor spectrum since tumors that do not frequently appear in p53$^{-/-}$ mice (adenocarcinomas and angiolipomas) arise when both p53 and FOXO3 are deleted [54].

FOXO mutations in cancer

Somatic alterations in FOXO genes, including chromosomal translocations and somatic point mutations, have been described in a limited number of tumor cases.

FOXO fusion proteins act as oncogenes

FOXO1 was first identified in alveolar rhabdomyosarcoma (ARMS) as a forkhead domain gene fused to *PAX3* as a result of a *t*(2;13) translocation. The gene was named forkhead in rhabdomyosarcoma (*FKHR*) and only later *FOXO1* [55]. A fusion between *PAX7* and FOXO1 was also described [*t*(1;13) translocation] [56]. These two fusion proteins contain an intact PAX DNA-binding domain (DBD, corresponding to the paired box and homeodomain) fused to the truncated forkhead box domain and the transactivation domain of FOXO1 [55, 56] (Fig. 3). Different models have been used to identify the oncogenic mechanism of cell transformation by the PAX3-FOXO1 fusion protein. Using shRNA targeting PAX3-FOXO1, it was shown that this fusion protein is essential for proliferation and transformation of the ARMS cells that express it. However, even though the fusion protein contributes to oncogenesis, it is not a robust oncogene and a high expression level is required to promote tumorigenesis [57]. In addition, different studies performed on transduced cells, transgenic or knock-in mice indicate that PAX3-FOXO1 alone is not sufficient to induce tumors and that additional genetic lesions are required [58, 59]. For instance, conditional knock-in *Pax3-Foxo1* mice do not develop tumors unless they also harbor conditional inactivation of the p53 or CDKN2A pathway. The disruption of these two pathways had already been implicated in human ARMS and seems to cooperate with FOXO fusion proteins to induce tumorigenesis [60, 61].

Due to the presence of an intact PAX DBD, these chimeric proteins transactivate genes from PAX-binding

