Post-translational modifications of FOXO family proteins (Review)

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Abstract. The Forkhead box O (FOXO) protein family is predominantly involved in apoptosis, oxidative stress, DNA damage/repair, tumor angiogenesis, glycometabolism, regulating life span and other important biological processes. Its activity is affected by a variety of posttranslational modifications (PTMs), including phosphorylation, acetylation, ubiquitination, methylation and glycosylation. When cells are subjected to different environments, the corresponding PTMs act on the FOXO protein family, to change transcriptional activity or subcellular localization, and the expression of downstream target genes, will ultimately affect the biological behavior of the cells. In this review, we will discuss the biological characteristics of FOXO protein PTMs.

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1. Introduction

In 1989, Weigel *et al* (1) first cloned the fork genes (fkh) from fruit flies, and discovered that the encoded protein was essential for the normal development of embryos (1,2). Currently, researchers have identified >100 types of Forkhead box (Fox) proteins present in almost all eukaryotes from yeast to humans (3). Fox protein families have a conserved DNA-binding region, which can specifically bind to the conserved DNA sequence 5'-TTGTTTAC-3' (4). The spatial structure of the Fox protein exhibits a 'helix-turn-helix' structure, which resembles a fork, thus, providing the name 'fork proteins' (5). Furthermore, through analyzing the similarity of the conserved region of the amino acid sequence, Fox proteins are divided into the 'A' and 'S' subtypes (6,7). At present, the FOXO protein family is one of the most widely studied protein families. In mammalian species, the FOXO subfamily includes FOXO1, FOXO3, FOXO4 and FOXO6. FOXO1, FOXO3 and FOXO4 are widely expressed in various tissues and organs (8), however the expression of FOXO6 is most often detected in the developing brain (9), and recent studies have demonstrated that FOXO6 is involved in cell growth and transformation of liver cancer and lung cancer (10,11). FOXO homolog genes, DAF-16 and dFOXO, exists in lower organisms, including *Caenorhabditis elegans*, *Drosophila* and sponges (12).

FOXO proteins control a series of cellular behaviors, including apoptosis, cell cycle, cell differentiation, cell proliferation, energy metabolism and autophagy (13), by activating or inhibiting downstream targets, via transcriptional regulatory functions. As FOXO proteins have important effects on the cell, changes in the expression of FOXO proteins are associated with the physiological or pathological processes of aging, angiogenesis, cancer, diabetes, infertility (14), immune system disorders (15) and neurodegeneration (16).

FOXO proteins are activated by various extracellular stimuli, including growth factors, cytokines and hormones. In order to further reveal the molecular mechanisms of FOXO proteins in transcriptional regulation, more studies have focused their attention on posttranslational modifications (PTMs). Currently, there are >400 types of protein PTMs have been determined, including phosphorylation, acetylation,

ubiquitination, methylation, glycosylation, small ubiquitin-like modification and nitrosylation. Different PTMs change the expression of FOXO target genes, and affect the activity, subcellular localization, DNA binding activity and half-life of FOXO proteins via different enzymatic reactions (17).

This review aims to summarize the PTM mechanisms that regulate FOXO proteins, and to further clarify the transcriptional regulatory role of FOXO proteins and detect novel drug targets for cancer therapy.

2. Phosphorylation of FOXO proteins

Phosphorylation, induced by a variety of protein kinases, is the most important PTM of FOXO proteins. Different enzymes regulate phosphorylation at different sites on FOXO proteins to produce different biological effects (Table I).

Akt serine threonine kinase (Akt) and serum/glucocorticoid regulated kinase (SGK). Akt (also termed protein kinase B) and SGK are major downstream targets of the phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) pathway, and furthermore, are the most extensively researched serine/threonine kinases involved in FOXO protein phosphorylation. These two kinases recognize the same phosphorylation substrate motif, RXRXXS/T (R, arginine; X, any amino acid; and S/T, serine/threonine). Akt phosphorylates FOXO1 at three sites, in particular, phosphorylation at Ser256 can change the positive charge of FOXO1 DNA binding domain to negative, thus, reducing the activity of FOXO1 (18,19). Akt and SGK phosphorylate FOXO3 at same sites, however SGK preferentially phosphorylates at Ser315, and Akt at Ser253. It has been reported that the process of FOXO3 phosphorylation is regulated by Akt and SGK synergistically (20).

In addition, the binding of FOXO with 14-3-3, which is a key protein involved in the translocation of FOXO proteins from the nucleus to the cytoplasm, requires FOXO phosphorylation at specific sites. For example, following phosphorylation, FOXO3 can combine with 14-3-3, this significantly changes the structure of the FOXO nucleus localization signal. Typically, binding to 14-3-3 increases nuclear export of FOXO proteins and decreases reentry into the nucleus (21,22). Additionally, the dissociation of FOXO3 from 14-3-3 is mediated by protein phosphatase 2, which dephosphorylates FOXO3 at Thr32/Ser253 (22). Studies have reported that phosphorylation of FOXO1 at Thr24 by Akt is necessary and sufficient for its binding to 14-3-3, however, when exclusion of FOXO1 from the nucleus id induced by insulin-like growth factor-1 (IGF-1), combining with 14-3-3 is not required. This result suggests that the translocation process of FOXO proteins is not only 14-3-3-dependent (23).

