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Review

FoxO transcription factors; Regulation by AKT and 14-3-3 proteins ☆

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ABSTRACT

The forkhead box O (FoxO) transcription factor family is a key player in an evolutionary conserved pathway downstream of insulin and insulin-like growth factor receptors. The mammalian FoxO family consists of FoxO1, 3, 4 and 6, which share high similarity in their structure, function and regulation. FoxO proteins are involved in diverse cellular and physiological processes including cell proliferation, apoptosis, reactive oxygen species (ROS) response, longevity, cancer and regulation of cell cycle and metabolism. The regulation of FoxO protein function involves an intricate network of posttranslational modifications and protein–protein interactions that provide integrated cellular response to changing physiological conditions and cues. AKT was identified in early genetic and biochemical studies as a main regulator of FoxO function in diverse organisms. Though other FoxO regulatory pathways and mechanisms have been delineated since, AKT remains a key regulator of the pathway. The present review summarizes the current knowledge of FoxO regulation by AKT and 14-3-3 proteins, focusing on its mechanistic and structural aspects and discusses its crosstalk with the other FoxO regulatory mechanisms. This article is part of a Special Issue entitled: PI3K–AKT–FoxO axis in cancer and aging.

Contents

1. Introduction

1.1. Identification of mammalian FoxOs

There are 4 mammalian FoxO members designated FoxO1/FKHR/FoxO1a, FoxO3/FKHRL1/FoxO3a, FoxO4/AFX and FoxO6, sharing high protein homology (reviewed in [1,2]; for the Fox gene nomenclature

☆ This article is part of a Special Issue entitled: PI3K–AKT–FoxO axis in cancer and aging.

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see [3]). The first identified mammalian member of FoxO was FoxO1, designated originally FKHR (forkhead in rhabdomyosarcoma), and located on chromosome 13 in humans [4]. This transcription factor was cloned while studying the t(2;13) chromosomal translocation in rhabdomyosarcoma, identifying a gene fusion of the transcription factor PAX3 with a protein having homology with transcription factors sharing the forkhead DNA binding domain. The PAX3/FKHR fusion was shown later to have oncogenic potential and enhanced transcriptional activity [5–7]. A subsequent study found that in a subset of rhabdomyosarcomas showing t(1;13) translocation, FKHR is found fused to PAX7, which shares high homology with PAX3 [8,9]. AFX (FoxO4) was the second forkhead domain transcription factor found rearranged in cancers [10]. In acute leukemias, AFX was found fused with the mixed-lineage leukemia (MLL) zinc finger transcription factor due to a t(X;11) translocation. Interestingly, the AFX fusion occurs in the same region as the FKHR fusion, resulting in a chimeric transcription factor containing the DNA binding domain of MLL and the transcription activation domain of AFX. FoxO3 (FKHRL1), located on chromosome 6, was identified in a study looking for FKHR homologues using the DNA binding domain of FKHR as the bait and it shares high homology with FoxO1 [11]. The newest member of the FoxO family, FoxO6, was identified using a degenerated PCR strategy and is located on chromosome 1 in humans [12]. FoxO6 is probably the most distant member of the FoxO family as discussed below. Though FoxO1/FKHR was identified and cloned in the mid 90s, its significance and functional aspects were realized only following the genetic characterization of its nematode homologue, DAF-16.

1.2. DAF-16, the C. elegans FoxO

As mentioned above, a key step in delineating FoxO function came from the *C. elegans* genetics field. DAF-16 was originally identified in genetic analyses of the *C. elegans* dauer larval stage [13]. The DAF-16 gene was situated downstream of the pheromone receptor DAF-2 [14]. Subsequent studies also connected this pathway to *C. elegans* longevity, showing that mutants of DAF-2, resulting in activation of DAF-16, live longer than normal animals [15]. Subsequent cloning of the DAF-16 gene and detailed pathway analysis delineated a signaling pathway starting from DAF-2 (insulin receptor like gene) and going through AGE1 (PI3-Kinase) and AKT to DAF-16 [16–18]. These studies underlined the significance of the pathway for metabolism and longevity control as well as the key role of DAF-16 in the pathway and the potential of its mammalian homologues to mediate signals from the insulin receptor. The studies indicated on negative control of DAF-16 function by AKT and also recognized the homology of DAF-16 to the mammalian FKHR gene, identifying three potential AKT phosphorylation sites conserved between DAF-16 and FKHR (Fig. 1). Since specific insulin-regulated transcription factors have not been identified at that time, the DAF-16 findings prompted a glut in studies focusing on the regulation of FKHR (FoxO) proteins by AKT in mammalian systems [19–27].

