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Regulation of FoxO protein stability via ubiquitination and proteasome degradation

Haojie Huang[1,*] and **Donald J. Tindall**[2,*]

[1]Department of Pathology, Stony Brook University, Stony Brook, NY 11790

[2]Department of Urology and Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN 55905

Abstract

Forkhead box O-class (FOXO) proteins are evolutionally conserved transcription factors. They belong to a family of proteins consisting of FOXO1, FOXO3a, FOXO4 and FOXO6 in humans. Increasing evidence suggests that FOXO proteins function as tumor suppressors by transcriptionally regulating expression of genes involved in cell cycle arrest, apoptosis, DNA repair and oxidative stress resistance. Activation of various protein kinases, including Akt, IκB kinase (IKK) and ERK, leads to phosphorylation of FOXO proteins and their ubiquitination mediated by E3 ligases such as SKP2 and MDM2 in human primary tumors and cancer cell lines. As a result, the tumor suppressor functions of FOXO proteins are either diminished or abrogated due to their ubiquitination proteasome degradation, thereby favoring cell transformation, proliferation and survival. Thus, ubiquitination and proteasome degradation of FOXO proteins plays an important role in tumorigenesis and represents a viable target for cancer treatment.

Introduction

Phosphatase and tensin homolog deleted in chromosome 10 (*PTEN*) is frequently mutated or deleted in a large spectrum of human tumor types [1,2]. PTEN functions primarily as a lipid phosphatase by antagonizing the effect of phosphoinositide 3-kinase (PI3K) [3,4]. Loss of PTEN increases the levels of phosphatidylinositol (3,4,5) trisphosphate (PIP3) in the plasma membrane, which in turn leads to activation of protein kinase B (PKB or Akt).

Akt plays a central role in cell survival by activating or inactivating a number of downstream effector proteins including FOXO transcription factors [5]. Increasing evidence suggests that FOXO proteins possess tumor suppression functions by regulating expression of genes involved in apoptosis, cell cycle arrest, oxidative stress resistance and DNA repair [5]. Activation of Akt due to frequent loss of PTEN or constitutively activation of PI3K in human tumors results in phosphorylation and inhibition of FOXO proteins [6]. Akt phosphorylation also induces nuclear export of FOXO proteins through the nuclear pore complex, which is dependent on 14-3-3 chaperone proteins and the exportin receptor, chromosomal region maintenance protein 1 (CRM1) [7,8]. Thus, the nuclear, transcription-

*Correspondence should be addressed to: tindall.donald@mayo.edu; or, haojie.huang@stonybrook.edu.

dependent tumor suppressor function of FOXOs is abolished due to Akt-mediated phosphorylation and nuclear exportation (Fig. 1).

Akt activation promotes FOXO protein degradation

In addition to Akt-mediation phosphorylation of FOXO, Akt activation promotes degradation of these proteins, and this process is inhibited by proteasome inhibitors [9]. Also, insulin induces FOXO1 protein degradation in HepG2 cells [10], and this degradation requires FOXO1 phosphorylation mediated by the PI3K/Akt pathway. Consistent with this, levels of FoxO1 are high in serum-starved normal chicken embryo fibroblasts, and FoxO1 protein undergoes rapid phosphorylation and degradation following platelet-derived growth factor treatment [11]. Moreover, Akt phosphorylation-dependent proteasome degradation of FoxO1 plays a key role in oncogenic transformation induced by the PI3K/Akt pathway. Thus, Akt inhibits the activity of FOXO by promoting phosphorylation and nuclear exportation of these proteins, and also by inducing their degradation by the proteasome. In addition to Akt, other kinases such as IκB kinase (IKK) and ERK also promote proteolysis of FOXO3a [12,13].

SKP2 works in concert with Akt to induce FOXO1 ubiquitination and proteasome degradation

The ubiquitin-proteasome system (UPS) plays an essential role in protein degradation. Before client proteins are recognized and targeted for degradation by the proteasome, ubiquitin is transferred and covalently attached to substrates via sequential activation of three enzymes including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (UBC, E2) and ubiquitin ligase (E3). Because there is only one E1 protein in mammals (and relatively few E2 proteins), substrate targeting specificity is believed to be mediated by E3. Based upon structure similarities E3 ligases are classified into two main classes: the RING-finger proteins and the HECT-domain proteins. The SKP1-CUL1-F-box protein (SCF) complex is a multi-subunit RING-finger E3 ligase, which targets FOXO1. CUL1 provides a scaffold function within this complex by recruiting the adaptor protein SKP1 and the RING-finger protein RBX1 (Fig. 2A). SKP1 binds to the F-box domain of the F-box containing proteins such as SKP2 and β-TrCP (Fig. 2A). Most SCF substrates are recognized by and bound to the F-box subunit. Through specific domains, such as the leucine-rich repeat (LRR) in SKP2 and the WD40 domain in β-TrCP, SCF complexes specifically recognize and bind to the substrates. Because substrate phosphorylation is essential for targeting of the substrates by SCF complexes, the phosphorylation sites are often called ‘phospho-degron’. CUL1 also binds to the RING-finger protein RBX1, which recruits UBC (E2) into the SCF complexes through its RING-finger domain. By binding to both substrate and the E2 enzyme, the SCF^{SKP2} E3 complex enables transfer of the ubiquitin protein onto the substrate (Fig. 2A).

