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FORUM REVIEW ARTICLE

Sense and Sensitivity: FOXO and ROS in Cancer Development and Treatment

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Abstract

Forkhead box O (FOXO) transcription factors are at the center of an emerging paradigm that links longevity, cell fate, and tumor development. Key to these processes is the ability of FOXO to regulate, and be regulated by, oxidative stress. Perturbation of the mechanisms that tightly couple reactive oxygen species (ROS) production, oxidative stress signaling, and FOXO activity to the subsequent cellular response is a pivotal step in cancer development and progression. Consequently, the ROS-FOXO pathway is a major therapeutic target in cancer, not only as it mediates the cellular response to chemotherapy, but also because it underpins drug resistance. As the intimate and reciprocal relation between FOXO and ROS is being unravelled, new opportunities arise to develop more-effective cancer treatments that circumvent resistance to the conventional cytotoxic drugs. *Antioxid. Redox Signal.* 14, 675–687.

Introduction

Forkhead box class O (FOXO) transcription factors are members of the forkhead box family of transcription factors comprising FOXO1, FOXO3a, FOXO4, and FOXO6. In invertebrates, only one FOXO is found, designated Daf-16 in *Caenorhabditis elegans* and dFOXO in *Drosophila melanogaster*. The FOXO transcription factors are involved in a wide spectrum of cellular functions, including cell proliferation, apoptosis, differentiation, regulation of oxidative stress, and DNA damage (9, 30, 37, 62), which are essential for cancer cell proliferation, survival, and progression. Compelling evidence indicates that deregulation of FOXO proteins is associated with tumorigenesis and cancer progression (37, 62). A comparison of mice with deletion of up to five FoxO alleles suggests that redundancy and developmental compensation exist among FoxO family members as well as unique lineage-specific roles (70). These models demonstrate that FoxOs are *bona fide* tumor suppressors *in vivo*, as deletion of all FoxOs induced thymic lymphomas and hemangiomas, whereas loss of individual genes gave rise to various neoplastic phenotypes.

However, these knockout mouse models also highlighted the role of the FoxO family in regulating the cellular levels of reactive oxygen species (ROS) and oxidative stress responses, in particular within the hematopoietic stem cell pool. This is consistent with previous findings in human cancer and immortalized cell lines, which also suggest that FOXO factors are both sensors of oxidative stress signals and effectors of the subsequent cellular response. The intimate and reciprocal interactions that govern FOXO function and the production and elimination of ROS, as well as the implications of these processes for tumorigenesis and cancer therapy, are discussed in this review.

Reactive Oxygen Species

The ROS, superoxide anion radical ($\bullet O_2^-$), hydrogen peroxide (H_2O_2), and hydoxyl radical (•OH), are highly reactive, diffusible, and ubiquitous molecules generated as inevitable by-products of aerobic respiration and metabolism. The most potent and reactive ROS is superoxide, which is formed by a single-electron reduction of molecular oxygen: $O_2 + e^- \rightarrow \bullet O_2^-$. Hydrogen peroxide is formed on additional reduction of oxygen as follows: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (3). Further reduction leads to the formation of OH, especially in the presence of metal ions through the Fenton or Haber-Weiss reactions. Hydroxyl radicals are extremely reactive with short half-life times. In neutrophils, myeloperoxidase catalyzes the formation of hypochlorous acid (HClO), whereas superoxide may also react with nitric oxide (NO) to form another reactive molecule, peroxynitrite (ONO_2^-): $\bullet O_2^- + NO \rightarrow ONO_2-$. Thus, the formation of superoxide anions can trigger a cascade of production of ROS, which can be extremely detrimental to the cell but also function as key signaling molecules. Approximately 1% to 2% of electrons in the mitochondrial respiratory chain leak to generate superoxide, primarily in reactions mediated by coenzyme Q and ubiquinone and its

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complexes (47). The mitochondria are considered the major cellular source of ROS *in vivo* (2). During electron transport in the endoplasmic reticulum, superoxide is generated by the leakage of electrons from NADPH cytochrome P450 reductase (17). In addition, superoxide may be generated by xanthine oxidase (100), a component of the xanthine oxidoreductase system, which is present predominantly as xanthine dehydrogenase in normal tissues, and converts into the ROS-generating xanthine oxidase in damaged tissues (100).

The lipoxygenases are another cellular source of ROS that catalyze the production of leukotrienes and ROS from arachidonic acid (86). In addition, lipoxygenase activity is required for ROS production by CD28 stimulation in T lymphocytes (52), and products of the lipoxygenase cascade, such as leukotriene B4 (LTB4), have been implicated in TNF-α–induced production of ROS (99). Considering the diverse cellular sources of ROS production and the elevated metabolic rates of the highly proliferative cancer cells, it is not surprising tumors often display altered ROS levels.

Oxidative stress is caused by imbalances in ROS production or exposure and cellular antioxidants (Fig. 1). Mammalian cells possess multiple mechanisms to remove ROS, including both enzymatic and nonenzymatic dietary antioxidants. Many antioxidants exert targeted functions. For example, glutathione peroxidase (*GPx1*) knockout mice are hypersensitive to toxins known to induce oxidative stress, such as paraquat (87). Other antioxidant mechanisms include glutathione-*S*-transferase (GST), and manganese-containing superoxide dismutase (MnSOD), which is located exclusively within the mitochondrial matrix, where it facilitates the dismutation of superoxide radical to hydrogen peroxide (82). This ROS is further detoxified by catalase to water and oxygen. Cell membranes are protected from lipid peroxidation, mainly by α-tocopherol and phospholipid hydroperoxide glutathione peroxidase (69). The thioredoxin-dependent peroxide reductases (PRX3 and PRX5) reduce hydrogen peroxide and lipid hydroperoxides (69). In addition, thioredoxin, thioredoxin reductase, and glutaredoxin are ubiquitous proteins that perform a multitude of functions besides their role in cellular antioxidant defenses (33). The thioredoxin system is essential for development, as disruption of the *TRX2* gene, which encodes for the mitochondrial isoform of thioredoxin, is embryonic lethal (68). In summary, the cellular impact of ROS depends largely on the balance between production and elimination.

