Forkhead box-O transcription factor: critical conductors of cancer’s fate

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Abstract

Cells have evolved elaborated mechanisms to coordinate the cellular answer of either survival or apoptosis. Recent concepts of human carcinogenesis have suggested disturbances in these cellular relays as a potential link to cellular dedifferentiation and uncontrolled proliferation. Forkhead box-O transcription factors (FOXOs) play an important role in tumour suppression by regulating the expression of genes involved in stress resistance, DNA damage repair, cell cycle arrest and apoptosis. The specific regulation of FOXO function is tightly controlled by posttranslational modifications such as phosphorylation, acetylation and ubiquitination. Loss of FOXO function has recently been identified in several human cancers. In this review, we will give an overview about recent progress in the understanding of function and regulation of FOXOs, as well as their role in carcinogenesis. Furthermore, we will discuss a potential clinical use of FOXOs by therapeutically restoring their tumour suppressive properties.

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Introduction

Eukaryotic cells, from yeast to mammals, respond and adapt to environmental challenges by an evolutionarily conserved endogenous system that balances the decision between cell repair and death by a network of signal transduction pathways. The balance between cell survival and death is crucial for cellular integrity. Forkhead box-O transcription factor (FOXO) transcription factors were recently identified as an important family of proteins that modulate the expression of genes involved in cell differentiation, resistance to oxidative stress, DNA damage repair, cell cycle arrest and apoptosis (Arden 2006, Reagan-Shaw & Ahmad 2006, van der Horst & Burgering 2007, Huang & Tindall 2007). FOXO transcription factors belong to the large family of forkhead proteins (Daitoku & Fukamizu 2007). In the past years, over 100 forkhead genes have been identified in a large variety of species, all sharing an evolutionarily conserved ‘forkhead’ DNA-binding domain. This DNA-binding domain consists of three α-helices, three β-strands and two wing-like loops (Boura et al. 2007), and binds to a 13 bp DNA duplex, containing the FOXO consensus binding sequence TTGTTTAC (Tsai et al. 2007). To date, four different isoforms of FOXOs are known in humans: FOXO1 (FKHR), FOXO3a (FKHRL1), FOXO4 (AFX1) and FOXO6.

The regulation of FOXOs depends predominantly on opposing signalling pathways that influence the subcellular distribution of FOXOs, as well as their activity and specificity. For instance, activation of the PI3K/Akt pathway results in an active export of FOXOs into the cytoplasm. By contrast, in response to stress, FOXOs are activated by the members of the c-Jun-N-terminal kinase (JNK) leading to nuclear FOXO accumulation.

It has been suggested that FOXOs may regulate gene expression in both ways, e.g. through activation or repression, depending on recruitment and interaction with either co-factors or -repressors (Boura et al. 2007, Huang & Tindall 2007).

In this review, we will first describe the present knowledge on FOXO function and regulation and then discuss the pathophysiological consequences of a loss of FOXO function in human cancers.
Functions of FOXO

Cell cycle control

In mammalian cells, proliferation is controlled by factors that regulate the transition at the G1/S and the G2/M checkpoints, which are responsible for the initiation and completion of DNA replication and control of cell division respectively. The regulatory function of FOXOs on cell cycle occurs at different stages.

First, ectopic expression of FOXO1, FOXO3a or FOXO4 in human cells blocks cell cycle transition at G1 phase by transcriptional upregulation of the cell cycle inhibitor p27kip1 (CDKN1B) (Dijkers et al. 2000, Medema et al. 2000). By subsequent binding of p27kip1 to the cyclin E/cdk2 complex, the entry of cells into S phase is prevented (Reynisdottir et al. 1995).

In addition, FOXO1 and FOXO3a inhibit G1/S phase transition by downregulation of cyclins D1 and D2 (Smith et al. 1996, Ramaswamy et al. 2002).