Changing the subcellular distribution of the FOXO proteins is not the only way to reduce its transcriptional activity. For example, FOXO1 transcriptional activity can be inhibited by activation of Akt signaling pathways induced by insulin, however, when the key site (Leu375) of the nuclear export signal, which regulates the transfer of FOXO1 to the cytoplasm, is mutated to alanine, FOXO1 loses the ability to relocalize, however, notably, insulin, remains able to inhibit FOXO protein activity (24). Furthermore, FOXO4 phosphorylation at Thr28 and Ser193 downregulates the transcription activity of the protein by suppressing DNA binding (22,25). In addition, Akt also induces the degradation of FOXO via the proteasomal pathway (26-28). The E3 ligase, S-phase kinase associated protein 2 (Skp2), suppresses FOXO1 transactivation, and eliminates its effect on inhibition of cell proliferation. The normal function of Skp2 phosphorylates the Ser256 site of FOXO1, and subsequently, causes degradation of FOXO1 via the proteasomal pathway (29).

Akt achieves its function of phosphorylating to regulating FOXO proteins though the interaction with various factors that form a complex loop (30). PKR-like ER kinase (PERK) phosphorylates FOXO1 at Ser298, Ser301 and Ser303, but prefers Ser298 site, which is not a target of Akt. Notably, PERK phosphorylates FOXO1 and increased the transcriptional activity, which is in contrast with the effect of Akt. This suggests that FOXO phosphorylation induced by PERK can counteract the phosphorylation effect of Akt. Research has demonstrated that PERK can directly upregulate the activity of FOXO proteins, or decrease its activity via indirect effects on Akt. Furthermore, the effects of PERK on FOXO protein increases the expression of insulin receptor, which increases the activity of AKT, and the activation of AKT, in turn, inhibits the activity of the PERK (31). Thus, the higher activation of AKT, the less PERK activity, comparably (32,33).

Mammalian sterile 20-like kinase (MST) and Jun-N-terminal kinase (JNK). MST has an important role in the regulation of cell size and apoptosis. Upon oxidative stress, MST1 binds and phosphorylates FOXO3 (34,35). Phosphorylation of FOXO3 mediated by MST1 disrupts its connection with 14-3-3, prompting the accumulation of it in the nucleus, and therefore, upregulates the expression of pro-apoptotic genes that induce neuronal cell death (36). Certain studies have reported that MST1 can affect the expression of downstream target genes of FOXO1 by similar mechanisms (37). Furthermore, the MST1-FOXO pathway has a significant role in drug treatment-induced cancer cell death (38). When cells are under to oxidative stress, the JNK-dependent signaling pathway causes phosphorylation of FOXO4, thereby inducing FOXO4 nuclear translocation and activity (39). JNK mediates the FOXO activity via the correct assembly of JNK interacting protein 1 (JIP1) complexes, which includes three substrates, mitogen-activated protein kinase (MAPK) kinase kinase 11, MAPK kinase 4 and JNK. The correct assembling of this complex is involved in the function of the JNK signaling pathway, thus, it can affect the activity of FOXO transcription factors directly. Additionally, Ras-like proto-oncogene A (RALA) is a small GTPases of the Ras superfamily and induces the correct assembly of the JIP1 scaffolding protein complex. Via reactive oxygen species (ROS) and RALA-dependent pathways, FOXO4 is induced and fully phosphorylated (39,40). The activation of FOXO induced by JNK, not only depends on its phosphorylation, but also on other interacting proteins or pathways. For example, JNK phosphorylates 14-3-3 and releases FOXO3 from 14-3-3, which antagonizes the effects of Akt signaling (41). In pancreatic HIT cells, the JNK signaling pathway may reduce the activity of Akt and, thus, reduce the phosphorylation of FOXO1 (42). It has been reported that JIP1 serves a key role

in the regulation of Akt activity, however the specific mechanism remains unclear (43).

Extracellular signal-regulated kinase (ERK)/p38. MAPKs include three members, ERK, JNK and p38. Under oxidative stress, FOXO1 is phosphorylated by ERK and p38 cooperatively (44). Accordingly, p38 can induce phosphorylation of FOXO3 following treatment with doxorubicin (45). Recent studies demonstrated that FOXO3 phosphorylation mediated by p38β, upregulates BCL2 interacting protein 3 expression, resulting in mechanistic target of rapamycin complex 1 inhibition and cell apoptosis (46). ERK1/2 can directly phosphorylate FOXO3a, and phosphorylated FOXO3a is subsequently degraded by MDM2 proto-oncogene (MDM2), which is similar to E3 ligase Skp2, to negatively regulate the transcriptional activity of FOXO3a (47). In addition, ERK can decrease the activity of the FOXO protein through phosphorylation of other proteins. Previous studies have reported the association between ERK, p66 shc adaptor protein 1 (p66shcA) and FOXO3. The phosphorylation of p66shcA Ser36 is required for phosphorylation of FOXO3a, which can be induced by ERK1/2 (48). In cardiac fibroblasts, activation of ERK1/2 can directly phosphorylate FOXO3a and regulate its activity, and ERK1/2 can also phosphorylate Skp2 in the same manner. Notable, a report previously demonstrated that Skp2 can inhibit the activity of FOXO3a and promote its degradation (48,49). SGK is another link between MAPK and FOXO proteins. When DNA is damaged, phosphorylated SGK1 reduces the activity of FOXO3a through ERK1/2. In addition, ERK and Akt can regulate the function of each other, for example, following oxidative stress, inhibiting PI3K can change the distribution of ERK1/2 phosphorylation in the cell. However, inhibition of ERK can also affect the phosphorylation of AKT (50).