FOXO and ROS: A Reciprocal Relationship

Regulation of FOXO transcription factors by ROS

FOXO activity is exquisitely regulated by posttranslational modifications. Cytoplasmic translocation and inactivation of FOXO on phosphorylation by PKB represents a paradigm of the mechanism of PI3K signaling. Other kinases, including JNK, ERK, and p38, also converge on FOXO to regulate its activity and stability. In addition to phosphorylation, FOXO proteins are subject to methylation, ubiquitination, mono-ubiquitination,

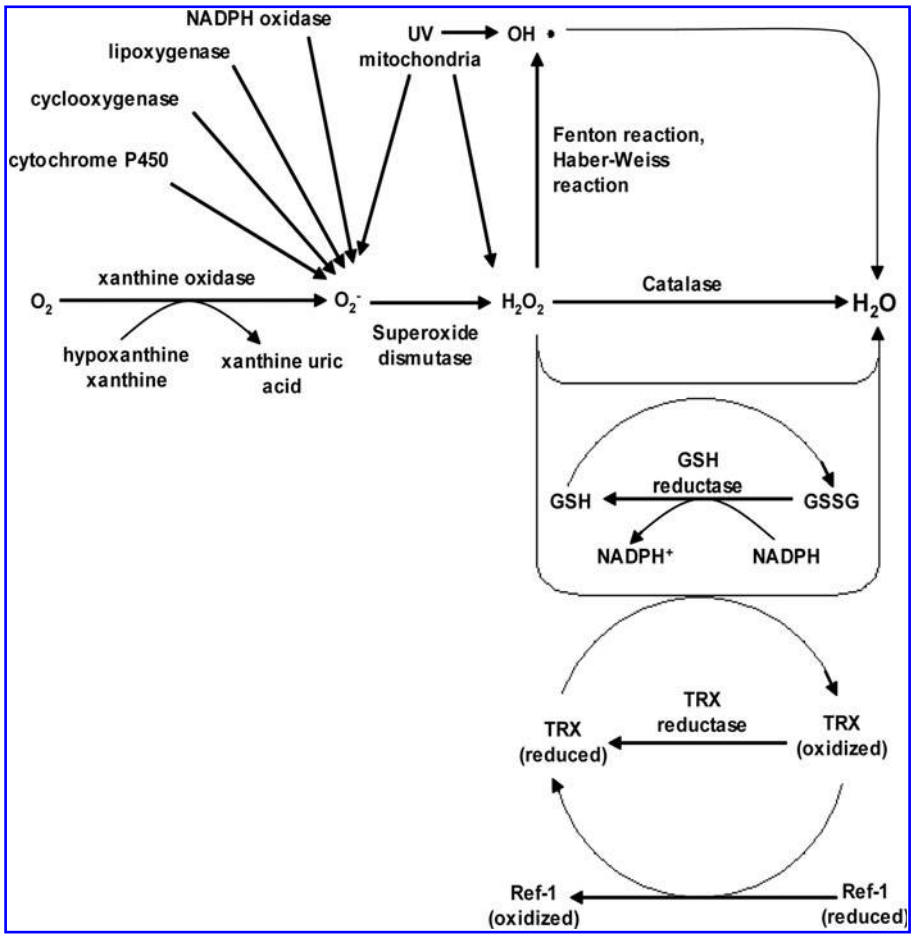

FIG. 1. Summary of ROS production and elimination. O2- is generated by cytochrome p450, lipoxygenases, cyclooxygenase, NADPH oxidase, the mitochondria, UV, and hypoxanthine/xanthine oxidase. H2O2 also is generated by the mitochondria and UV. Superoxide dismutase converts O2- into H2O2, which is degraded by glutathione (GSH) peroxidase and catalase to H2O. Thioredoxin (TRX) may also reduce H2O2 and refold oxidized proteins. H2O2 produces the highly reactive OH• by the Fenton and Haber-Weiss reactions.

O-GlcNAcylation, and acetylation (12). The consequence of these posttranslational modifications on the activity, cell localization, and stability of FOXO varies. ROS function both as direct regulators of FOXO factors, and also indirectly through modulation of growth factor–receptor activity, protein tyrosine and serine/threonine kinases and phosphatases, G proteins, lipid metabolism that interact with the factors upstream of FOXO, as described earlier. Through these mechanisms, ROS can both inhibit and activate FOXO in a context-dependent manner.

Inhibition of FOXO transcription factors by ROS

The best characterized of all FOXO regulatory pathways is the PI3K-PKB–mediated inhibition of FOXO activity, and at physiologic levels, ROS signaling is frequently associated with growth factor–receptor activation and stimulation of cellular metabolism and growth through the PI3K-PKB pathway. Hydrogen peroxide (H_2O_2) can activate the PI3K-PKB pathway through exerting insulin-mimetic effects by activation of the insulin receptor and phosphorylation of insulin receptor substrate-1 (IRS-1) (36), or by triggering phosphorylation of the endothelial/placental growth factor receptors (EGFR/PGFR) (14), or the association of the Shc-Grb2-SOS complex with EGFR (78). ROS can also activate PI3K-PKB signaling by inducing phosphorylation of the heat-shock protein HSP27 in a CAMKII-dependent manner (66), resulting in scaffolding of MK2 to the PKB signal complex, or by inhibiting the PI3K negative regulator PTEN (phosphatase and tensin homologue) (84). All of these pathways would be expected to converge on FOXO phosphorylation and inactivation, and this may explain the proliferative effects of physiologic levels of ROS.

Oxidative stress may also inactive FOXO though enhancing the activity of many kinases. Of particular relevance to FOXO transcription factors is the activation of MAPK family members, including ERK (67), p38 (35), and JNK (25), by ROS. The activation of p38 and JNK is thought to involve the MEKKK ASK1, which is negatively regulated by redox-sensitive thioredoxin (96). Oxidative stress can inactivate thioredoxin, thereby freeing ASK1 to activate p38 and JNK. Phosphorylation of FOXO1 and FOXO3a by ERK and p38 inhibit their transcriptional potential (19), but not always (10).

ROS are also by-products of the lipoxygenase and arachidonic acid pathways, and as such, they link lipid metabolism with the FOXO pathway. For example, oxidants activate phospholipase A2 (PLA2) (102), which catalyzes the hydrolysis of the sn-2 fatty acyl bond of phospholipids to yield fatty acids and lysophospholipids. The PLA2 reaction is the primary pathway through which arachidonic acid (AA) is released from phospholipids. Epoxyeicosatrienoic acids (EETs) are eicosanoids synthesized from arachidonic acid by the cytochrome P450 epoxygenase pathway and are known to inactivate FOXO, which in turn reduces the expression of its target gene $p27^{Kip1}$ and endothelial cell proliferation (76).

Oxidative stress may also inhibit FOXO through the nuclear factor kappa B (NF-κB) pathway. NF-κB signaling is enhanced in response to oxidative stress, which in turn can lead to phosphorylation and inhibition of FOXO3a in an IκB kinase (IKK)-dependent manner (39). Conversely, FOXO can increase the expression of IκBα (103), a canonic pathway-inhibitory protein. In addition, NF-κB and FOXO are linked in oxidative stress by the FOXO target *GADD45* (71).