Secondly, at the G2/M boundary, FOXO3a prevents cell cycle transition by induction of cyclin G2 and Gadd45a (growth arrest and DNA damage-inducible protein 45), interfering with cyclin/CDK complexes (Furukawa-Hibi et al. 2002, Martinez-Gac et al. 2004).

Thirdly, FOXOs may induce a state of cellular quiescence (G0) by transcriptional upregulation of the Rb family member p130 (RBL-2). The FOXO isoforms FOXO3a and FOXO4 directly bind to consensus sites in the promoter of the p130 gene, e.g. in murine and human colon carcinoma cells, thereby activating its transcription (Kops et al. 2002b). In G0 cells, p130 represses the expression of genes required for re-entry into the cell cycle through activation of the anti-proliferative transcription factor E2F4 (Smith et al. 1996).

Apoptosis

Activation of FOXOs can directly promote the transcription of genes involved in the extrinsic and/or intrinsic apoptotic pathways.

In a pioneer study by Brunet et al. it was shown in different cell lines that Fas ligand (FASLG), a major player in the extrinsic apoptosis pathway, is a downstream target of FOXO3a. Thus, the expression of a constitutively activating FOXO3a mutant in Jurkat T-lymphocytes induced apoptosis in a FASLG-dependent manner (Brunet et al. 1999). Modur et al. (2002) showed that restoration of FOXO function (FOXO1 and FOXO3a) in prostate cancer cells resulted in the upregulation of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL; TNFSF10), which is another important mediator of the extrinsic apoptosis pathway. Furthermore, the transcription of the pro-apoptotic Bim (BCL2L11) is induced in human T-cells after FOXO3a stimulation (Stahl et al. 2002). Bim is believed to promote the mitochondrial release of cytochrome c, via the inhibition of Bcl2, thereby activating the intrinsic apoptosis pathway (Puthalakath et al. 1999, Adams & Cory 2007). Moreover, FOXO3a has been demonstrated to induce PUMA (BBC3) transcription (You et al. 2006) in human lymphoid cells and mouse embryonic fibroblast cells, conferring increased sensitivity for proapoptotic signals.

In addition, FOXOs may repress the transcription of prosurvival genes of the BCL2 family. For instance, FOXO4 has been shown to inhibit the expression of the prosurvival gene Bcl-xL in HeLa cells (Tang et al. 2002).

DNA repair

FOXOs are emerging as important regulators of genes involved in DNA damage repair. DNA repair is initiated by the capability of FOXO3a and FOXO4 to induce the transcription of Gadd45α, which harbours base excision activity and maintains genomic integrity, e.g. through DNA demethylation and cell cycle control (Holland et al. 1999, Smith et al. 2000, Furukawa-Hibi et al. 2002, Tran et al. 2002, Kobayashi et al. 2005). As a result, FOXO3a-induced DNA repair is severely impaired in Gadd45α-deficient fibroblasts (Tran et al. 2002).

Recent data have suggested that FOXO3a can also promote DNA repair in a non-transcriptional manner. Tsai et al. reported a direct interaction between FOXO3a and the ataxia telangiectasia mutated (ATM) protein in response to DNA damage. They showed that exposure of LNCAP prostate cells to ionizing radiation led to covalent binding of FOXO3α to ATM, resulting in an autophosphorylation of ATM on Ser1981, which in turn was blocked by knock-down of FOXO3a (Tsai et al. 2008).

Detoxification

FOXO transcription factors play a crucial role in the fine-tuning of the antioxidative system. An increase in reactive oxygen species leads to FOXO3a- and FOXO1-mediated upregulation of antioxidative proteins, such as manganese superoxide dismutase (MnSOD) and catalase (Kops et al. 2002a, Adachi et al. 2007, Alcendor et al. 2007).

Recently, peroxiredoxin III (Prx III) has been identified as another FOXO3a target gene induced by oxidative stress in cardiac fibroblasts. Inhibitory phosphorylation of FOXO3α through PI3K/Akt
prevented binding of FOXO3a to the Prx III promoter, while FOXO3a overexpression increased Prx III promoter activity (Chiribau et al. 2008).