2. Regulation of FoxO proteins by AKT

2.1. Historical perspective

The first study showing regulation of a mammalian FoxO by AKT was a study by Brunet et al. published in early 1999 [19]. This study demonstrated that AKT can phosphorylate FoxO3/FKHRL1 on the three predicted sites: T32, S253 and S315 both *in vitro* and *in vivo* and that this phosphorylation resulted in the nuclear exclusion of FoxO3. Accordingly, cell treatment with PI3K agonists such as IGF-1 or serum induced FoxO3 phosphorylation and nuclear exclusion while PI3K inhibition induced FoxO3 dephosphorylation and nuclear accumulation. The study also demonstrated that T32 and S253 phosphorylations mediated FoxO3 binding to the adapter protein 14-3-3z, suggesting that 14-3-3 proteins facilitated FoxO3 nuclear/cytoplasmic shuttling. The study also identified DNA sequences within the IGFBP1 (insulin responsive sequence, IRS) and FAS ligand (forkhead responsive element, FHRE) promoters that can mediate FoxO3 binding and showed that AKT phosphorylation regulates the transcriptional activity of FoxO3. Finally, the study showed that FoxO3 can mediate survival signaling downstream of AKT and that its over-activation can induce apoptosis. A study by Kops et al [20], appearing at the same time as the above study, demonstrated a similar regulation of FoxO4/AFX phosphorylation and transcriptional activity by AKT. These

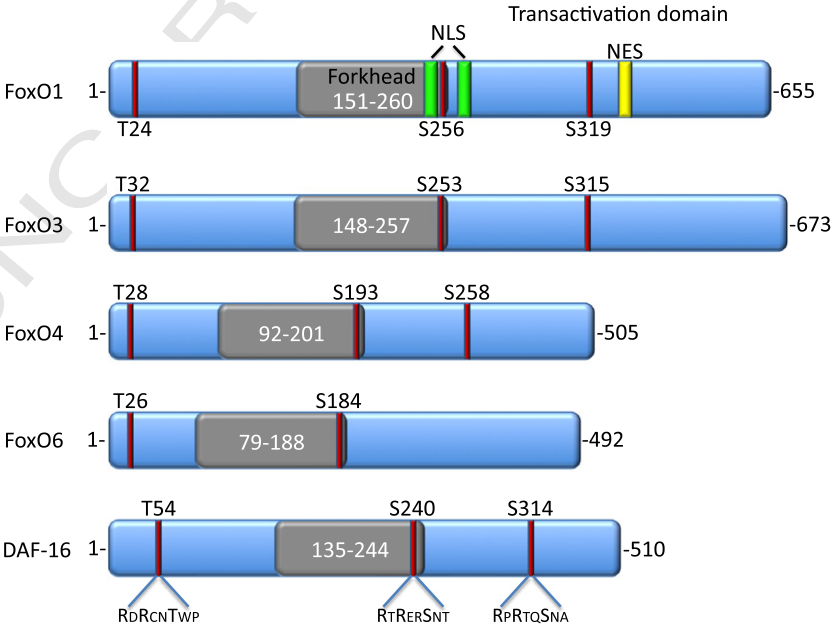

Fig. 1. Conserved AKT phosphorylation sites in FoxO proteins. Depiction of mammalian and *C. elegans* FoxO isoforms and the corresponding AKT phosphorylation sites. Indicated are also the locations of the forkhead domain and the nuclear export (NES) and nuclear localization sequence (NLS).

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studies were followed by numerous studies demonstrating the ability of AKT to phosphorylate FoxO1/FKHR and the other FoxO members, corroborating this key regulatory mechanism [21–27]. These studies were consequently confirmed also with DAF-16 and *Drosophila* FoxO, demonstrating the conservation of this regulatory mechanism through evolution [28–35].