FOXO1 is specifically bound by SKP2 in a number of cell types [14]. Consistent with the role of Akt in FOXO1 degradation, SKP2 binding of FOXO1 requires Akt-mediated phosphorylation of FOXO1 at serine 256 [14]. SKP2 also induces polyubiquitination and degradation of FOXO1. Importantly, SKP2-induced ubiquitin-dependent proteasome degradation requires Akt phosphorylation of FOXO1 at serine 256 [14]. Therefore, like other well-studied substrates of $SCF^{\beta\text{-}TrCP}$ and SCF^{SKP2} complexes, such as IκBα, β-Catenin and $p27^{KIP1}$, recognition and binding of FOXO1 by SKP2 requires the phospho-degron motif that contains the serine 256 phosphorylation site (Fig. 2B). Although to date it is unclear which lysine residue(s) in FOXO1 is specifically targeted by SCF^{SKP2} for ubiquitination, these residues are likely localized within the first 260 amino acids in the NH2-terminus of FOXO1 [14]. Interestingly, among the known SCF targeting substrates,

including IκBα, β-Catenin and $p27^{KIP1}$, the ubiquitin accepting lysines are usually located 9–14 amino acids within the region NH2-terminal to the phospho-degron motif (Fig. 2B). Thus, it is possible that SCF^{SKP2} utilizes a similar mechanism in targeting FOXO1 for ubiquitination and degradation (Fig. 2B).

Regulation of SKP2-mediated ubiquitination and proteasome degradation of FOXO1 by other pathways

In addition to inducing phosphorylation of FOXO1, which is an essential step for SKP2-mediated ubiquitination and proteasome degradation of FOXO1, Akt may contribute FOXO1 degradation by other means. Loss of PTEN increases the levels of SKP2 mRNA, and this effect of PTEN is Akt-dependent [15]. SKP2 levels and stability are regulated by another E3 ligase anaphase-promoting complex/cyclosome and its activator Cdh1 (APC/C^{Cdh1}) [16,17]. Akt phosphorylates SKP2 at serine 72; however, the functional significance of this phosphorylation is being debated. Two reports suggest that this phosphorylation promotes cytoplasmic localization of SKP2 and impairs APC/C^{Cdh1}-mediated degradation of SKP2 in the nucleus [18,19]. However, another report suggests that Akt-mediated phosphorylation of SKP2 at serine 72 does not affect the subcellular localization of SKP2 [20]. Moreover, it has been shown that conditional knockout of the nuclear cofactor CBP in thymocytes increases SKP2 protein levels and promotes development of T cell lymphomas [14,21]. Intriguingly, in addition to being an important transcription coactivator, CBP is a key functional component of the APC/C^{Cdh1} E3 ligase [22]. Thus, it can be speculated that loss of CBP impairs APC/C^{Cdh1} E3 activity and thereby promotes stabilization of SKP2 [14,21]. Importantly, high levels of the SKP2 protein are inversely correlated with low levels of the FOXO1 protein in CBP-knockout T cell lymphomas [14]. SKP2-mediated degradation of FOXO1 has also been shown in other tumor types [23,24]. Thus, many cancer relevant pathways converge on the regulation of the levels of SKP2 protein, which may in turn affect FOXO1 protein levels via the ubiquitin proteasome pathway.

Monoubiquitination and deubiquitination of FOXO proteins

The single molecule RING-finger E3 ligase murine double minute 2 (MDM2) promotes ubiquitination of various FOXO factors including FOXO1, FOXO3a and FOXO4, suggesting that MDM2 acts as a general E3 ligase for FOXO protein degradation [13,25,26]. Activation of ERK by the Ras/Raf pathway leads to FOXO3a phosphorylation and downregulation [13]. ERK-phosphorylated FOXO3a is further subjected to MDM2-mediated ubiquitination and proteasome degradation [13]. Intriguingly, knockout or knockdown of MDM2 alone increases FOXO3a protein levels, and this effect was shown to be mediated by MDM2-induced polyubiquitination of FOXO proteins [26] whereas another study showed that MDM2 catalyzes multiple mono-ubiquitination of FOXO4 rather than poly-ubiquitination [25]. Monoubiquitination of FOXO4, which promotes its nuclear localization, was also observed in cultured cells in response to oxidative stress [27]. Moreover, monoubiquitinated FOXO4 can be deubiquitinated by the deubiquitinating enzyme herpesvirus-associated ubiquitin-specific protease (HAUSP)/USP7 [27]. Based on these findings, it has been proposed [25] that ERK-phosphorylated FOXO proteins, especially FOXO3a, can be monoubiquitinated by a priming E3 ligase such as MDM2 and that this induces nuclear localization of FOXO proteins (Fig. 1). This process may be reversed by the deubiquitination of FOXO proteins mediated by HAUSP/USP7. Under certain conditions, e.g., when MDM2 levels are high, FOXO proteins become polyubiquitinated (Fig. 1). It has also been proposed that monoubiquitinated FOXO protein can be further converted into a polyubiquitinated form by branching E3 ligases such as SKP2 [25].