In addition, FOXOs have been found to interact with a number of other transcription factors, thereby modifying their transcriptional activity and specificity, for instance, of SMAD, RUNX3 and p53, which has been excellently reviewed elsewhere (Gomis et al. 2006, Allen & Unterman 2007, van der Horst & Burgering 2007). Figure 1 shows a summary of known functions of FOXO proteins. Note that not all FOXO isoforms may be expressed in all tissues, and that their function may differ in an isoform-specific way.

### Regulation of FOXOs

Forkhead activity is controlled at both the transcriptional and posttranslational levels (Huang & Tindall 2007). Recent work has suggested that the FOXO isoforms FOXO1 and FOXO3a are at least partly under transcriptional control of the transcription factor E2F1. The proximal gene promoters of FOXO1 and FOXO3a harbour an evolutionarily conserved E2F1-binding site (Nowak et al. 2007). Furthermore, induction of E2F1 in human glioblastoma cells promotes the transcription of FOXO1 and FOXO3a in vitro (Nowak et al. 2007). Additionally, regulation of FOXO gene transcription by nutritional and hormonal status was found in rat hepatocytes in vivo. A diminished nutrient availability and artificially induced insulin resistance led to increased mRNA expression of FOXO1, FOXO3a and FOXO4 (Imae et al. 2003).

The activity of FOXOs is tightly regulated by posttranslational modifications (PTMs) such as phosphorylation, acetylation and ubiquitination (Fig. 2; Van Der Heide et al. 2004, Vogt et al. 2005, van der Horst & Burgering 2007, Huang & Tindall 2007, Reagan-Shaw & Ahmad 2006). PTMs of FOXOs ensure the short-term integration of prosurvival and -apoptotic signals, while transcriptional and translational modifications predominantly control the long-term adaptation to a changing cellular micromilieu.

In the following section, we will summarize PTMs that regulate FOXO activity. This will be of substantial value for a better understanding of the role of FOXOs in carcinogenesis.

### Inhibitory phosphorylation of FOXOs

One prerequisite for the transcriptional regulatory function of FOXOs is their nuclear localization. In
various mammalian cells, the FOXO isoforms FOXO1, FOXO3a and FOXO4 were demonstrated to underlie inhibitory phosphorylation on isoform-specific phosphorylation sites, resulting in their nuclear exclusion and a consecutive loss of their transcriptional function. Several pathways mostly related to mitogenic signalling can inactivate FOXOs in a phosphorylation-dependent manner. In particular, the PI3K/Akt signalling cascade, which is a downstream target of insulin signalling, is known to be a major inhibitor of FOXO function (Biggs et al. 1999, Brunet et al. 1999, Kops et al. 2002b). For example, Akt phosphorylates mammalian FOXO1 at sites Thr24, Ser256 and Ser319 (Biggs et al. 1999). FOXO3a can be phosphorylated by Akt on Thr32, Ser256 and Ser319 (Kashii et al. 2000), whereas inactivation of FOXO4 is associated with Akt-mediated phosphorylation on Thr32 and Ser197 (Matsuzaki et al. 2005b).

Upon PI3K/Akt-induced phosphorylation of FOXO1, FOXO3a and FOXO4, two binding sites for proteins of the 14-3-3 family are generated, which results in the disruption of FOXO-DNA binding (Brunet et al. 2002). Successive to the association with 14-3-3 proteins, the binding of exportins, e.g. Crm1 and Ran, to isoform-specific FOXO nuclear export sequences enables the shift of FOXOs into the cytoplasm (Brunet et al. 2002). Moreover, binding of 14-3-3 proteins suppresses the nuclear re-import of FOXOs by masking their nuclear localization sequence (Obsilova et al. 2005, Hermeking & Benzinger 2006).