Cyclin-dependent kinases (CDKs). The initiation of the cell cycle depends on the activity of complexes of cyclins and CDKs. CDK2 can specifically phosphorylate FOXO1 at Ser249, and induces transfer of FOXO1 to the cytoplasm, ultimately reducing its transcriptional activity (51). In addition to CDK2, CDK1 can also phosphorylate FOXO1 at Ser249 (52). Research has demonstrated that, in prostate cancer cells, CDK1 phosphorylates FOXO1 at Ser249, thereby, inhibiting the transcriptional activity of FOXO1 and reducing the effects of FOXO1 on mitosis. FOXO1 phosphorylation induced by CDK1 not only eliminates the effect of FOXO1 on cell death, but also reduces the inhibitory effects of FOXO1 on the proliferation of malignant transformed cells (53). In addition to CDK1 and CDK2, a recent study suggested that the CDK5 can influence the transcriptional activity of FOXO1 directly. Different extracellular stimuli, including oxidative stress, ischemia reperfusion and serum starvation, can induce CDK5, which leads to phosphorylation of FOXO1 at Ser249. Furthermore, the CDK5/P25 complex significantly increases the binding of FOXO1 and 14-3-3 protein, thereby promoting FOXO1 retention in the cytoplasm, and ultimately, inhibits its transcriptional activity. Phosphorylation of FOXO1 at Ser249 mediated by CDK5 can inhibit the retention of FOXO1 in the cytoplasm induced by downregulation of Akt activity. This research suggests that CDK5 may regulate the function of FOXO1 by direct and indirect mechanisms (54).

Adenosine monophosphate-activated protein kinase (AMPK). In mammals, AMPK phosphorylates human FOXO3 at six regulatory sites (Table I) (55). The inactivation of p38α triggers nuclear translocation of FOXO3a in an AMPK-dependent manner, and leads to subsequent activation of FOXO3a targets genes, which induces autophagy, cell-cycle arrest and cell death (56). AMPK activity is necessary for defending against ROS-induced injury, and the molecular mechanism responsible for this effect. AMPK directly phosphorylates human FOXO1 at Thr649, which is critical for FOXO1 nuclear localization, stabilization and transcriptional activity, and the phosphorylation event leads to increased expression of the anti-oxidant enzymes, manganese superoxide dismutase (MnSOD) and catalase (57).

AMPK-FOXO3a signaling is critical for regulation of energy homeostasis and oxidative stress in cells (58). It has been reported that the activation of AMPK by 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) results in increased FOXO1 mRNA and protein levels, and strongly enhances FOXO1 transcriptional activity (59). FOXO3a is also induced via AMPK, and this effect promotes its nuclear and mitochondrial translocation, resulting in significantly reduced ROS levels, enhanced aerobic respiration and maintaining energy homeostasis (60). Furthermore, prolonged activation of AMPK by AICAR enhances the nuclear import of FOXO3a and improves the adaptability of cells to oxidative stress (61). During hypoxia, AMPK is important for in regulating FOXO proteins in cells. For example, in anoxic myocardial cells, upregulation of AMPK can decrease the phosphorylation level of FOXO1 and FOXO3, and exclusion of them from the nucleus, leading to sequential activation of target genes, such as catalase (62). It has been demonstrated that AMPK phosphorylates the FOXO homologue, DAF-16, at multiple sites and activates DAF-16-dependent transcription (63). Notably, DAF-16 can induce the expression of genes encoded by AMPK, which slows aging in *Caenorhabiditis elegans*. This suggests that DAF-16 and AMPK form a positive feedback loop (64). In mammals, however, the research about this feedback loop has not detected any changes in the γ substrate of AMPK depending on FOXO protein activation (65). However, it cannot be ruled out that FOXO proteins tissue-specifically modulate the gene transcription of AMPK, as human FOXO3 and FOXO4 directly induce liver kinase B1, which phosphorylates AMPK and enhances its bioactivity (66). Thus, it is hypothesized that a FOXO-AMPK pathway feedback loop may exist in mammalian cells.

In addition to directly phosphorylating and forming a feedback loop with FOXO proteins, AMPK can also modulate other proteins in order to regulate FOXO proteins. For example, AMPK, by increasing the levels of NAD^+, increases the activity of histone deacetylation enzyme, sirtuin 1 (SIRT1), which consequently leads to the activation and acetylation of FOXO1 and FOXO3 (67).