2.2. Mechanistic aspects of FoxO regulation by AKT

As illustrated in Fig. 1, the regulatory AKT phosphorylation sites are shared by all mammalian FoxO members and are conserved through evolution. All FoxO proteins, with the exception of FoxO6, contain three AKT phosphorylation sites (FoxO6 lacks the carboxy terminal site [12]). Notably however, The AKT consensus phosphorylation motif defined by Alessy et al, RxRxxS/T [36,37], can be phosphorylated also by other AGC family kinases [38] such as PKA, PKC, SGK and PAK family kinases. Indeed, SGK was shown to phosphorylate FoxO3 on the AKT phosphorylation sites, though with different site preference than AKT: both phosphorylated the T32 site equally well, however, SGK showed preference for the S315 site and AKT for the S253 site [39]. PKAa was also shown recently to phosphorylate FoxO1 on the AKT phosphorylation sites in vascular endothelial cells [40]. To what extent other AGC family kinases participate in FoxO regulation through phosphorylation of these sites and under what cellular conditions remains to be determined.

Regarding the functional consequences of AKT phosphorylation, it appears that these phosphorylations serve primarily as docking points for 14-3-3 binding and do not affect protein function directly, e.g. DNA binding affinity. This notion was inferred initially from DAF-16 studies and later from mammalian FoxO studies [28,41–43]. Crystallography studies also do not suggest direct effect of these phosphorylations on FoxO protein function [44]. However, since 14-3-3 deficient models are lethal it has been difficult to distinguish between direct effects of phosphorylation versus 14-3-3-mediated effects. One approach to address this question is discussed bellow in Section 3.

Other open questions relating to mechanistic aspects of FoxO regulation by AKT relate to the cellular compartment of the phosphorylation event, the binding of AKT to FoxO and isoforms specificity. Regarding the phosphorylation location, though initial studies on AKT activation offered a model where AKT activation occurs at the plasma membrane followed by translocation of active AKT to the nucleus, the current view is that AKT can be also directly activated in the nucleus by nuclear pools of PI3K involving phosphorylation by PDK1 and DNA-PK [45–47]. Thus, it is plausible that FoxO proteins can be phosphorylated both in the cytoplasm and nucleus and that for different conditions different pools of AKT may target FoxO proteins at different locations. It is established however that FoxO proteins phosphorylated at the AKT sites can be detected primarily in the cytoplasm, while nuclear FoxO is devoid of phosphorylation at these sites, suggesting that even if FoxO proteins are being phosphorylated in the nucleus, their half-life in this compartment is short.

As to AKT-FoxO interaction, it has been observed that endogenous AKT and FoxO can be found in a complex [48], however, the interaction between the two proteins has not been studied in detail. In this regard, the binding of AKT has not been thoroughly investigated to any of its numerous targets [38,49,50]. A recent study from our group addressed this point to some extent, establishing that the three AKT phosphorylation motifs are not involved in AKT-FoxO interaction, suggesting to the existence of a distant docking point on FoxOs for AKT binding that remains to be defined [51].

Little is known about the preference of AKT isoforms to specific FoxO isoforms. Isoform specific AKT knockouts models have not been thoroughly investigated yet as to the status of specific FoxO isoform phosphorylation levels or activity. Knockdown of either AKT1 or AKT2 in Hella cells seems to reduce FoxO3 phosphorylation equally well [43].

3. Regulation of FoxO proteins by 14-3-3

14-3-3 proteins are a family of evolutionary conserved modulator proteins that regulate multiple signaling pathways in the cell through binding to specific Ser/Thr-phosphorylated motifs on target proteins (reviewed in [52–54]). Mammals express 7 14-3-3 isoforms that can form homo and hetero dimers. Known 14-3-3 binding sites include two defined motifs: RSxpS/TxP (mode 1) and RxxxpSxP (mode 2) as well as several other phosphorylated sequences and some non-phosphorylated ones [55–58]. Upon target binding, usually as a dimer, 14-3-3 proteins can affect the function of the target protein by several means, including directly modulating the enzymatic activity of the target protein, its protein stability, cellular localization or its association with other proteins [54,59,60]. Besides FoxO proteins, many other AKT targets have been shown to be regulated by 14-3-3, including BAD [61], TSC2 [62], ataxin-1 [63], p27Kip1 [64], YAP [65], tuberin [66], PRAS40 [67], MDMX [68] and SRPK2 [69]. This sharing of targets is due to the overlap between the recognition motifs of AKT and 14-3-3: RxRxxS/T for AKT and RSxpS/TxP for 14-3-3.