However, Yan et al. (2008) recently revealed a mechanism that allowed FOXO1 to re-enter the nucleus after Akt-induced translocation into the cytoplasm: the protein phosphatase 2A (PP2A) disrupts the binding of FOXO1 and proteins of the 14-3-3 family by dephosphorylating the Akt phosphorylation sites Thr32 and Ser256, leading to nuclear relocation of FOXO1 and reactivation of its proapoptotic transcriptional function in response to growth factor withdrawal.

In striking contrast to the other mammalian FOXO isoforms, Akt-mediated phosphorylation of FOXO6 on Thr26 and Ser184 appears to inhibit its activity without a subsequent translocation into the cytoplasm. Studies in HEK239T cells revealed that FOXO6 lacks the conserved C-terminal Akt motif, which mediates the 14-3-3 protein-dependent shuttling into the cytoplasm (Van Der Heide et al. 2005). Moreover, recent data on the crystal structure of the mammalian forkhead DNA-binding domain suggest that Akt-mediated
phosphorylation itself diminishes the affinity of FOXOs for DNA binding through modulation of van der Waals forces (Tsai et al. 2007).

Besides PI3K/Akt, further kinases involved in mitogenic signalling have been identified to mimic PI3K/Akt-mediated phosphorylation and inhibition of FOXO3a and FOXO4. For example, Ikkβ, an important mediator of NF-κB signalling, phosphorylates FOXO3a on Ser644, resulting in the nuclear exclusion of FOXO3a (Hu et al. 2004, Hu & Hung 2005). A similar effect has been demonstrated for the serum- and glucocorticoid-inducible kinase SGK1 (Arteaga et al. 2007). Moreover, it has been shown that FOXO1 can be inactivated by the cyclin-dependent kinases CDK2 and CDK1 via increased phosphorylation at Ser249 in prostate cancer cells (Huang et al. 2006, Liu et al. 2008).

**Activating phosphorylation of FOXOs**

In response to oxidative and genomic damage, FOXO transcription factors are activated by the Raf/JNK and ERK pathways. This involves FOXO phosphorylation at sites that are distinct from those targeted by PI3K/Akt and other mitogenic signalling cascades (Asada et al. 2007). Stress-induced phosphorylation of FOXOs results in a nuclear retention of FOXOs and activation of their transcriptional regulatory functions (Vogt et al. 2005, van der Horst & Burgering 2007, Huang & Tindall 2007).

For example, studies by Essers et al. (2004) provide evidence in murine cells, that H$_2$O$_2$-dependent activation of JNK increases phosphorylation of FOXO4 on Thr447 and Thr451, resulting in nuclear accumulation of FOXO4 and increased transcription of the FOXO targets MnSOD and catalase. Additionally, the stress-induced protein kinase MST1 was shown to activate FOXO3a in a phosphorylation-dependent manner. Using an in vitro model of mammalian neurons, Lehtinen et al. (2006) found that MST1 phosphorylates FOXO3a upon oxidative stress on Ser207, thereby inhibiting the interaction between FOXO3a and 14-3-3 proteins.

Furthermore, Greer et al. (2007) demonstrated in human kidney cells that glucose deprivation increases the phosphorylation of FOXO3a on six specific phosphorylation sites in an AMP kinase-dependent manner. When phosphorylated at these specific residues, FOXO3a does not change its subcellular distribution but changes the expression of its target genes. For instance, AMP kinase-mediated phosphorylation of FOXO3a significantly induced the transcription of Gadd45a in mice.