IκB kinase (IκK). IκK, a central regulator of nuclear factor-κB (NF-κB), is involved in controlling cell proliferation, survival, the prevention of apoptosis and tumorigenesis. The IκK complex includes two catalytic subunits, IκK-α and IκK-β, and a regulatory subunit, IκK-γ. The functions IκK-α

and IκK-β are similar, but the effect of IκK-γ on FOXO3 remains unclear. FOXO3 phosphorylation at Ser644 induced by IκK-β promotes its retention in the cytoplasm, and thus, has a pivotal role in chemotherapeutic resistance. Furthermore, the overexpression of IκK-β results in an accumulation of endogenous FOXO3 in the cytoplasm and degradation via the ubiquitin proteasome pathway. Also, using anti-NEMO binding domain peptide significantly inhibits the IκK complex, which induces the relocalization of FOXO3 to the nucleus. Overall, the phosphorylation of FOXO by IκK decreases its stability, and also changes its subcellular location (68). IκK-ε/IκK-ι is a member of the IκK family, also known as IKBKE. Previous studies have demonstrated that inflammatory stimuli, including interferon (IFN) and viruses, activate IκK-α/β and IKBKE. The IKBKE kinase region has only ~27% similarity with IκK-α and IκK-β, but it can phosphorylate FOXO3a at Ser644, leading to the degradation of FOXO3a and export from the nucleus, thus reducing the transcriptional activity of FOXO3a (69). Additionally, phosphorylation of FOXO3a by IKBKE does not rely on the Akt pathway (70). Recent research suggests that IKBKE, which activates Akt, can directly phosphorylate Akt at Thr308 and Ser473, therefore, IKBKE regulates FOXO3a activity directly, and also regulates the activity of FOXO3 indirectly via the Akt pathway (71). In addition, it has been demonstrated that phosphorylation of FOXO3a caused by IKBKE decreases the expression of IFN-β to participate in the regulation of the immune response (72). In acute myeloid leukemia cells, FOXO3 is in a constitutively inactive state due to its cytoplasmic localization, which is not dependent on the PI3K/Akt or ERK/MAPK pathways, instead, NF-κB, which is a key regulator of cell survival, sustains a constitutively active state (73). IκK-specific inhibition upregulates FOXO3 and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) (74). It has also been reported that FOXO3a activates IκK/NF-κB pathways though inducing the B-cell CLL/lymphoma 10 protein (BCL10), which is an upstream regulatory factor of IκK/NF-κB (75). In this case, the regulation of FOXO3 and IκK is reciprocal.

3. Acetylation of FOXO proteins

Similarly to phosphorylation, acetylation also regulates various functions of different FOXO proteins. Deacetylases and acetylases have been reported to modify FOXO proteins to change their DNA-binding activity, stability and interaction with other proteins.

Deacetylation. The effect of acetylation on FOXO proteins is controlled by the histone acetyltransferases and histone deacetylases (HDACs). HDACs, which remove the acetyl groups from histones though enzymatic reaction, are pivotal for regulating gene expression. HDACs include class I (HDAC1, 2, 3 and 8), class II a (HDAC4, 5, 7 and 9), class II b (HDAC6 and 10), class III and class IV (HDAC11). The class III HDACs, also termed sirtuins, possess NAD-dependent catalytic sites and traditional inhibitors of HDACs cannot inhibit sirtuin function (76). Mammals have seven sirtuins, with sirtuin (SIRT) 1-3 exhibiting strong NAD^+-dependent deacetylation activity, whereas SIRT4-7 exhibit strong deacetylation and are more accurately described as NAD^+-dependent deacylases (77). SIRT2 is predominantly localized in the cytoplasm, whereas SIRT3, SIRT4 and SIRT5 are predominantly in the mitochondria, and SIRT1, SIRT6, SIRT7 in the nucleus (78). FOXO proteins are deacetylated by HDACs and sirtuins simultaneously. Certain studies indicate that class I and II HDACs regulate FOXO nuclear localization and transcriptional activation in response to nutrient deprivation (79). However, compared with class II, class I HDACs preferentially regulate the activity of FOXO proteins. Generally, HDAC1 is able to complex with HDAC2 to exert normal functions. In addition, HDAC4/5 recruits HDAC3 and FOXO proteins to form a complex, allowing it to be deacetylated and activated (80). It has been reported that HDAC1 is a primary regulator of FOXO in skeletal muscle and a key regulator of the atrophy. Furthermore, HDAC1-mediated deacetylation of FOXO in the cytosol may be a major signal that leads to decreased phosphorylation and nuclear localization of FOXO (79). Research has demonstrated that reducing FOXO3a acetylation may lead to FOXO3a-dependent transcriptional activation and induce atrophy of muscle fibers (81,82). There is substantial literature demonstrating that SIRT1 can deacetylate FOXO1, FOXO3a and FOXO4, and subsequently, stimulate the expression of anti-oxidants, including MnSOD and thioredoxin (83), and through an auto-feedback loop also potentiate SIRT1 expression (84). A previous study demonstrated that SIRT1 has a primary role in the regulation of endothelial progenitor cell apoptosis induced by oxidative stress, and that SIRT1 integrated with FOXO3a directly to inhibit FOXO3a by deacetylation (85). SIRT1 and SIRT2, through deacetylating FOXO3, promote the degradation of FOXO3 via the ubiquitin-proteasome pathway. Studies have demonstrated that Skp2, which is the substrate of E3 ubiquitin ligase, prefers to combine with acetylated FOXO3, where Lys242, Lys259, Lys290 and Lys569 sites of acetylated FOXO3 are targets for ubiquitination (86), thus, acetylation of FOXO3a exposes the lysine groups, and thus, promotes FOXO3a polyubiquitination.