Induction of FOXO activity by ROS

The effect of ROS or oxidative stress or both on FOXO activity is dependent on cellular context and the duration and intensity of ROS accumulation, which in turn will influence the relative amplitude and duration of kinase activation. Consistent with this idea, the ability of PKB to target FOXO is influenced by the level of ROS. For example, treatment of human 293 cells with high concentrations of hydrogen peroxide results in a reduction in inhibitory phosphorylation of FOXO1 by PKB, and therefore FOXO1 activation (63). MST1 (mammalian Sterile 20-like kinase 1) is activated in response to oxidative stress. When activated, MST1 phosphorylates FOXO1 and FOXO3a (7), thereby increasing FOXO activity by disrupting 14-3-3 binding, allowing nuclear accumulation, before other proteins remove the inhibitory phosphate groups (101). Similarly, the oxidative stress–activated MAPK, JNK, is known to increase FOXO activity by several mechanisms, including direct phosphorylation at Thr^{447} and Thr^{451} in FoxO4 (25), by phosphorylating and inhibiting the 14-3-3 proteins that sequester FOXO in the cytoplasm (89), and indirectly, by repressing PKB activity (91). Thus, similar upstream mechanisms—the activation of p38 and JNK through the oxidative stress–mediated MEKKK-MEKK pathway—converge on FOXO to yield a specific cellular response, involving both inhibition and stimulation of target-gene expression. Another kinase, MST1 (mammalian Sterile 20-like kinase 1), is also activated in response to oxidative stress. When activated, MST1 phosphorylates FOXO1 and FOXO3a (7), thereby increasing FOXO activity by disrupting 14-3-3 binding, allowing nuclear accumulation, before other proteins remove the inhibitory phosphate groups (101) (Fig. 2).

FOXO sensing ROS directly

Numerous transcription factors are redox sensitive, some of which confer either oncogenic or tumor-suppressive functions. Direct redox regulation is mediated principally by the oxidation state of the sulfhydryl group (RSH) on cysteine residues, which are easily oxidied to form a disulfonic bond (RSSR), sulfenic acid (RSOH), sulfinic acid (RSO2H), or sulfonic acid (RSO3H). ROS interact with sulfhydryl groups of the cysteine residues on proteins and oxidize them to form either intramolecular or intermolecular disulfide bonds (74). Recent evidence also suggests that FOXO proteins are subjected to redox regulation (18). For example, ROS induce the formation of disulfide bridges between cysteine residues of FOXO4 and its acetyltransferase p300/CBP, resulting in the cross-linking of FOXO4 to p300/CBP (18), which in turn is essential for the p300/CBP-mediated acetylation and regulation of FOXO4. Acetylation of FOXO by p300/CBP also increases its stability by reducing polyubiquitination (57), a modification implicated in oxidative stress responses (12). These findings indicate that FOXO proteins are both ROS sensors and mediators of the oxidative stress response, suggesting that these transcription factors function as a "homeostat" for the intracellular redox status.

Regulation of cellular ROS by FOXO factors

FOXO proteins regulate the intracellular redox environment through several mechanisms (Fig. 3). For example, activation of FOXO upregulates MnSOD (SOD2) and catalase,

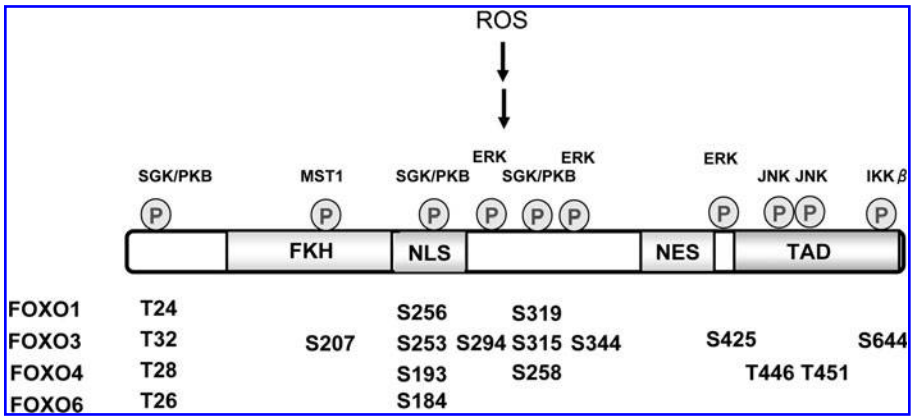

FIG. 2. Domain structure of FOXO proteins with phosphorylation sites targeted by ROS signaling. All FOXO proteins contain a Forkhead homology domain (FKH), a nuclear-localization sequence (NLS), a nuclear-export signal (NES), and a *trans*-activation domain (TAD). A number of kinase phosphorylation sites, mostly conserved between FOXO proteins, are downstream of ROS signaling. Phosphorylation by MST and JNK increase FOXO3 activity, whereas PKB, SGK, IKKβ, and ERK are inhibitory.

as well as other antioxidative stress proteins, such as sestrin 3 and PINK1 (58). Further, FOXO-mediated repression of its target gene *TXNIP*, which encodes for thioredoxin-interacting protein, enhances thioredoxin activity and decreases cellular ROS levels in glucose-treated endothelial cells (20). The biologic significance of FOXO in regulating the cellular redox environment is exemplified by knockout studies in mice. FOXO-dependent signaling is required for the long-term regenerative potential of the hematopoietic stem cell (HSC) compartment. FoxO(1/3/4)-deficient bone marrow is characterized by defective long-term repopulating activity that correlates with increased ROS levels and apoptosis of HSC (94). These observations indicate that the role of FOXO in stress resistance and longevity in *C. elegans* is evolutionarily conserved in mammalian cells (16). In this context, the interaction of FOXO factors with histone deacetylates (HDACs), such as SIRT1, is critical. SIRT1 binds and deacetylates FOXO, thereby promoting the expression of antioxidant genes, such as *MnSOD*, while simultaneously inhibiting FOXO-dependent apoptotic gene expression (12). Given the breadth and diversity of FOXO functions, particularly in relation to regulation of the life span, cellular redox status, and oxidative stress responses, it is perhaps not surprising that deregulation of this system contributes to tumorigenesis in many tissue types.