**Acetylation**

The transcriptional activity and specificity of FOXOs have been shown to be tightly controlled by acetylation and deacetylation in the nucleus. Nuclear FOXOs can bind to various proteins, influencing FOXOs’ acetylation status on conserved lysine residues in vitro and in vivo (Daitoku et al. 2004, van der Horst et al. 2004, Frescas et al. 2005, Perrot & Rechler 2005, Arden 2006): for example, in response to oxidative stress, nuclear FOXO3a associates with the histone acetylase CREB-binding protein (CREBBP) and P300 (Daitoku et al. 2004, Perrot & Rechler 2005). This association is required for the assembly of an active initiation complex, responsible for FOXOs’ transcriptional function. Furthermore, CREBBP acetylates FOXO3a and simultaneously recruits nuclear proteins possessing deacetylating functions (Giannakou & Partridge 2004). Thus, the ‘acetylation signature’ of FOXO3a can be modified by deacetylating partners, enabling the fine-tuning of target gene expression (Greer & Brunet 2008). For instance, subsequent to H$_2$O$_2$ exposure, pre-acetylated FOXO3a is increasingly deacetylated by mammalian sirtuin proteins (SIRTs). This in turn triggers the FOXO-mediated expression of antioxidative target genes and inhibits the expression of proapoptotic targets, e.g. Bim and FASL6 (Brunet et al. 2004). Furthermore, it has been shown that deacetylation of FOXO1 by SIRTs alters the susceptibility of FOXOs to PI3K/Akt signalling in vitro by sensitizing FOXO1 for phosphorylation by Akt (Matsuzaki et al. 2005a).

However, future studies are needed to elucidate the importance of FOXO deacetylation as a prerequisite for phosphorylation by Akt, and to decipher in which way this is mandatory for PI3K/Akt-FOXO signalling.

**Ubiquitination**

Two functionally opposing types of FOXO ubiquitination have been discovered and involve polyubiquitination by SKP2 (Huang et al. 2005) and monoubiquitination by yet unidentified binding partners (van der Horst et al. 2004, Vogt et al. 2005).

Huang et al. demonstrated in murine cells that overexpression of SKP2, an oncogenic subunit of the Skp1/Cul1/F-box protein ubiquitin complex, significantly decreased the cellular content of FOXO1. Thereby SKP2 induced the polyubiquitinyation of FOXO1, leading to its proteasomal degradation. Interestingly, an Akt-mediated phosphorylation at FOXO1 site 256 is required for SKP2 action (Huang et al. 2005, Arden 2006, Reagan-Shaw & Ahmad 2006).

By contrast, monoubiquitination of unphosphorylated cytoplasmic FOXOs is thought to trigger the
nuclear import and activation of FOXOs by mechanisms that are still incompletely understood (Arden 2006, van der Horst et al. 2006).

\(\beta\)-catenin binding
Recently, FOXOs have been reported to associate directly with cytoplasmatic \(\beta\)-catenin upon exposure to oxidative stress in HEK239T cells (Essers et al. 2004). \(\beta\)-catenin is a prominent member of the canonical Wnt pathway and has been described to act as a coactivator of several transcription factors such as TCF/LEF, thereby promoting proliferation and dedifferentiation (Hoppler & Kavanagh 2007). In turn, binding of \(\beta\)-catenin to FOXOs was found to augment their transcriptional activity, resulting in the upregulation of anti-proliferative genes, such as \(p27^{kip1}\) and proapoptotic genes, such as Bim (Essers et al. 2005, Almeida et al. 2007). Interestingly, there is evidence that the pool of cytoplasmatic \(\beta\)-catenin is limited, resulting in a competitive binding of FOXOs and other transcription factors (TCF/LEF) to \(\beta\)-catenin (Manolagas & Almeida 2007). Thus, the binding of \(\beta\)-catenin to FOXOs augments their activation, while in parallel opposing the transcriptional activity of TCF/LEF and vice versa (Hoogeboom et al. 2008). An imbalance consequential to oxidative stress tipping target gene expression towards the FOXO axis has been recently proposed, but further studies are required to fully elucidate the physiological and pathophysiological consequences of a limited \(\beta\)-catenin pool for FOXO- and TCF/LEF-dependent target gene expression (Almeida et al. 2007, Manolagas & Almeida 2007, Hoogeboom et al. 2008).

The role of FOXOs in cancer
The ability of FOXOs to control cell survival and cell death suggests that FOXOs may function as tumour suppressors. Indeed, loss of FOXO function has been observed in a number of human cancers and may represent a common feature of carcinogenesis. Generally, FOXOs can be deregulated through genetic defects and altered PTMs (Fig. 3). It is conceivable that each type of cancer may harbour an individual signature of FOXO deregulation determining its unique phenotype.