3.1. Regulation of FoxO localization

The initial work by Brunet et al [19] demonstrated that two of the three AKT phosphorylation sites on FoxO3, T32 and S253 cooperatively mediated the binding to the 14-3-3z isoform. The authors proposed that 14-3-3 binding might be responsible for the regulation of FoxO3 nuclear localization, as AKT activation induced FoxO3 accumulation in the cytoplasm while its inhibition resulted in FoxO3 accumulation in the nucleus. Accordingly, FoxO3 mutants lacking the AKT phosphorylation sites were strictly nuclear. This initial observation was further investigated in a follow up study demonstrating that 14-3-3 proteins contribute to FoxO3 accumulation in the cytoplasm following phosphorylation by AKT, both by increasing nuclear export of FoxO3, functioning in conjunction with two nuclear export sequences present at the carboxy terminus of FoxO3 and by inhibiting FoxO3 reimport to the nucleus by potentially masking two nuclear localization sequences (NLS) present near the S253 14-3-3 binding site ($_{248}$RRR$_{250}$ and $_{269}$KKK$_{271}$) [41]. The ability of 14-3-3 to confer conformational changes on FoxO NLS has been also demonstrated using crystallography structural studies, using FoxO4 NLS as a model [70].

While phosphorylation mediates the binding of 14-3-3, dephosphorylation mediates the dissociation of the complex. PP2A has been implicated in FoxO3 dephosphorylation at the T32 and S253 sites [43,71]. PP2A inhibitors or its knockdown can stabilize FoxO3 phosphorylation in the presence of AKT inhibition [43]. This effect also results in stabilization of the FoxO3-14-3-3 complex. In addition, PP2A inhibition attenuates FoxO3 relocalization to the nucleus in response to AKT inhibition as well as increased FoxO3 transcriptional activity. Interestingly, this study suggests that PP2A is not responsible for regulating FoxO1 or FoxO4, pointing out to isoform specific regulation by phosphatases. Though multiple 14-3-3 isoforms have been shown to bind and regulate FoxO proteins, including 14-3-3 sigma, epsilon [41,72] and zeta [19,73] it has not been established whether there are subtle differences in FoxO regulation by the different isoforms, especially since 14-3-3 proteins form both homo and hetero-dimers.

3.2. Regulation of FoxO DNA binding

The effect of AKT phosphorylation through induction of 14-3-3 binding on FoxO DNA binding was described initially with DAF-16 [28]. This study reconstructed *in vitro* DAF-16 DNA binding and demonstrated that 14-3-3 binding to DAF-16 completely blocked the ability of DAF-16 to bind DNA. This study also demonstrated that AKT phosphorylation in-itself did not have an effect on DAF-16 DNA binding but required the binding of 14-3-3 to the phosphorylated

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sites to block the DNA binding. This inhibition required a dimeric 14-3-3, suggesting that a 14-3-3 dimer through simultaneous binding to the T32 and S253 sites could mask the forkhead DNA binding domain. This notion was proved in later studies with mammalian FoxO4 and FoxO3, demonstrating the ability of 14-3-3 to mask the DNA binding domain of FoxO [42,44,74,75]. These studies also confirmed that AKT phosphorylation in-itself does not confer conformation changes in the DNA binding domain that could affect DNA binding, rather, the generation of the 14-3-3 binding sites is the critical effector of the phosphorylation event (for more details see the review by Obsil et al in this issue [76]). Other posttranslational modifications of FoxO1 were reported however to affect FoxO DNA binding, for example, acetylation and phosphorylation by MST1 [77]. Interestingly, it appears that the PI3K-AKT pathway regulates FoxO DNA binding and transcriptional activity also in a FoxO phosphorylation-independent manner, since FoxO mutants lacking the AKT phosphorylation sites, though constitutively localize to the nucleus, have low DNA binding and transcriptional activity under conditions of high PI3K-AKT activity [28]. This observation suggests to the existence of a PI3K-AKT-regulated FoxO cofactor/s required for high affinity DNA binding and transcriptional activity.