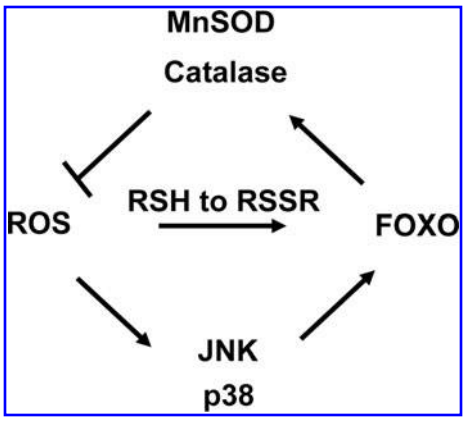

FIG. 3. Summary of the ROS and FOXO feedback signaling loop. FOXO proteins can induce the expression of antioxidant defense enzymes, including MnSOD2 and catalase, to reduce ROS levels and oxidative stress. Conversely, ROS can promote FOXO activity indirectly by activating JNK, p38, and other kinases and directly through targeting sulfhydryl group (RSH) on FOXO cysteine residues, which are easily oxidized to form a disulfonic bond (RSSR).

The ROS-FOXO Axis in Cancer

Hyperactivation of the PI3K/PKB pathway is a hallmark of many cancers and often is attributed to the loss of the tumor-suppressor gene *PTEN* (22). Consequently, inactivation of FOXO proteins is an early event in tumorigenesis, and, in this context, FOXO proteins can be classified as tumor suppressors. In agreement, mice lacking FoxO(1/3/4) develop hemangiomas and lymphoproliferative diseases, conditions associated with early neoplasms (70).

In untransformed cells, ROS are generated at low levels as a result of normal cell metabolism, but usually eliminated effectively by the potent cellular antioxidant defense system. ROS also are produced in response to activated growth factor–receptor signaling (4,13). These low levels of ROS may stimulate cell proliferation (Fig. 3). In contrast, cancer cells produce elevated levels of ROS, reflecting the increased metabolic rate, which can culminate in a continuous state of oxidative stress (92). A sustained increase in ROS production contributes to tumorigenicity and cancer progression by promoting genomic instability through increased DNA damage and reduced mismatch repair (85). In addition, the perturbed redox status may lead to activation of key signaling components important for cell proliferation and survival (73) and inhibition of FOXO3a (Fig. 4). Consistent with a role for ROS during tumorigenesis, Ras-transformed fibroblasts demonstrate elevated ROS (44), and overexpression of the superoxide-generating oxidase Mox1 is sufficient to transform immortalized NIH3T3 fibroblasts (88). Thus, sustained oxidative stress can confer growth and survival advantages to cancer cells by activating signaling pathways that promote cell proliferation and transformation. Repression of FOXO protein activity may be pivotal for ROS-induced transformation; expression of a dominant-negative FOXO3a mutant that lacks the transcriptional activation domain of transcription blocks ROS-induced cell death (63). In addition, the induction of expression of a number of proapoptotic FOXO genes, including *Bim* and *BCL-6*, in response to hydrogen peroxide, can be blocked by either silencing endogenous FOXO3a (46) or expression of the dominant-negative mutant (63). Cancer cells have evolved mechanisms that could potentially be used to

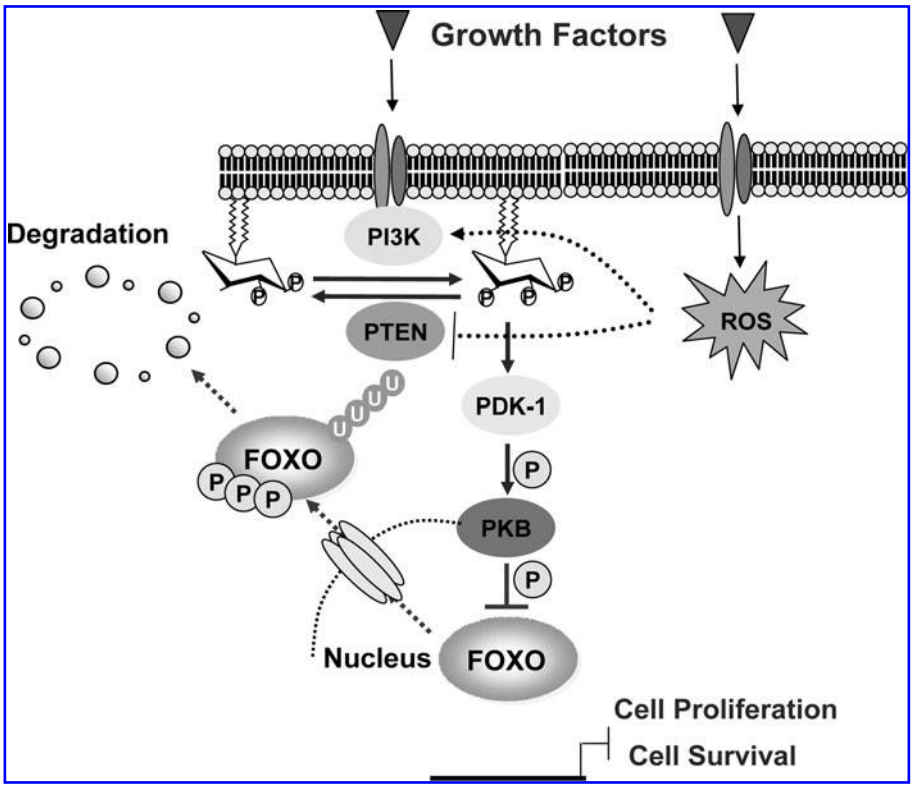

FIG. 4. Model for mechanisms by which growth factors integrate their signals through ROS with the PI3K-PKB-FOXO signaling cascade to promote cell proliferation and survival. In response to growth-factor stimulation, low levels of ROS activate PI3K and inhibit PTEN to activate PKB and repress FOXO activity.

escape this relation between ROS accumulation and FOXO-dependent *Bim*-induced apoptosis. For example, evidence suggests that colorectal carcinoma cells are addicted to ERK-mediated repression of *Bim*, which will in part be achieved by ERK-induced degradation of FOXO3a (98) and would be expected to reduce their sensitivity to oxidative stress.

In nonmalignant cells, the oxidative stress defense response is mediated in part by FOXO-dependent induction of MnSOD, catalase, and GADD45 expression. This antioxidant defense response has also been shown to be important for regulation of longevity by the FoxO orthologue Daf-16 in the nematode worm *C. elegans* (61). Consistent with a role for FoxO proteins in reducing ROS levels, hematopoietic stem cells (HSCs) isolated from *FoxO1/3/4* triple-knockout mice show increased intracellular ROS levels (94). Hyperactivation of the PI3K-PKB pathway during tumorigenesis disables the ability of FOXO to detoxify ROS, leading to a build-up of intracellular ROS levels, continuous oxidative stress, and cancer progression. However, increased cellular ROS levels can also activate stress-activated kinases, including JNK, that enhance the transcriptional potential of FOXO proteins (24). Together, these findings point to a tightly regulated feedback loop between ROS and FOXO proteins, with ROS regulating and sensing FOXO activity and FOXO factors controlling intracellular ROS levels.