Figure 3 Oncogenic signalling cascades and molecular events contributing to loss of FOXO function. FOXOs can be deregulated through genetic defects on the one hand and posttranslational modifications on the other hand. Thus, each type of cancer might harbour an individual signature of FOXO deregulation determining its unique phenotype. For example, inhibitory phosphorylation of FOXOs by mitogenic signalling cascades, e.g. PI3K/Akt, has been observed in various human cancers. Furthermore, genetic defects can disrupt FOXO function. In addition, proteasomal degradation has been shown to impair FOXO expression in human cancers.
Genetic defects of FOXOs

Several FOXO gene alterations have been identified in human cancers: for instance, a chromosomal fusion of the C-terminal domain of FOXO1 and the N-terminal domain of the transcription factors PAX3 or PAX7 was found in human alveolar rhabdomyosarcoma (ARMS; Bois & Grosveld 2003, Bois et al. 2007). These chimeras constitutively promote the transcription of PAX3 and PAX7 target genes respectively, which are associated with increased proliferation and cell motility (Kikuchi et al. 2008). In addition, the transcription of tumour suppressive FOXO target genes is thought to be impaired (Arden 2006). In line with this concept, studies revealed that PAX3 upregulation itself was not sufficient to induce ARMS in mice, but required FOXO1 downregulation for malignant transformation (Anderson et al. 2001). Accordingly, Bois et al. (2005) demonstrated that loss of FOXO1 expression is a common feature of ARMS, while re-expression of FOXO1 in ARMS-derived cells induced apoptosis and cell cycle arrest.

Moreover, Dong et al. (2006) have demonstrated that human prostate cancers harbour a loss of heterozygosity on chromosome 13q14, which is the known gene locus of FOXO1. Thereby, loss of FOXO1 gene expression was directly correlated with increased cell proliferation in cancer cells.

Similar to PAX/FOXO1 transgenes, chromosomal breakpoints occurring in human acute myeloid leukaemia were found to involve FOXO3a and FOXO4. For example the mixed lineage leukaemia gene (MLL) can fuse with FOXO3a and FOXO4, thereby putatively disturbing their physiological tumour suppressive function (Parry et al. 1994, So & Cleary 2003).

Alteration of posttranslational FOXO protein modification

As outlined above, FOXOs are modified by a multitude of posttranslational events, which directly influence their transcriptional activity and specificity. With regard to the importance of PTMs for FOXO activity, it can be assumed that disruption of this sophisticated network results in loss of FOXO function and may trigger carcinogenesis.

PI3K/Akt pathway

The PI3K/Akt signalling pathway has emerged as a central mitogenic pathway in various types of mammalian cancer (Zbuk & Eng 2007) and there is amble evidence for a direct correlation between PI3K/Akt activation and FOXO inactivation in cancers (Kim et al. 2007, Yip et al. 2008).

Generally, three different molecular mechanisms were found responsible for a constitutive activation of PI3K/Akt signalling and loss of FOXO function in malignancies.

First, physiological inhibitors of the PI3K/Akt cascade such as the tumour suppressor phosphatase PTEN can be lost due to genetic mutations or decreased expression leading to consecutive Akt activation. Consequently, FOXO1 and FOXO3a are phosphorylated and translocated into the cytosol (Modur et al. 2002). Consistent with these findings, Emerling et al. (2008) reported an overactivation of PI3K/Akt signalling in PTEN−/− LNCAP prostate cancer cells, resulting in the nuclear exclusion and inhibition of FOXO3a activity. Using murine astrocytes, de la Iglesia and coworkers revealed a direct interrelationship between loss of PTEN- and Akt-mediated cytoplasmatic translocation of FOXO3a (De la Iglesias et al. 2008).