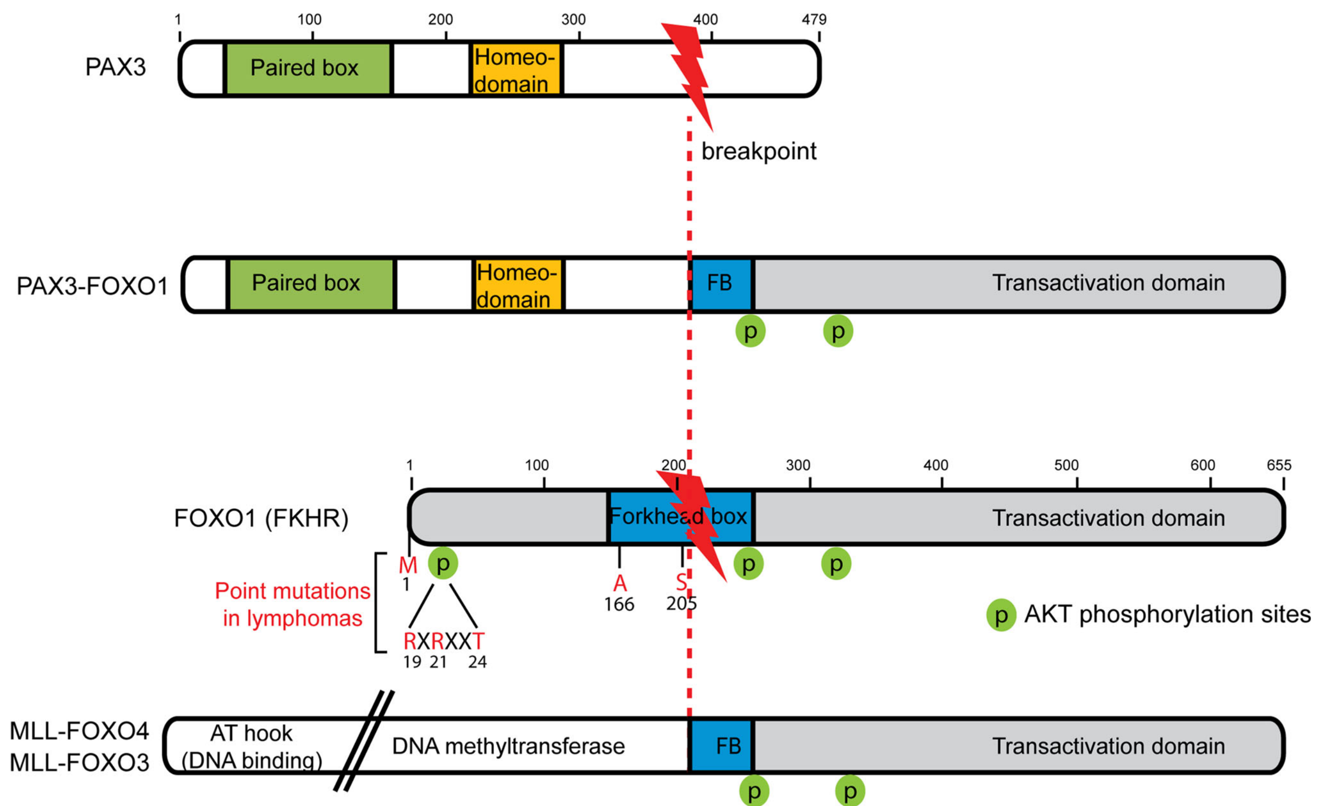

Fig. 3 FOXO genomic alterations and mutations. The PAX3/7-FOXO1 fusion protein occurs following a breakpoint at the chromosomal region corresponding to the C-terminal part of PAX3/7 and the forkhead box domain of FOXO1. This generates a fusion protein that contains the PAX DNA-binding domain (corresponding to the paired box and homeodomain), the truncated forkhead box domain and the FOXO1 transactivation domain. The MLL-FOXO3/4 fusion protein corresponds to the DNA-binding domain and DNA methyltransferase domain of MLL fused to the truncated forkhead box domain of FOXO3 or 4 and the transactivation domain. Point mutations have been identified in FOXO1 in non-Hodgkin B-lymphomas

sites, but their transcriptional activity is more potent compared to wild-type PAX3 or PAX7 proteins [62]. The enhanced activity of the fusion proteins could explain tumorigenesis, at least in part, through altered transcription of target genes. For example, N-Myc, which is up-regulated by PAX3-FOXO1, cooperates with the fusion protein to transform cells [57, 59]. The anti-apoptotic factor BCL-XL, which is also up-regulated by PAX3-FOXO1, seems to be important for ARMS cell survival [58, 61]. However, many of the identified genes still need to be validated. The loss of one *FOXO1* allele due to the chromosomal translocation was also expected to contribute to tumorigenesis. However, *FOXO1* haplo-insufficiency does not accelerate tumor development in mice with PAX3-FOXO1 expressed in terminally differentiating muscle cells [61].

FOXO4 and FOXO3 were next identified in fusion proteins with MLL (mixed lineage leukemia, encoded by the *KMT2A* gene) in acute leukemia [*t*(X;11) and *t*(6;11) translocations, respectively]. Similar to the PAX3/7-FOXO1 fusion proteins, MLL-FOXO3/4 proteins contain the C-terminal part of FOXO with its transactivation domain [63, 64] (Fig. 3). *MLL* translocations are often associated with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). Many other partners of MLL in fusion proteins have been identified. It was suggested that truncated MLL contributes to leukemogenesis regardless of the fusion partner as illustrated with mice expressing the MLL-LacZ fusion protein, which develop hematological tumors. Nevertheless, the partner could either provide a transactivation domain to MLL or stabilize the truncated MLL protein [65]. In the case of fusions involving FOXO, the conserved transactivation domain seems to be critical for the oncogenic potential of these MLL fusions in myeloid progenitors [66, 67] (Fig. 3). Interestingly, it was also shown that MLL-FOXO4 antagonizes the transcriptional activity of endogenous FOXO3 and represses its ability to induce apoptosis [66].