Role of ROS in Chemotherapy-induced and Physiologic Apoptosis

ROS are key intermediates in the process of apoptosis induced by numerous agents (53). For example, TNF-α induces cell death in a ROS-dependent manner (31), and ROS are implicated in TGF-β–mediated cell death (81). In addition, depletion of essential IL-3 from the medium of B lymphoma BaF3 cells triggers ROS production followed by cell death, which can be prevented by antioxidants or BCL-2 overexpression (38). The BCL-2 family of proteins may play an important role in protecting cells from ROS-induced cell death by preventing mitochondrial permeability and loss of $\Delta\psi m$, which would result in further mitochondrial ROS production (11). Given the abnormal oxidative environments of cancer cells, and the complicated relation between ROS and tumorigenesis, it is perhaps not surprising that ROS are important effectors of chemotherapeutic drugs (Fig. 5).

Good evidence suggests that the chemotherapeutic agents, such as doxorubicin and cisplatin, trigger apoptosis in cancer cells, as well as cause cytotoxic side effects in nonmalignant tissues, at least partly through the induction of cellular oxidative stress (95). Contrary to the tumor-promoting activities of ROS, the acute increase in intracellular ROS induced by chemotherapeutic drugs triggers cell-cycle arrest and apoptosis when it outstrips cellular antioxidant defenses. Surprisingly, cancer cells are more sensitive to an acute increase in ROS levels than are nontransformed cells. For example, increased cellular ROS levels induced by arsenic trioxide specifically eliminate cancer cells (72). This is because oncogenic transformation elevates ROS to such high levels that a further acute increase triggers reactivation of the apoptotic program in cancer cells (95). Conversely, normal cells are less sensitive to these therapeutic prooxidants because of their relatively low basal ROS levels and high antioxidant capacity. In other words, the excessive levels of ROS in cancer cells can be their "Achilles heel" when it comes to anticancer treatments. Thus, ROS can stimulate proliferation and promote the cellular transformation phenotype but also induce oxidative apoptosis, especially in cancer cells.

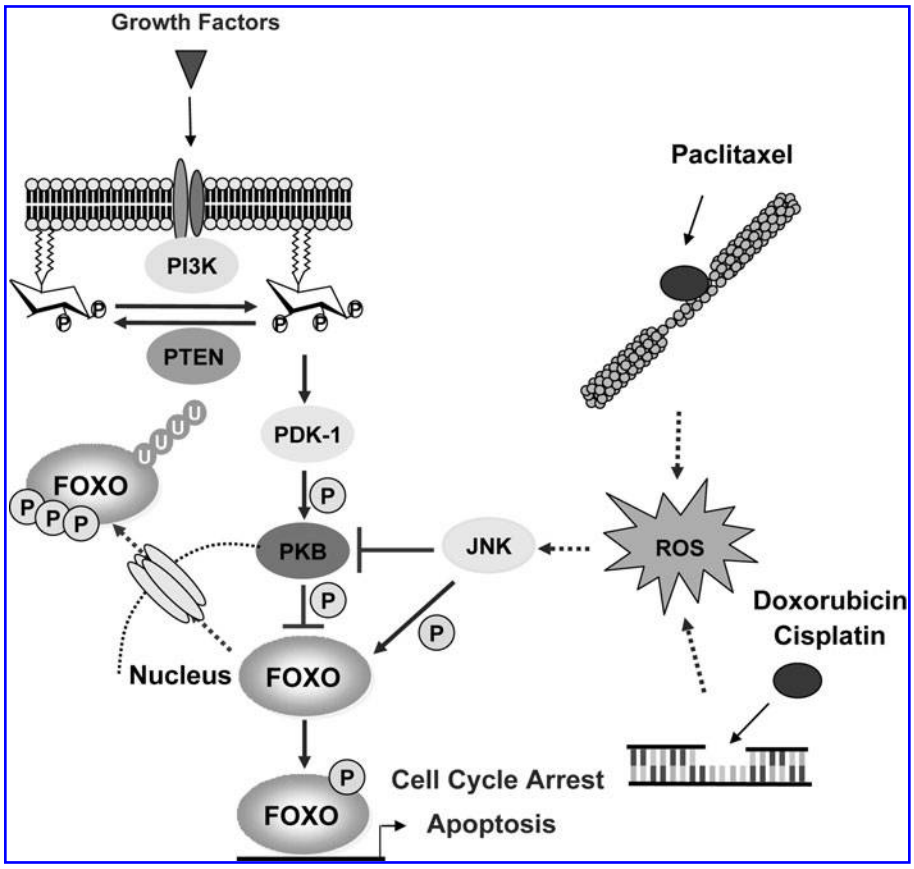

FIG. 5. Model for mechanisms by which chemotherapeutic drugs integrate their signals through ROS with the PI3K-PKB-FOXO signaling cascade to induce cell-cycle arrest and apoptosis. Chemotherapeutic drugs, such as paclitaxel (taxol), cisplatin, and doxorubicin, ultimately integrate their signals with the PI3K-PKB-FOXO signaling cascade through ROS. Taxenes, such as paclitaxel (taxol), stabilize microtubules and, as a consequence, interfere with the normal microtubule assemble/disassemble dynamics during the cell-division cycle. Cisplatin is a platinum-derived compound that binds to DNA and causes the formation of platinum-DNA adducts, which ultimately trigger apoptosis (programmed cell death). Doxorubicin is an example of anthracycline antibiotics, which function by intercalating DNA and causing DNA damage. High levels of ROS inhibit PI3K and PKB activity and activate FOXO to trigger cell-cycle arrest and apoptosis.

The Role of FOXO Proteins and ROS in Cancer Drug Responses

ROS and FOXO as targets for cancer treatment

The principal mechanism of action for many traditional anticancer drugs is the induction of DNA damage (28). For example, epirubicin intercalates into DNA and represses topoisomerase II activity, whereas platinum-based compounds cause cross-linked DNA adducts (79). Another class of common classic anticancer drugs are taxanes, which include paclitaxel (Taxol) and docetaxel. These taxanes are spindle poisons that disrupt normal microtubule function through stabilizing GDP-bound tubulin within the microtubule. Because microtubules are involved in cell division, taxanes are essentially mitotic poisons. Although all these chemotherapeutic agents act primarily in different manners, the cytostatic and cytotoxic responses invariably involve cell-cycle arrest or apoptosis induced by FOXO proteins. FOXO3a is a key mediator of the cytotoxic effects of cisplatin in colon cancer. Treatment of drug-sensitive colon cancer cells with cisplatin induces FOXO3a dephosphorylation, nuclear translocation, and activation, leading to cell death (27). Conversely, FOXO3a activity is not modulated by cisplatin in the resistant colon cancer cells. In agreement, the PKB specific inhibitor API-2/triciribine, which is currently assessed in clinical trials, dephosphorylates FOXO3a and resensitizes resistant colon cancer cells to cisplatin treatment. Conversely, silencing of FOXO3a expression by using small interfering RNA can protect drug-sensitive colon carcinoma cells from cisplatin-induced cell death (27).