Secondly, some human tumours harbour transgenes, such as RET/PTC, ZNF198-FGFR1 or BCR-ABL, that confer constitutive activation of PI3K/Akt signalling. For example, Jung et al. (2005) could demonstrate in human embryonic kidney (HEK) cells that the overexpression of the RET/PTC chimera led to increased activation of Akt signalling and subsequently inhibited the transcriptional activity of FOXO1. Moreover, the ZNF198-FGFR1 fusion protein has been shown to stimulate the PI3K/Akt cascade in acute myeloid leukaemia and lymphoma, resulting in FOXO3a Thr32 phosphorylation and a 14-3-3 protein-dependent nuclear exclusion of FOXO3a (Dong et al. 2007). Likewise, in chronic myeloid leukaemia (CML), the chimeric BCR-ABL fusion protein has been found to confer FOXO3a inactivation through Akt-mediated phosphorylation on the phosphorylation sites Thr32 and Ser253 (Birkenkamp et al. 2007).

Third, PI3K copy gains or overexpression of the catalytic subunit p110α are frequently found in human malignancies (Kikuno et al. 2007) and are directly associated with increased phosphorylation of FOXO1 and FOXO3a as shown in human colon cancers (Samuels et al. 2005).

Furthermore, the astrocyte-elevated gene-1, AEG-1, has been shown to confer PI3K/Akt activation in several tumours. For instance, human prostate cancers cells display elevated levels of AEG-1 resulting in Akt activation and aberrant cell proliferation. Interestingly, knock-down of AEG-1 in prostate cancer cell lines, such as LNCAP and PC-3, decreased the level of phospho-Akt and simultaneously increased the fraction of nuclear FOXO3a, as well as FOXO p27kip1 target gene transcription (Kikuno et al. 2007).
Inhibition of nuclear FOXO export | Disruption of persisting FOXO inhibition | Induction of FOXO expression | Influencing FOXO acetylation
---|---|---|---
Competitive inhibition of 14-3-3 proteins (R18) | Inhibition of PI3K (LY294002, grifolin) | Paclitaxel | Activation of SIRT1 (resveratrol)
Inhibition of PI3K/AKT1 (wortmannin) | Inhibition of tyrosine kinases (imatinib; mesylat) | | Inhibition of SIRT1 (nicotinamid)

**Ikβ/NF-κB pathway**

Besides the PI3K/Akt pathway, additional mitogenic cascades have been found to impair FOXO function in cancer. Inactivation of FOXOs due to overexpressed Ikβ was reported in breast cancers lacking activation of the PI3K/Akt cascade. Hu et al. (2004) and Hu & Hung (2005) reported that the level of Ikβ/NF-κB activation was directly correlated with the amount of phosphorylated FOXO3a in the cytoplasm. Interestingly, Ikβ-mediated phosphorylation of FOXO3a did not only induced cytosolic dislocation of FOXO3a, but also triggered its proteasomal degradation in HEK293T cells (Hu et al. 2004).

**RAS/ERK pathway**

Furthermore, both the transcriptional activity and protein expression of FOXO3a have been shown to be impaired after ERK activation. Overexpression of ERK in the human hepatoma cell line Hep-3BX significantly reduced the nuclear content of FOXO3a, as well as its total cellular expression. ERK phosphorylates FOXO3a on Ser294, Ser344 and Ser425, which resulted in a FOXO3a translocation into the cytosol and MDM2-mediated ubiquitination. When treated with the ERK inhibitor U0126, the cells showed a restored expression of FOXO3a and an increased transcription of the FOXO targets p27kip1 and Bim. Moreover, induction of a FOXO3a mutant harbouring constitutive phosphorylation on ERK phosphorylation sites (i.e. causing FOXO inactivation) in the human breast cancer cell line 435 led to increased cellular proliferation when compared with the cells expressing the phosphorylation resistant FOXO3a mutant. Transplantation of these cells into nude mice showed that the phosphorylated FOXO3a protein did not suppress tumour growth, whereas the non-phosphorylated FOXO3a protein inhibited uncontrolled proliferation (Yang et al. 2008).