Recently, a novel *t*(X;19) translocation involving *FOXO4* was identified in Ewing-like sarcoma, leading to the formation of a CIC-FOXO4 fusion protein [68, 69].

FOXO1 point mutations

As mentioned above, FOXO1 is frequently down-regulated and considered as a tumor suppressor in Hodgkin lymphomas. In B-cell non-Hodgkin lymphoma cases, recurrent somatic mutations were identified in *FOXO1*, especially in the start codon and at T24 [70]. The prevalence of *FOXO1* mutations was the highest (close to 10 %) in diffuse large B-cell lymphoma. Half of the mutations are located in the N-terminal region (M1, R19, R21 and T24) and disrupt the consensus phosphorylation site of AKT (RXRXXT) or switch the initiation codon to a methionine located after T24, thus preventing FOXO1 phosphorylation at that site (Fig. 3). This affects FOXO1 interaction with 14-3-3 proteins and its nuclear export [71]. Mutations were also found in the DNA-binding domain of FOXO1 but were not further characterized. Patients with tumors that present a *FOXO1* mutation have a significantly lower overall survival compared with patients that have wild-type *FOXO1*. However, the functional impact of these FOXO1 mutations is not clear. One could speculate that blocking FOXO1 phosphorylation by AKT at T24 may disrupt its tumor suppressor activity while keeping functions that are beneficial to tumor cells. Mutated FOXO1 may also block the remaining wild-type FOXOs in a dominant negative manner.

FOXOs as pro-tumoral factors

A number of recent reports have challenged the tumor suppressor role of FOXOs in leukemia, colon cancer and breast cancer, introducing a more complex picture.

FOXOs in cancer stem-like cells

As mentioned above, FOXO phosphorylation in leukemia cells was initially shown to favor their proliferation and survival. However, in line with the essential role of FOXOs in hematopoietic stem cells, several studies pointed to a positive role of FOXOs in the maintenance of leukemia-initiating cells (LICs), which are characterized by their ability to self-renew, to reinitiate leukemia and to resist to therapy. In chronic myelogenous leukemia (CML), the oncogenic fusion kinase BCR-ABL constitutively activates the PI3K-AKT pathway, which phosphorylates FOXOs in the bulk of leukemic cells. By blocking BCR-ABL, tyrosine kinase inhibitors (TKI) such as imatinib induce a cell growth arrest and apoptosis, at least in part by reactivating FOXOs [72]. This therapy induces long term remission in CML but does not efficiently eliminate LICs, which drive CML recurrence. Naka and colleagues showed that, despite BCR-ABL activation, FOXO3 remains active in the nucleus of LICs and plays an essential role in leukemia maintenance [73]. Paradoxically, FOXO3 deletion actually induced LIC apoptosis. FOXO3 activation in these cells was ascribed to AKT inhibition by TGFβ signaling. Accordingly, FOXO3 depletion or TGFβ receptor inhibition increased imatinib efficacy in a mouse model. A follow-up study identified BCL-6 as a FOXO target gene that represses p53, enhances self-renewal of CML LICs and plays an important role in their survival [74]. Altogether, these reports demonstrate the existence of a TGFβ-FOXO3-BCL6 pathway that promotes leukemia persistence.

A similar role of FOXOs in leukemia-initiating cells was described in acute myeloid leukemia. In a murine model of AML induced by the MLL-AF9 fusion gene, LICs feature low AKT phosphorylation and active FOXO [75]. In these cells, depletion of FOXOs or enforced AKT activation induces leukemic cell maturation and increased cell death. This was also observed in human AML cell lines. In human AML primary samples, FOXO nuclear localization was highly variable. By analyzing the transcriptome of a large AML cohort, a subgroup of patients with AML could be clustered using a specific FOXO target gene signature, indicating that FOXOs may play a role in a significant proportion of AML cases [75]. In this respect, Santamaria et al. had previously observed an inverse correlation between the level of FOXO3 expression and the overall survival of patients with AML [76].