Doxorubicin has been shown to induce ROS production via one-electron reduction to the corresponding semiquinone free radicals, which then react rapidly with oxygen to generate superoxide radical anions (65). Subsequent activation of stress-activated MAPK p38 and JNK phosphorylates and enhances FOXO activity (65). In agreement, FOXO4 has been shown to sensitize cancer cells to doxorubicin-mediated cytotoxicity (54). Studies on breast cancer cell lines revealed that a correlation exists between the expression of FOXO proteins and the sensitivity to paclitaxel, with more responsive cell lines expressing higher levels (90). For example, it has also been shown that exposure of breast cancer cells to taxanes, such as paclitaxel, triggers cell death by upregulation of FOXO3a and downstream target Bim (90, 91). This finding also indicates a potential prognostic role for both FOXO3a and Bim in predicting paclitaxel responsiveness in breast cancer. Similarly, FOXO1 has also been implicated in the cytotoxic stress and drug-resistance induced by paclitaxel in ovarian cancers (32). FOXO3a also induces cell death in primary endometrial cells and hemopoietic cell lines exposed to oxidative stress (46), and its expression in CML cell lines leads to cell-cycle arrest followed by apoptosis (23, 26). In addition, JNK activates FOXO3a in paclitaxel-treated breast cancer cells (91), by antagonizing inhibitory PKB-dependent inactivation of FOXO3a (89), by direct phosphorylation and activation of FOXO (25), and by phosphorylating and inhibiting the FOXO-chaperone protein 14-3-3 (89).

Chemotherapeutic drugs are known to induce oxidative stress by stimulating ROS production directly and indirectly (95). DNA damage activates ATR (ataxia telangiectasia

mutated and Rad3 related), which triggers the damage-repair response and activates the G_2/M checkpoint (15). During normal cell-cycle progression, and in the absence of a DNA insult, FOXO1 is inhibited on phosphorylation of Ser^{249} by CDK1 and 2, thus promoting cell-cycle progression (40, 51). On DNA damage, however, FOXO1 escapes inactivation because of inhibition of CDK2 via Chk1 and Chk2 (40), which in turn promotes cell-cycle arrest by downregulating cyclins B1 and B2 (93), or upregulating cyclin G2, a cyclin B-CDK1 inhibitor (55). If the ROS-induced DNA-damage is too extensive, FOXO induces the expression of proapoptotic mediators, including Bim and bNIP3, as well as TRAIL and FasL (29). However, the induction of cell death by ROS also involves several other mechanisms that are indirectly linked to FOXO factors. For example, ROS may induce cell death through the production of ceramide (97). Furthermore, ceramide increases oxidative damage in HL-60 leukemic cells due to inhibition of catalase by caspase-dependent proteolysis (45), establishing a positive-feedback loop between ROS and ceramide. A downstream consequence is p38 MAPK activation and, hence, it is possible that FOXO activation also plays a role in the ceramide response. ROS also play a critical role in p53-induced cell death (75) and link to FOXO in this manner. For example, a splice variant of $p52^{Shc}$/$p46^{Shc}$, $p66^{Shc}$ has been found to act as a downstream target of p53, and is indispensable to the ability of stress-activated p53 to induce elevation of intracellular oxidants, cytochrome *c* release, and apoptosis (59). Recent evidence suggests that $p66^{Shc}$ links α_1-adrenergic receptors to a ROS-dependent PKB-FOXO3a phosphorylation pathway in cardiomyocytes (34), whereby PKB signaling phosphorylates/inactivates FOXO and downregulates *MnSOD*.

In addition to the induction of apoptosis, cancer therapy sometimes aims to induce the differentiation of cancer cells, and depart from a cancer cell stem-like phenotype. Both ROS and FOXO play important roles in differentiation; for example, highly phosphorylated FOXO3a is an adverse prognostic factor in acute myeloid leukaemia, in part through deregulation of differentiation. In this context, it is likely that the regulation of oxidative stress by FOXO factors, rather than FOXO by ROS is critical. The role that ROS play in the FOXO-mediated regulation of differentiation is not fully understood. However, FOXO3a has previously been reported to induce erythroid differentiation of the leukemic K562 cells through repressing the transcription of the Inhibitor of Differentiation 1 (*ID1*) gene by binding directly to the *ID1* gene promoter (5). This induction of FOXO3a and downregulation of ID1 expression are essential for imatinib-induced differentiation (5).

ROS and FOXO in cancer drug resistance

As outlined earlier, FOXO proteins, and particularly FOXO3a, have a crucial role in mediating the cytostatic and cytotoxic effects of anticancer drugs (30, 37). However, and rather paradoxically, high levels of active nuclear FOXO3a are found in multidrug-resistant leukemic cells (42, 43). In these cells, the resistance is a consequence of prolonged drug exposure, raising the possibility that continuous elevated FOXO activity may be critical in acquired drug resistance.