**Skp2 polyubiquitination**

Increased proteasomal degradation of FOXOs may constitute another mechanism contributing to loss of FOXO function in cancers. In fact, the proteasomal machineries run out of control in many malignancies. For instance, the E3-ubiquitin ligase Skp2 has been found overexpressed in lymphomas and breast, colorectal, thyroid and lung cancers (Dehan & Pagano 2005). Recently, Goto et al. reported a correlation between Skp2 expression and FOXO1 downregulation in endometrialdervived cancer cells. Furthermore, the authors observed a decreased protein expression of FOXO1 (and Gadd45a target gene expression) in the biopsies of human endometrial cancers and suggested that Skp2 might represent an important factor for tumorigenesis by accelerating the proteasomal degradation of FOXOs (Goto et al. 2008).

**Perspective: FOXOs as drug targets for cancer treatment**

Recent approaches to suppress tumour growth by inducing cell cycle arrest and apoptosis in a FOXO-dependent manner have shown promising results (Sunters et al. 2003, Hussain et al. 2006, Dong et al. 2007, Jin et al. 2007a,b). Interventions on different regulatory stages of FOXO function resulting in either restoration or augmentation of FOXO function seem especially useful for drug-targeting:

First, disruption of constitutive PI3K/Akt signalling by specific kinase inhibitors can save FOXOs from inactivating phosphorylation. The possibly best-established example is the treatment of CML with the tyrosine kinase inhibitor STI571 (gleevec), which inhibits PI3K/Akt and thus FOXO3a phosphorylation and restores its transcriptional activity as reflected by Bim upregulation (Fernandez et al. 2004, Essafi et al. 2005). Similarly, it was shown that treatment of human osteosarcoma cells with the PI3K/Akt inhibitor grifolin reduces the fraction of phosphorylated and inactivated FOXOs, thereby increasing the rate of apoptosis (Alexia et al. 2006, Jin et al. 2007a).

Secondly, inhibition of the nuclear export of FOXOs by inhibitors of the 14-3-3 protein family and related exportins, such as the peptide R18, can increase FOXO transcriptional activity. Using a model of human leukaemia cells, Dong et al. (2007) demonstrated that treatment of cells with R18 downregulates the expression of 14-3-3 proteins, which in turn leads to...
increased nuclear accumulation of FOXO3a and restored transcription of its anti-proliferative targets p27kip1 and Bim.

Thirdly, inhibition of the proteasomal degradation of FOXOs could replenish the cellular content of FOXOs, and might thus represent an interesting mode to improve the chemo- and radiation-inducible rates of apoptosis. However, so far there are no studies on this topic available in the literature.

Fourthly, induction of FOXO expression potentially decreases cellular proliferation and dedifferentiation. Treatment of human breast cancer cells with paclitaxel has been shown to increase FOXO3a expression and transcription of the FOXO target gene Bim, which confers apoptosis in breast cancer cells (Sunters et al. 2003).

Fifthly, modulation of the acetylation status of FOXO may provide another useful method to influence FOXO target gene expression. In this context, it has been found that deacetylation of FOXO promotes FOXO-dependent gene transcription (Frescas et al. 2005, Kobayashi et al. 2005). In SV40-transformed hepatocytes, resveratrol, which is a small activator of NAD-dependent deacetylase (sirtuin), causes FOXO1 translocation into the nucleus, suggesting that FOXO interaction with SIRT1 might contribute to its subcellular localization. By contrast, the sirtuin inhibitor nicotinamid prevented SIRT1 binding (Frescas et al. 2005). Table 1 summarizes several putative strategies to augment FOXOs function as tumour suppressor.

In conclusion, FOXO transcription factors have emerged as key regulators of cellular fate. Loss of FOXO function occurs at different levels of FOXO regulation and is a novel molecular feature of cancer. In vitro and in vivo data suggest that restoration of FOXO function may represent a promising avenue in cancer treatment. However, future studies are mandatory to elucidate whether this represents an appropriate scheme to positively influence the clinical outcome of cancer patients.

Declaration of interest
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