In conclusion, whereas compound deletion of *Foxo1*, *3* and *4* in mice induces a myeloproliferative disorder, FOXOs also play an essential role in the self-renewal of leukemia-initiating cells (Fig. 4). Following these studies, a key question is whether FOXOs may play similar roles in other types of cancer initiating cells. In this respect, opposite results were reported in glioblastoma, colon cancer and breast cancer. Indeed, FOXO3 was shown to induce the differentiation of glioblastoma stem-like cells upon treatment with a combination of inhibitors targeting PI3K, mTOR and MEK [22]. In breast cancer stem-like cells, FOXO3 activation by inhibitors of PI3K or AKT1 leads to apoptosis and loss of the stem cell phenotype, defined as the ability to form mammospheres in vitro and tumors in mice [77, 78]. FOXO inhibition by AKT in these models also promotes resistance to chemotherapy [78]. Conversely, FOXO3 activation by an AKT inhibitor in colon cancer stem-like cells induces apoptosis [79].

FOXOs in cell invasion and metastasis

As mentioned above, several studies have demonstrated that active FOXO3 induces apoptosis in colon cancer cells [79, 80]. However, concomitant activation of β-catenin and FOXO3 was shown to prevent cell death and induce metastasis of xenografts in mice [80]. Similarly, β-catenin also confers resistance to PI3K and AKT inhibitors, which

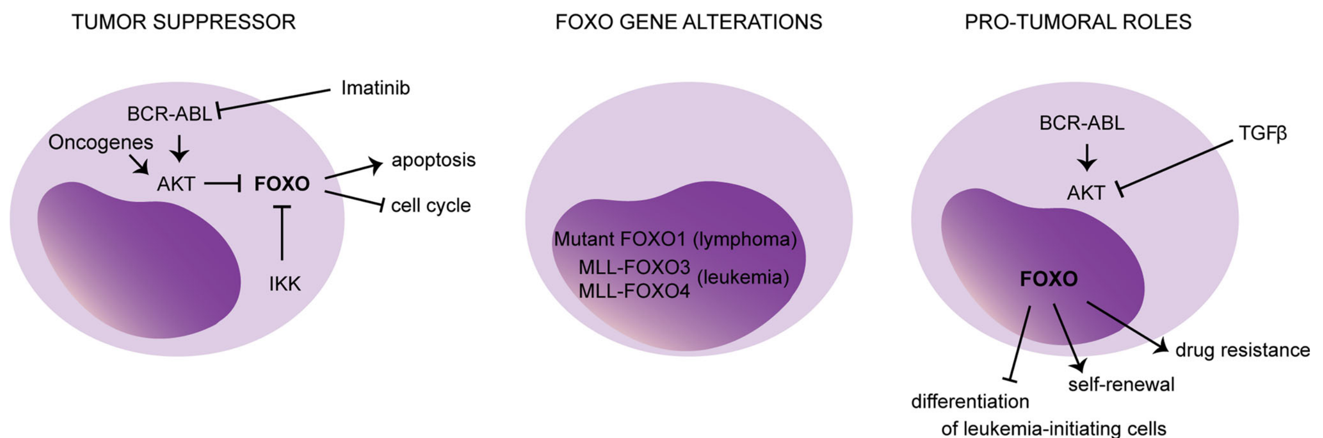

Fig. 4 FOXOs in leukemia. FOXOs are inactivated by AKT and IKK in CML and AML, respectively (*left*). Tyrosine kinase inhibitors, such as imatinib and nilotinib, reactivate FOXOs in CML to promote apoptosis and cell cycle arrest. In some acute leukemia cases, FOXO3 and FOXO4 are found in fusion protein with MLL (*middle panel*). FOXOs also play a pro-tumoral role by promoting the self-renewal of LICs (*right*)

activate FOXOs [81]. In human colon carcinoma, high nuclear levels of FOXO3 and β-catenin correlate with metastatic stage and shorter survival. In the presence of β-catenin, FOXO3 acts as a metastasis inducer rather than a tumor suppressor. This model could potentially explain other pro-tumoral activities of FOXO.