SIRT1 mediated multitudinous biological effects though deacetylation of FOXO proteins. It was reported that SIRT1 increases the activity of FOXO proteins by deacetylation, however, others have demonstrated that the functions of FOXO proteins are negatively correlated with deacetylation induced by SIRT1 (87). Indeed, deacetylation of FOXO proteins increases the transcription activity (88). Kitamura *et al* (88) reported that compared with wild-type FOXO1, mutated FOXO1, that cannot be acetylated, has stronger transcriptional activity. However, it has been recently demonstrated that acetylated FOXO proteins are easily degraded by the ubiquitin-proteasome pathway, which abolishes the increased transcriptional activity of FOXO proteins induced by deacetylation (86). To summarize, this mechanism may be a crucial step for balancing the effect of FOXO on transcription. Typically, SIRT1 is located in the nucleus, however, SIRT1 has also been reported to be detected in the cytoplasm or mitochondria (89). In fact, SIRT1, when located in the cytoplasm, promotes autophagy and nuclear localization (90). However, SIRT2 is predominantly localized in the cytoplasm. In the nucleus, SIRT1 induces autophagy though deacetylation of FOXO1 and activates FOXO1 transcriptional activity. When

SIRT1 is deactivated, acetylated FOXO1 is exported to the cytoplasm, and combines with the autophagy related 7 protein to accelerate autophagy. Whereas, SIRT2-induced deacetylation of FOXO1 inhibits autophagy (91). Therefore, SIRT1 and SIRT2 exhibit opposing effects on the regulation of certain cell behaviors, even though they both deacetylate FOXO1. Resveratrol can increase the SIRT1 deacetylation activity, which reduces FOXO1 and FOXO3a acetylation and increases the nucleus localization, resulting stronger transcriptional activity. Resveratrol activates AMPK, which increases the deacetylase activity of SIRT1, and further increases the FOXO1 transcription activity (92,93). Studies have demonstrated that certain proteins form complexes with FOXO-SIRT1. The protein four and a half LIM domains 2 (FHL2) improves the interaction between SIRT1 and FOXO1. In prostate cancer cells, FOXO1 activity is inhibited by FHL2, which promotes the deacetylation of FOXO1 by SIRT1 (94). FoxO1 protects pancreatic beta cells against oxidative stress by forming a complex with the promyelocytic leukemia protein and the NAD-dependent deacetylase SIRT1, resulting in activation of neurogenic differentiation 1 and MAF bZIP transcription factor A, which are two Insulin2 gene transcription factors (90). In addition, lysine demethylase 5B (KDM5) protein can also form a complex with HDAC4 and combine with FOXO proteins, thereby promoting FOXO deacetylation. The complex binds to target genes with FOXO promoter regions to promote resistance to expression of oxidative stress proteins. In the absence of KDM5, HDAC4 can still form a complex with FOXO1 and induce its deacetylation; however, FOXO1 recruitment to a subset of target genes, which have a role in oxidative stress resistance, is attenuated. This decreased ability of FOXO1 induces the decreased transcriptional activation of a subset of oxidative stress resistance genes (95).

SIRT2 deacetylates FOXO3 and increases the DNA-binding activity of FOXO3 resulting in upregulation of FOXO3 target genes, including $p27^{kip1}$, MnSOD and BCL2 like 11 (Bim) (96). In 3T3-L1 adipocytes, FOXO1 is deacetylated by SIRT1, which increases the DNA-binding of FOXO1 to the promoter of peroxisome proliferator activated receptor (PPAR)γ, and subsequently, suppresses PPARγ expression (97). Inhibition of SIRT2-mediated deacetylation of FOXO1 can increase the retention of FOXO1 in the cytoplasm (98). Furthermore, SIRT3 is the major deacetylase in the mitochondrion (99). SIRT3 deacetylates FOXO3a, which strengthens the DNA-binding of FOXO3a, but does not change the mitochondrial location of FOXO3a (100). It has been demonstrated that FOXO3a forms a complex with SIRT3 and mitochondrial RNA polymerase, binding to mitochondrial DNA, and further increasing the expression of oxidative phosphorylation-associated proteins, ultimately, increasing mitochondrial respiration (59). It has been reported that SIRT6 is pivotal for regulating DNA repair and telomerase (101). In mouse embryonic fibroblasts, SIRT6 inhibition can increase the acetylation level of FOXO3a. However in breast cancer cells, overexpression of SIRT6, which reduces the acetylation level of FOXO3a, weakens the transcription activity of FOXO3a and generates resistance to epirubicin and paclitaxel, which suggests that inhibition of SIRT6 to upregulate the activity of FOXO3a may be a promising therapeutic strategy for breast cancer (78).