A number of mechanisms confer resistance in cancer cells, including enhanced oxidative stress defense, amplification of cell-survival signals, increased DNA-damage repair, and altered cellular drug uptake, efflux, or metabolism (30). It is well established that FOXO proteins can induce expression of catalase, *MnSOD*, and *GADD45* in response to oxidative stress and DNA damage (60). It is therefore not surprising that FOXO proteins, through regulating the expression of these target genes, can counteract the oxidative stress and DNA damage induced by chemotherapy. In addition, activation of FOXO proteins may also be playing a role in buffering the oxidative stress driven by high ROS and proliferative levels in cancer cells. Consistent with this idea, we recently reported that doxorubicin-resistant leukemic cells display higher levels of active FOXO3a (42, 43). Subsequent analysis demonstrated that the nuclear unphosphorylated FOXO3a was the primary drive behind the hyperactivation of the PI3K-PKB pathway in these cells, thereby promoting survival and resistance to doxorubicin and other drug treatments (42, 43). *PIK3CA*, the gene encoding p110α, the catalytic subunit of class 1A PI3K, was identified as a direct FOXO3a target gene responsible for amplification of the PI3K-PKB pathway in drug-resistant CML cells (43). Similarly, FOXO1 is also capable of enhancing PKB phosphorylation in hepatocytes, by repressing the expression of Tribble 3 (TRB3), a pseudokinase capable of binding and inhibiting PKB activity (56). The FOXO orthologue in *D. melanogaster* has also been shown to be able to induce the expression of the insulin receptor (dInR) (77), resulting in increased PI3K activity and cell growth under low nutrient conditions. Furthermore, the transcripts of IGFR1 (insulin-like growth factor 1 receptor), a known upstream regulator of insulin and PI3K pathway, are induced on FOXO3a activation in the K562 leukemic cells (43). Consistent with these observations, recent DNA microarray studies identified *IGFR1* and *PIK3CA* as FOXO gene targets in a colon carcinoma cell line (21) and *IGFBP1* and *INSR* (insulin receptor) in human endometrial stromal cells (93). Together, these findings provide unequivocal evidence that FOXO transcription factors can engage in an evolutionarily conserved feedback mechanism that enhances the activity of the upstream PI3K-PKB pathway, although the specific underpinning mechanisms may be highly cell specific. This FOXO-PI3K-PKB feedback loop may constitute an important survival mechanism when cells are exposed to oxidative or cytotoxic stress (30, 43, 77). However, the FOXO-PI3K-PKB loop is evidently uncoupled and the homeostatic mechanism disrupted in drug-resistant cancer cells, resulting in constitutive nuclear FOXO3a activity in conjunction with PI3K-PKB hyperactivity (30, 43).

In addition to promoting cell survival, FOXO proteins can also enhance drug resistance by activating multidrug-resistant genes, such as *ABCB1,* important for drug efflux and metabolism in resistant cancer (42). The *ABCB1* gene product P-glycoprotein is a cellular membrane transporter that mediates ATP-dependent efflux of a wide variety of hydrophobic anticancer drugs, including taxanes (*e.g.*, paclitaxel and docetaxel), anthracyclines (*e.g.*, doxorubicin, daunorubicin, and epirubicin), and other chemotherapeutic agents that enter cells freely by passive diffusion (42).

Collectively, these results provide crucial evidence for the crucial role of FOXO proteins in the development of acquired drug resistance (Fig. 6). However, the molecular mechanism that allows drug-resistant cells to tolerate high levels of active FOXO protein, without triggering an apoptotic response, is as yet undefined but likely to involve posttranslational modifications, such as phosphorylation and acetylation, capable of

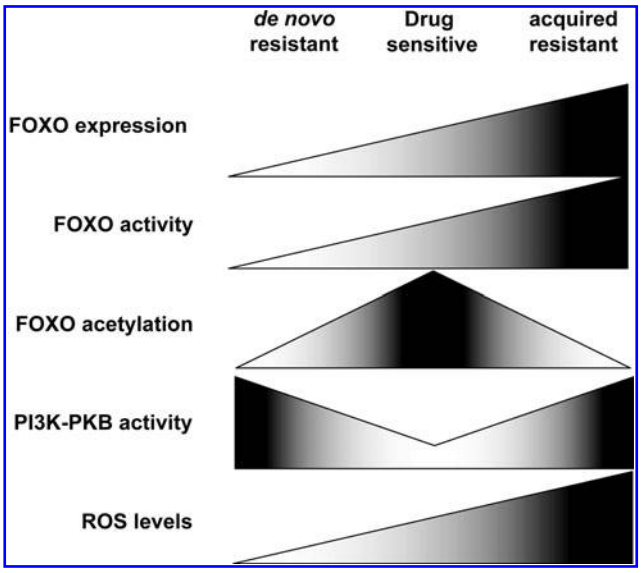

FIG. 6. Diagram describing the dual role of FOXO transcription factors in regulating drug sensitivity and resistance. The hypothetical levels of FOXO expression, activity, acetylation, PI3K-PKB activity, and ROS are shown in chemotherapeutic drug–sensitive and –resistant cancer cells.

altering the nature of the gene networks under FOXO control. For example, phosphorylation of FOXO by JNK promotes its acetylation, leading to activation of proapoptotic genes. Conversely, recruitment of SIRT1 deacetylates FOXO proteins, and favors the activation of FOXO target genes involved in cellular defenses against oxidative stress (8). This mechanism may also underpin the relative resistance of normal cells to the cytotoxic effects of oxidative stress and chemotherapy.

Targeting the FOXO-ROS axis: SIRT inhibition

The activity of FOXO proteins is regulated in part by acetylation. SIRTs (sirtuins) are NAD^+ (nicotinamide adenine dinucleotide-positive)-dependent class III HDACs (6). SIRT1, the mammalian homologue of the yeast Sir2, can enhance cell survival by deacetylating FOXO proteins, thereby altering their transcriptional output (8). In this respect, SIRT1 has a direct role in tumorigenesis. For example, SIRT1 can activate oxidative stress defense and DNA-repair mechanisms, thus promoting survival of the cancer cell (1). SIRT1 also antagonizes cellular senescence in human primary diploid fibroblasts (41). Consistent with a role in cancer development, SIRT1 is overexpressed in a range of malignancies, including lymphomas, leukemia, sarcomas, prostate, lung and colon cancer, as well as in drug-resistant cancers (49). It has been proposed that cancer and drug-resistant cells are "addicted" to high levels of SIRT1 activity, rendering them more vulnerable to SIRT inhibition compared with normal cells. For example, SIRT1-dependent deacetylation of FOXO drives the expression of antioxidant genes, including *MnSOD*, catalase, and *GADD45*, suggesting that SIRT1 inhibition may restore the tumor-suppressor functions of FOXO, especially in drug-resistant cells. Synthetic SIRT inhibitors with high potencies, including EX527 (64), sirtinol, and salermide (48), have now been developed, and these compounds, in combination with conventional cytotoxic agents, may potentially circumvent drug-resistant cancers (Fig. 7).