FOXOs have also been involved in the promotion of breast tumor cell invasion. In breast cancer cells, the role of FOXO3 in cell migration and invasion has been linked to the estrogen receptor α (ERα) status. Indeed, in $ER\alpha^{+}$ cells, FOXO3 cooperates with 17β-estradiol to reduce cell invasiveness, while in $ER\alpha^{-}$ cells, FOXO3 tends to increase it [82]. During cancer progression, the tumor mass increases and causes serum deprivation leading to FOXO import to the nucleus and activation. Surprisingly, in this context, it was reported that FOXO3 induces the expression of the matrix metalloproteinases-9 and -13 (MMP-9 and MMP-13) to promote migration and cellular invasiveness [83]. Similarly, in human breast cancer cells, FOXO1 induces the transcription of MMP-1 and, by doing so, enhances the cellular invasive potential. This regulation could involve the phosphatase CDC25A and CDK2. In this context, in a murine model, CDC25A promotes metastasis of breast cancer cells [84].

In conclusion, these studies point to a paradoxical role of FOXOs in breast, colon and myeloid cancers, in which FOXOs are capable of inducing cell death, but may also promote cancer progession.

FOXOs and cancer therapy

FOXO factors are well-established mediators of cancer cell death induced by chemotherapeutic agents. However, several studies showed that they can also be involved in drug resistance. Consequently, FOXO factors may play a paradoxical role in cancer therapy, as summarized in Table 2.

FOXOs and drug response

In breast cancer cells treated with 5-fluorouracil, an anti-cancer agent used in therapy, there is an accumulation of HuR (human antigen R, also called ELAV-like RNA-binding protein 1), an RNA-binding protein which binds to and stabilizes FOXO1 mRNA to promote apoptosis [85]. Paclitaxel, which is also used for the treatment of breast cancer, was shown to stimulate apoptosis via FOXO3-induced Bim expression [86]. Similarly, as described above, FOXOs mediate the apoptotic effect of imatinib in BCR-ABL leukemia cells via Bim induction [87]. Other well-known anti-cancer drugs (e.g. Trastuzumab, Lapatinib and Tamoxifen) also activate FOXOs to mediate apoptosis in cancer cells (for a comprehensive review, see [88]). ONC201/TIC10 is a dual inhibitor of AKT and ERK that targets chemotherapy-resistant colorectal cancer stem cells via induction of TRAIL expression by FOXO3 [79]. These different studies highlight the importance of FOXO expression and activation in the apoptotic response to treatment (Table 2).

Other compounds, such as resveratrol, sulforaphane and α-tocopheryl succinate, have also demonstrated anti-cancer activities involving FOXO-mediated apoptosis [89–91].

FOXOs and drug resistance

FOXO1 increases the expression of the multidrug resistance protein 1 (MDR1, also called ABCB1) in adriamycin-resistant breast cancer cells [92]. Another study performed in K562 leukemia cells resistant to doxorubicin

Table 2 Regulation of drug response by FOXOs

Cancer	Drug	Drug response or resistance	FOXO effect	References
Breast	Adriamycin	Resistance	MDR1 expression	[92]
Breast	5-Fluorouracil	Response	FOXO1 stabilization apoptosis	[85]
Breast	Paclitaxel	Response	Bim expression apoptosis	[78, 86]
Breast, HER2+	Lapatinib	Resistance	Epigenetic up-regulation of MYC	[100]
HER2+ cells	AKT inhibitor	Resistance	RTK expression	[95]
CML BCR-ABL+	Imatinib	Response	Bim expression apoptosis	[87]
CML blast crisis	Nilotinib	Response	Erythroid differentiation	[101]
K562 cells	Doxorubicin	Resistance	MDR1 expression	[93]
K562 cells	Doxorubicin	Resistance	p110α expression	[94]
Ovary	Paclitaxel	Resistance	MnSOD expression	[98]
Renal cell carcinoma	PI3K and AKT inhibitors	Resistance	Rictor expression	[96]
Colorectal cancer stem-like cells	ONC201/TIC10	Response	TRAIL expression	[79]
Colon cancer	PI3K and AKT inhibitors	Resistance	In the presence of high nuclear β-catenin	[80, 81]
Diffuse large B-cell lymphomas	SYK and AKT inhibitors	Response	Apoptosis and metabolic effects	[102]