Acetylation. Calcium response element-binding binding protein (CBP)/p300 acetylates FOXO1, FOXO3 and FOXO4 (81,102-104). Acetylated FOXO proteins have lower DNA-binding activity compared with normal levels (102). For example, FOXO4 combines with CBP following treatment of human embryonic kidney (HEK)-293T cells with peroxide stress, and acetylated FOXO4 exhibits lower transcription activity (105). Acetylated FOXO proteins tend to be located in the cytoplasm, as acetylated FOXO proteins are more sensitive to phosphorylation induced by Akt (106). The phosphorylation of FOXO proteins induced by Akt following insulin treatment is always accompanied by the acetylation of FOXO proteins (107). Increasing evidence suggests that the acetylation of FOXO proteins regulates its transcriptional activity by altering DNA binding, interaction with transcriptional regulatory proteins, and changes to stability and subcellular localization. FOXOs protein acetylation has weakens its transcriptional activity, however, in pancreatic β cells, the acetylation of FOXO1 induced by stress increases its stability and prevents the ubiquitin-proteasome pathway degradation (88). In pancreatic cancer cells, capsaicin enhances the expression of CBP/p300, and reduces SIRT1, SIRT2 and SIRT3. Capsaicin promotes acetylation of FOXO1 and increases nuclear entry, eventually enhancing the DNA-binding. Furthermore, acetylation occurs prior to phosphorylation of FOXO proteins (108). However, the acetylation of FOXO1 caused by p300 does not change its stability. Both wild-type p300, and p300 with an acetyltransferase deletion mutation, enhance the half-life of FOXO1 in HEK-293T and H4IIE rat hepatoma cells, which suggests that acetylation itself is not the key factor required for FOXO1 stability (107).

In addition, FOXO1-CoRepressor (FCoR) protein, which has acetyltransferase activity, influences the interaction between FOXO1 and SIRT1 deacetylase to maintain FOXO1 acetylation and inhibit the activity of FOXO1 in the rat adipocyte (109). It has been demonstrated that the interaction of FOXO1 and SIRT1 is induced by FHL2, which enhances the deacetylation of FOXO1 (94). Currently, FCoR is the only protein known to inhibit the interaction between FOXO1 and SIRT1.

4. Ubiquitination of FOXO proteins

Polyubiquitination. The degradation of FOXO proteins depends on the ubiquitin-proteasome pathway. E3 ubiquitin ligase is the key factor involved in FOXO protein ubiquitination and Skp2 is a subunit of the Skp1/cullin 1/F-box protein ubiquitination complex. FOXO1 combines with Skp2 when phosphorylated at the Ser256 loci. Skp2 is the predominant ubiquitin ligase for FOXO1, and induces its polyubiquitination and degradation (18). C-terminus of Hsc70-interacting protein (CHIP) is highly expressed in the heart and blood vessels, and the C-terminus has a cochaperone/ubiquitin ligase with a dual function. Under the stimulus of TNF-α, CHIP promotes the smooth muscle cells to degrade and ubiquitination (110). Ring finger and WD repeat domain 2 (COP1), a ring-finger E3 ubiquitin ligase regulated by insulin, has a key role in survival of mammalian cells. In hepatocellular carcinoma cells, COP1 degrades FOXO1 and reduces the expression of FOXO1 target genes, including glucose-6-phosphatase and phosphoenolpyruvate carboxykinase (111).

Table I. Summary of the post-translational modification of FOXO proteins.

Author, year	Modification	Enzyme	Subtype	Site(s)	Effect on FOXO	Refs.
Huang *et al*, 2005	Phosphorylation	Akt	FOXO1	T24,S256,S319	↓	(18)
Zhang *et al*, 2002						(19)
Brunet *et al*, 2001			FOXO3	T32,S253,S315	↓	(20)
Obsilova *et al*, 2005			FOXO4	T28,S319,S258	↓	(22)
Obsil *et al*, 2003						(25)
Brunet *et al*, 2001		SGK1	FOXO3	T32,S253,S315	↓	(20)
Zhang *et al*, 2013		PERK	FOXO1	S298,S301,S303	↑	(31)
Xiao and Yuan, 2010		MST1	FOXO1	S212	↑	(34)
Xiao and Yuan, 2010			FOXO3	S207	↑	(34)
Zeng and Hong, 2008						(35)
Zeng and Hong, 2008			FOXO4	S196	↑	(35)
Essers *et al*, 2004		JNK	FOXO4	T447,T451	↑	(39)
Asada *et al*, 2007		ERK/p38	FOXO1	S246,S284,S295,S326 S413,S415,S429,S46, T475,T557	↑	(44)
Yang *et al*, 2008			FOXO3	S294,S344,S425,	↓	(47)
Yuan *et al*, 2008		CDK1	FOXO1	S249	↑	(52)
Huang *et al*, 2006		CDK2/5	FOXO1	S249	↓	(51)
Zhou *et al*, 2015						(54)
Greer *et al*, 2007		AMPK	FOXO3	T179,S399,S413,S555 S588,S626	↑	(55)
Yun *et al*, 2014			FOXO1	T649	↑	(57)
Shen and Hahn, 2011		IκK	FOXO3	S644	↓	(69)
Bertaggia *et al*, 2012	Acetylation	CBP/p300	FOXO3	K242,K259,K262,K271	↓	(81)
Brunet *et al*, 2004				K290,K569		(103)
Matsuzaki *et al*, 2005			FOXO1	K242,K245,K262	↓	(102)
Fukuoka *et al*, 2003			FOXO4	K186,K189,K408	↑	(104)