Role of proteasome degradation of FOXO proteins in cell transformation and tumorigenesis

Activation of FOXO proteins upregulates expression of genes involved in cell cycle arrest, apoptosis and DNA repairs, implying that these proteins function as tumor suppressors [5]. Indeed, forced expression of FOXO1 and FOXO3a in PTEN-mutated prostate and kidney cancer cells induces apoptosis and cell cycle arrest, respectively [14,28]. The anti-tumor function of FOXO1 is largely diminished in cells overexpressing SKP2 [14]. In contrast, co-expression of SKP2 with an Akt phosphorylation-resistant mutant of FOXO1 promotes neither the proteasome degradation of this mutated form of FOXO1, nor the mutant-induced death of prostate cancer cells [14]. Thus, degradation of FOXO1 contributes to Akt-mediated cell growth and survival, as well as SKP2-induced tumorigenesis [23]. These findings were confirmed in an independent study using chicken embryo fibroblasts (CEF) as a working model [11], where it is well established that PI3K and Akt induce transformation. Intriguingly, FoxO1 expression is suppressed in CEF transformed by PI3K and Akt, and this effect is mediated by PI3K/Akt-induced proteasome degradation of FoxO1 [11]. Importantly, phosphorylation-dependent proteasome degradation of FoxO1 plays an important role in oncogenic transformation by PI3K/Akt [11].

Surprisingly, FOXO3a protein is localized in the cytoplasm of some breast tumors lacking Akt phosphorylation or activation [12]. The cytoplasmic localization FOXO3a is regulated by IκB kinase (IKK) phosphorylation of FOXO3a, which also induces proteasome degradation of FOXO3a [12]. Moreover, cytoplasmic localization of FOXO3a is correlated with IKK expression as well as poor survival in breast cancer. Importantly, constitutive expression of IKK promotes cell proliferation in cultured cancer cells and tumorigenesis in mice, but these effects of IKK can be reversed by FOXO3a [12]. Thus, IKK phosphorylation of FOXO3a also serves as a vital mechanism in promoting cell growth and tumorigenesis.

Another well-established cancer relevant kinase cascade is the Ras-MEK-ERK pathway, where activation of ERK induces phosphorylation of FOXO3a at multiple residues [13]. ERK phosphorylated FOXO3a can be recognized by the E3 ligase MDM2, resulting in ubiquitination and proteasome degradation of FOXO3a [13]. Also, ERK/MDM2-mediated proteasome degradation of FOXO3a plays an important role in promoting cell proliferation and tumorigenesis [13].

In summary, FOXO proteins act as tumor suppressors by transcriptionally regulating expression of a large number of genes that promote apoptosis, cell cycle arrest, oxidative stress resistance and DNA repair. Importantly, the anti-tumor functions of these transcription factors are often abolished by kinase-mediated phosphorylation and E3 ligase-mediated ubiquitination and proteasome degradation. Thus, deregulation of FOXO proteins may play an essential role in promoting cancer cell growth and survival.

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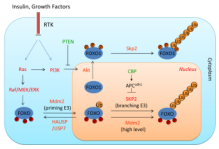

Figure 1.
Regulation of FOXO proteins by phosphorylation-dependent ubiquitin proteasome degradation pathways. Stimulation of cells by growth factors and insulin results in the activation of Akt and ERK through the Ras- and PI3K-dependent pathways. This in turn leads to phosphorylation of FOXO transcription factors. Akt-mediated phosphorylation promotes the localization of FOXO1 and other FOXO proteins in the cytoplasm, where the substrate-binding F-box protein SKP2 in the SCF^{SKP2} E3 ligase binds to and induces ubiquitination and subsequent proteasome degradation of FOXO1. Activation of ERK by the Ras-Raf-MEK cascade also leads to phosphorylation of FOXO proteins, such as FOXO3a. This phosphorylation further facilitates the recognition of FOXO3a by the E3 ligase MDM2, which promotes either monoubiquitination or polyubiquitination in the presence of high levels of MDM2 or other E3 ligases such as SKP2. Monoubiquitination of FOXO proteins such as FOXO4 is subjected to deubiquitination mediated by the deubiquitination enzyme HAUSP/USP7.

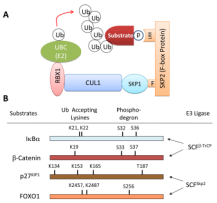

Figure 2.

Ubiquitination of FOXO1 by the SCF^{SKP2} E3 complex. A, a diagram showing the components of the SKP1-CUL1-F-box protein SKP2 (SCF^{SKP2}) ubiquitin E3 ligase complex. F, F-box. LRR, leucine rich repeats. B, a putative positioning model for the destruction domain in the known substrates of $SCF^{\beta\text{-}TRCP}$ and SCF^{Skp2} complex E3 ligases. One prediction of this positioning model is that ubiquitin (Ub) accepting lysine residues are usually located 9–14 amino acids upstream of the phosphodegron site(s) in the substrates of the SCF E3 ligases.