Many anti-cancer drugs induce FOXO activation to promote cell cycle arrest and apoptosis. In some cases, in response to anti-cancer agents or inhibitors, FOXO induces the expression of genes that favor cell survival and proliferation and thereby plays a role in drug resistance

confirmed the regulation of MDR1 expression by FOXO3 in response to doxorubicin [93].

A second mechanism of resistance involves the activation of feedback loops by FOXOs, promoting resistance. In K562 cells that are resistant to doxorubicin, FOXO3 enhances p110α expression, increasing PI3K activity and promoting resistance to the drug [94]. In HER2 positive tumor cell lines in which the PI3K-AKT pathway is hyperactivated, treatment with an AKT inhibitor increases FOXO activity, as expected. But FOXOs, and in particular FOXO3, then induce the expression of several tyrosine kinase receptors (HER3, IGF-1R and Insulin receptor). In line with these observations, in a xenograft model, inhibitors of AKT and tyrosine kinase receptors act synergistically to reduce tumor growth [95]. Again, in renal cell carcinoma, the inhibition of the PI3K-AKT pathway increases FOXO activity, which induces the expression of Rictor, a member of the mTORC2 complex, leading to AKT phosphorylation and activation, and subsequently to drug resistance. In a xenograft model, FOXO1 and 3 knockdown potentiates the effect of PI3K and AKT inhibitors for renal tumor suppression [96]. In line with these reports, we already mentioned that FOXO3 fails to induce apoptosis of colon cancer cells if β-catenin is over-activated, which confers resistance to PI3K and AKT inhibitors [80]. This could be overcome by WNT pathway inhibitors [81].

In addition, some anti-cancer drugs exert their cytotoxic effects by promoting oxidative stress in cancer cells [97]. Since FOXOs can induce the expression of antioxidant enzymes, this can counterbalance the effects of anti-cancer agents by protecting cancer cells from their cytotoxic effects. For instance, paclitaxel increases the level of H_2O_2 in sensitive-ovarian cancer cells and, by doing so, induces apoptosis. However, paclitaxel-resistant ovarian cancer cells strongly express FOXO1, which protects them from oxidative stress-induced apoptosis by regulating the expression of SOD2 [98] (Table 2).

Most results were obtained using cell lines or murine models. They give interesting indications regarding the role of FOXOs in drug response and drug resistance and reinforce the notion that FOXO functions in cancer are ambiguous. However, future studies need to be performed with primary tumor cells to validate these mechanisms.

Conclusion

Although the tumor suppressor role of FOXO factors is supported by ample experimental evidence, this has not yet been confirmed by cancer genetics. So far only rare alterations involving FOXO genes have been identified in tumors compared to classical tumor suppressors, and most of these alterations confer a gain rather than a loss of function. On the one hand, FOXO anti-tumoral function is supported by the well-established anti-proliferative activity of these factors, which are inhibited by major oncogenic pathways in numerous tumor models. On the other hand, FOXO factors have been involved in resistance to anti-cancer drugs, maintenance of leukemia-initiating cells and colon cancer metastasis. The balance between anti- and pro-tumoral activities of FOXOs may rely on interactions with other pathways, such as WNT/β-catenin or TGFβ. These paradoxical effects of FOXO have been unraveled

using advanced cancer mouse models, which can recapitulate most aspects of cancer initiation and progression. Correlating mouse results with human cancer samples is also essential to clarify the roles of FOXOs. Understanding the functions of FOXO in cancer is of critical importance to improve the treatments that are currently being developed to block the PI3K pathway. Altogether, these data highlight the complexity of FOXO functions in cancer and suggest that FOXOs may be elusive direct targets for cancer therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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