Table I. Continued.

Author, year	Modification	Enzyme	Subtype	Site(s)	Effect on FOXO	Refs.
Beharry *et al*, 2014	Deacetylation	HDAC1/2	FOXO1/3	NM	↑	(79)
Mihaylova *et al*, 2011		HDAC4/5	FOXO1/3	NM	↑	(80)
Salminen *et al*, 2013		SIRT1	FOXO1/3/4	NM	↑↓	(83)
Motta *et al*, 2004						(87)
Wang *et al*, 2007		SIRT2	FOXO1/3	NM	↑	(96)
Jing *et al*, 2007						(98)
Peserico *et al*, 2013		SIRT3	FOXO3	NM	↑	(60)
Kim *et al*, 2010						(100)
Khongkow et at, 2013		SIRT6	FOXO3	NM	↓	(78)
Huang *et al*, 2005	Polyubiquitination	Skp2, COP1, CHIP	FOXO1	ND	↓	(18)
Li *et al*, 2009						(110)
Kato *et al*, 2008						(111)
Yang *et al*, 2008		MDM2	FOXO3	ND	↓	(47)
Brenkman *et al*, 2008	Monoubiquitination	MDM2	FOXO4	ND	↑	(113)
van der Horst *et al*, 2006		USP7	FOXO4	ND	↓	(114)
Yamagata *et al*, 2008	Arginine methylation	PRMT1	FOXO1	R248,R250	↑	(118)
Xie *et al*, 2012	Lysine methylation	Set9	FOXO3	K270,K271	↓	(120)
Calnan *et al*, 2012						(121)
Kuo *et al*, 2008	Glycosylation	ND	FOXO1	ND	↑	(122)
Housley *et al*, 2008						(123)
Butt *et al*, 2012		ND	FOXO3	ND	↑	(124)
Ho *et al*, 2010		ND	FOXO4	ND	↑	(125)

FOXO, Forkhead box O; Akt, Akt serine/threonine kinase; SGK1, serum/glucocorticoid regulated kinase 1; PERK, PKR-like ER kinase; MST1, mammalian sterile 20-like kinase 1; JNK, Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; CDK, cyclin-dependent kinase; AMPK, adenosine monophosphate-activated protein kinase; IκK, IκB kinase; CBP, calcium response element-binding binding protein; HDAC, histone deacetylase; SIRT, sirtuin; COP1, ring finger and WD repeat domain 2; Skp2, S-phase kinase associated protein 2; CHIP, C terminus of Hsc70-interacting protein; MDM2, MDM2 proto-oncogene; USP7, ubiquitin specific protease 7; PRMT1, protein arginine methyltransferase 1; Set9, SET domain containing lysine methyltransferase 7; T, threonine; S, serine; K, lysine; NM, not mentioned; ND, not determined; R, arginine; ↓, downregulated; ↑, upregulated.

In addition, other kinases may promote FOXO protein degradation though phosphorylating different FOXO loci. ERK regulates the phosphorylation of FOXO3 at Ser294, Ser344 and Ser425 sites that mediate the binding of FOXO3 and MDM2, which results in the polyubiquitination and degradation of FOXO3 (47). Additionally, Ser644 phosphorylation of FOXO3 by IκB facilities ubiquitination. and ultimately, induces degradation of FOXO3 (112). In conclusion, the phosphorylation of FOXO proteins induced by Akt and IκB is important for mediating polyubiquitination. In addition, FOXO1 acetylation increases the stability following oxidative stress by preventing the polyubiquitination of FOXO1 (88), this result suggests that the activity of FOXO proteins are regulated by various acetylation and ubiquitination events.