Conclusion and Perspective

The cellular redox environment depends on a balance between ROS production and elimination, two processes regulated by FOXO transcription factors. Conversely, ROS also control FOXO activity. Although physiologic levels of ROS are involved in growth-factor signaling, accumulation of free radicals is also an evolutionarily conserved mechanism of initiating apoptosis in normal biologic processes, as well as in response to cytotoxic drugs. The high rate of proliferation in cancer cells requires a considerable expenditure of energy, increased metabolism, and thus enhanced ROS production, which in turn modulates FOXO activity and promotes disease progression. Yet chronically elevated ROS levels also underpin the ability of some chemotherapeutic drugs and prooxidants to induce apoptosis selectively in malignant cells while leaving normal cells relatively unaffected. In other words, whereas the ROS-FOXO homeostat is reset in cancer cells, it remains a major target for cancer treatment and for resensitizing drug-resistant malignancies. Recent advances in cancer research have led to the development of more-specific or "targeted" therapies; for example, small molecules or biologic agents that selectively interfere with molecules involved in tumor cell survival, growth, angiogenesis, and invasion

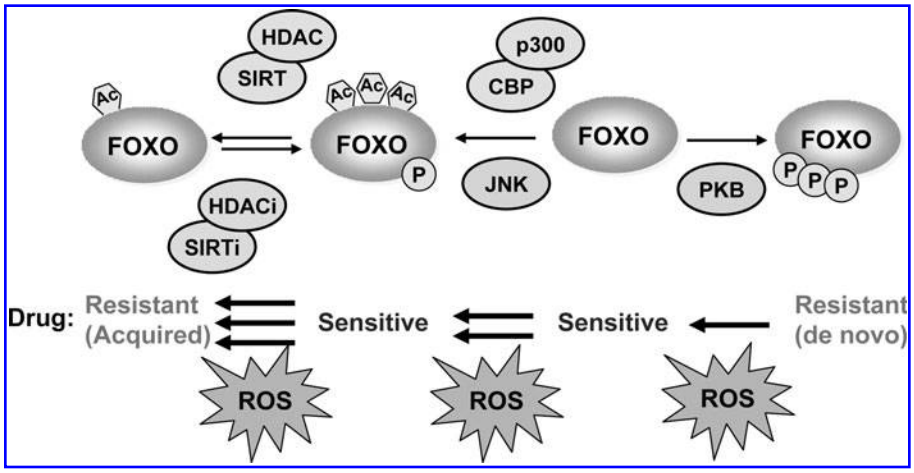

FIG. 7. The posttranslational modifications and regulators of FOXO that determine drug sensitivity and cell fate in response to chemotherapy and oxidative stress. Posttranslational modifications: Ac, acetylation; P, phosphorylation.

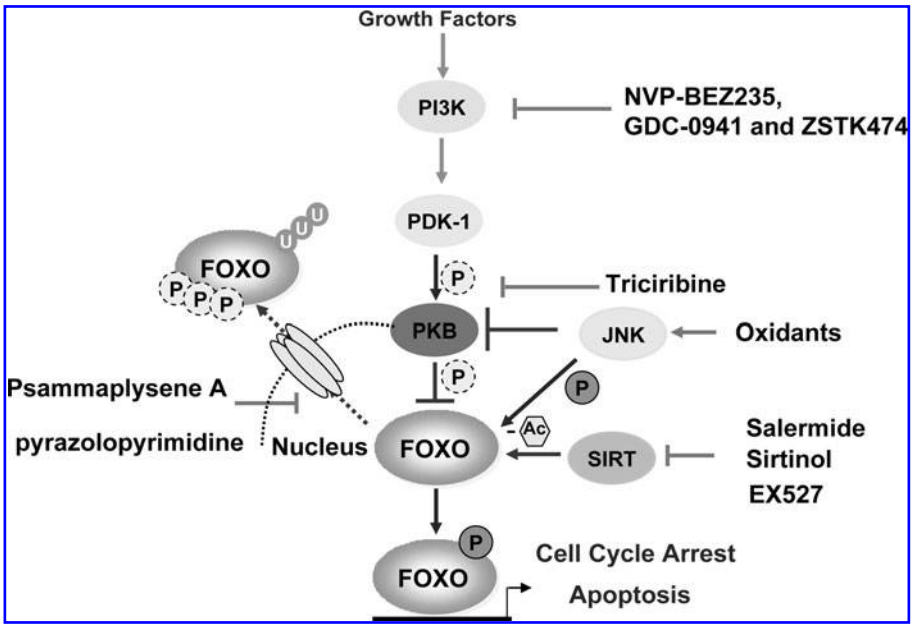

FIG. 8. Potential therapeutic strategies for targeting the FOXO to restore cell-cycle and cell-death control and to resensitize cancer cells to chemotherapy.

(80). Targeted activation of FOXO3a, for example, in response to pyrazolopyrimidine derivatives, is already being evaluated in preclinical studies for its potential to improve the effectiveness of conventional chemotherapy (50). Another such compound is the marine sponge *Psammaplysilla* sp.–derived bromotyrosine product psammaplysene A (83). It is a naturally occurring inhibitor of FOXO1 nuclear export and has been demonstrated to trigger apoptotic cell death in FOXO1-expressing endometrial cancer cell lines and to sensitize colorectal cancer cells to the cytotoxic effects of cisplatin (27). Similarly, PI3K and PKB inhibitors (*e.g.*, NVP-BEZ235, GDC-0941, ZSTK474, and triciribine) can also be used to target cancer cells that have enhanced levels of PI3K-PKB activity and are addicted to the survival signals (22). SIRT inhibitors could be valuable to restore the tumor-suppressor activity of FOXO proteins and to resensitize resistant malignancies to cytotoxic drugs (Fig. 8). Moreover, additional analysis of the ROS-FOXO axis may well yield biomarkers that could be used for monitoring drug action and in personalized cancer care.

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Abbreviations Used

ABC = (ATP)-binding cassette
ASK1 = apoptosis signal-regulating kinase
BCL-6 = B-cell lymphoma 6
Bim = Bcl-2-interacting mediator of cell death
CaMKII = Ca^{2+}/calmodulin-dependent protein kinase II
CDK = cyclin-dependent kinase
e^- = electron
ERK = extracellular signal-regulated kinase
FasL = Fas ligand
FOXO = forkhead box class O
GADD45 = growth arrest and DNA damage
Grb2 = growth factor receptor-bound protein 2
H^+ = hydron
HDAC = histone deacetylase
HSP = heat-shock protein
IGFBP1 = insulin-like growth factor–binding protein 1
JNK = c-Jun N-terminal kinase
MEKKK = MEK kinase kinase
MK2 = MAPK-activated protein kinase-2
MnSOD = manganese superoxide dismutase
O-GlcNAc = O-linked *β-N*-acetylglucosamine
p38 = p38 mitogen-activated protein kinase
PI3K = phosphoinositide 3-kinase
PKB = protein kinase B (also called AKT)
ROS = reactive oxygen species
SIRT1 = silent mating type information regulation 2 homologue 1
SOS = son of sevenless guanine nucleotide exchange factor
Thx2 = thioredoxin-2
TNF-α = tumor necrosis factor-alpha
TRAIL = tumor necrosis (TNF)-related apoptosis-inducing ligand

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