Mono-ubiquitination. FOXO proteins activities are also regulated by mono-ubiquitination. However, different from the degradation of FOXO proteins caused by polyubiquitination, mono-ubiquitination increases the activity of FOXO proteins. Typically, MDM2 is considered to be an E3 ubiquitin ligase and can promote the polyubiquitination of FOXO1 and FOXO3. However, in response to oxidative stress, MDM2 induces mono-ubiquitination for FOXO4, thus, increasing the FOXO4 nuclear relocalization and transcriptional activity (113). However, this effect can be reversed by Herpes-virus-associated ubiquitin specific protease (also termed USP7). USP7 enhances the exclusion of FOXO4 from the nucleus, but does not affect the half-life of FOXO proteins. Furthermore, under oxidative stress conditions, peptidylprolyl cis/trans isomerase, NIMA-interacting 1 (Pin1) binds to FOXO4, and attenuates FOXO4 monoubiquitination and transcriptional activity. Pin1 exerts its effects by preventing nuclear FOXO4 accumulation via stimulation of the activity of the deubiquitinating enzyme HAUSP/USP7, which interacts with and deubiquitinates FOXO4 in response to oxidative stress (114). Additionally, a study reported that USP7 mediates the mono-deubiquitination of FOXO1 in liver cells. USP7-mediated mono-deubiquitination of FOXO1 results in suppression of FOXO1 transcriptional activity through decreased FOXO1 occupancy on the promoters of gluconeogenic genes (115).

In myocardial cells, atrogin-1 (also termed F-box protein 32) inhibits the phosphorylation of FOXO1/3 induced by insulin or IGF-1 and promotes its nuclear entry to activate the FOXO transcriptional activity. For canonical ubiquitin-proteasome degradation, target ubiquitin chains are linked by Lys48 and target proteins for the proteasomal degradation. By contrast, it has been reported that the deposition of Lys63-linked ubiquitin chains on FOXO proteins cannot initiate proteasome degradation. Atrogin-1 modifies the ubiquitination of FOXO proteins in the unconventional manner of Lys63-linked ubiquitin chains. As a consequence, this mechanism enhances FOXO protein transcriptional activity via ubiquitination rather than initiating the ubiquitin-proteasome degradation. Notably, atrogin-1 is also a target gene of FOXO proteins. FOXO proteins with unconventional ubiquitination increase the expression of atrogin-1, which results in a positive feedback loop (116). Furthermore, it has been reported that HDAC6 is not a traditional deacetylation enzyme, and it is directly involved in the protein ubiquitin-proteasome degradation pathway though via its ubiquitin connecting region. HDAC6 is able to interact with atrogin-1 and regulate FOXO protein ubiquitination (117). This finding further demonstrates that FOXO proteins are regulated by acetylation and ubiquitination.

5. Methylation of FOXO proteins

Protein arginine methyltransferase (PRMT1) methylates FOXO1 at Arg248 and Arg250. This modification inhibits the phosphorylation of FOXO1 at Ser253 induced by Akt, blocking the exclusion of FOXO1 from nucleus, and consequently, increases apoptosis in response to oxidative stress. However, knock-out of PRMT1 promotes translocation of FOXO proteins from the nucleus to the cytoplasm, thus, accelerating the ubiquitin-proteasome degradation pathway by inducing poly-ubiquitination (118). Methylated FOXO1 remains in the nucleus, where it activates the expression of downstream targets genes. Lysine methylation, induced by other methyltransferases, is important for regulating histones and other proteins (119). Certain studies demonstrated that the methyltransferase, SET domain containing lysine methyltransferase 7 (Set9), methylates FOXO3 at Lys270 and Lys271. Methylated FOXO3 has lower DNA-binding activity and transactivation, and this effect is independent of phosphorylation induced by Akt (120,121). Set9 mediates lysine methylation to suppress the expression of Bim and apoptosis of nerve cells by inducing FOXO3 in response to oxidative stress. Set9 affects FOXO3 specifically, whereas other FOXO protein subtypes are not methylated by Set9 (121).

6. Glycosylation of FOXO proteins

Glycosylation is a highly complex process that attaches glycans to protein, lipids or other organic molecules. Proteins are glycosylated by two different types of glycosylation; N- and O-glycosylation. FOXO1 is a substrate for O-glycosylation, which induces upregulation of glucose-6-phosphatase (122) and other gluconeogenic genes expression (123), however it does not influence the nuclear-cytoplasm shuttling of FOXO proteins. This finding suggest that glycosylation is a primary PTM for regulating FOXO1 activity. Additionally, it has been reported that partial Ser/Thr sites of FOXO3 may be targets for O-glycosylation (124). Furthermore, in response to oxidative stress, O-linked N-acetylglucosamine transferase glycosylates FOXO4, which enhances the transcriptional activity of FOXO4 (125).

7. Conclusion

The present review describes the recent findings regarding PTMs of FOXO proteins, and an increasing number of reports indicate that different stimuli activate different FOXO proteins PTMs, and FOXO transcriptional activity is not regulated by a single PTMs. Furthermore, with the same stimulus, different PTMs may exert opposing effects to ultimately maintain FOXO protein activity at a particular level. Certain enzymes that modify the FOXO proteins are also downstream target genes of the FOXO transcription factors. Thus, these enzymes create a positive feedback loop to regulate the FOXO proteins. Occasionally, different modifications affect each other via association with certain

factors, therefore, influencing the activity of FOXO proteins directly and indirectly. In conclusion, FOXO protein PTMs have a complex topological relationship and further investigation of the association between disease and FOXO PTMs is required.

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