3.3. Regulation of FoxO transcriptional activity and protein stability

The consequence of FoxO phosphorylation by AKT and concomitant binding of 14-3-3 is reduced FoxO transcriptional activity [19,28]. This result represents probably the sum of limited FoxO presence in the nucleus and its reduced DNA binding activity, however, since FoxO proteins have been shown to affect transcription also by serving as cofactors for other transcription factors [1,78,79], it is plausible that 14-3-3 biding may interfere with the ability of FoxO to bind other target proteins. This point is of importance since FoxO proteins have been shown to participate in several important transcriptional complexes, for example, with estrogen receptor [80–82], p53 [83,84], myc [85], RUNX1 [86], Smad3/4 [87] and Hif-1-a [88]. In this regard, the ability of 14-3-3 to affect its target's participation in protein complexes is a well-documented phenomenon [54,60]. As regards the effect of 14-3-3 binding on the ability of FoxO proteins to interact with transcriptional regulators, the available data is scarce. Of note, an initial report suggesting that AKT phosphorylation/14-3-3 binding primarily regulates DNA binding but not transcriptional activity *per se*, could be somewhat misleading, since the fragment used in this study for examining the transcriptional activity of FoxO was missing the 14-3-3 binding sites, thus it did not provide a conclusive information on the role of 14-3-3 binding in regulating FoxO transcriptional activity in the context of full-length FoxO [28]. A separate study suggested also that FoxO1 transcriptional activity could be regulated by insulin independent of its DNA binding region and phosphorylation by AKT [89,90].

14-3-3 proteins have been shown to affect the stability of several of their target proteins as well the half-life of the phosphorylated form, suggesting that it can protect the target protein from both degradation and dephosphorylation [52,54,60,91–94]. Indeed, 14-3-3 has been shown to protect FoxO3 dephosphorylation at the AKT sites, which is mediated by PP2A [43]. Though there is no published data regarding the effect of 14-3-3 binding on FoxO protein stability, when one examines the available literature, it could be noticed that FoxO mutants lacking the AKT phosphorylation sites show significantly lower steady-state expression levels than wildtype FoxOs [19,23,95]. Our recent results confirm this observation by demonstrating that increased 14-3-3 expression enhances the expression levels of both total FoxO protein and its phosphorylated form through a mechanism that involves both protection from dephosphorylation and degradation [51]. This function of 14-3-3 suggests that availability of unbound 14-3-3 in the cell may dictate the fate and dynamics of phosphorylated FoxO proteins toward either fast recycling/reshuttling to the nucleus, stabilization in the cytoplasm or degradation. The abundance of binding-capable 14-3-3 in the cell is tightly regulated based on cell cycle stage and environmental conditions, for example by the regulation of its availability through interaction with intermediate filaments during cell cycle progression [54,60,96–99], phosphorylation by stress-activated kinases, such as JNK [73,100,101], or kinases that abrogate 14-3-3 dimerization such as PKA [102], SDK [103] and MAPKAPK2 [104]. Thus, it is plausible that the abundance of binding-capable 14-3-3 in the cell could dictate FoxO protein levels and the magnitude of their activation, allowing fine-tuning of the pathway based on changing cell conditions [51].

4. Cross-talk with other pathways

Other mechanisms besides the established AKT-14-3-3 system have been shown to regulate FoxO functions and can directly modulate FoxO function with some of them also cross talking with the AKT-14-3-3 pathway. These regulatory mechanisms include additional phosphorylation events (Fig. 2), acetylation, methylation and ubiquitination [1,105,106]; see also Dobson, M. and Tzivion, G. FoxO3. *UCSD-Nature Molecule Pages (2011):* http://www.signaling-gateway.org/molecule/query?afcsid=A000945 (doi:10.1038/mp.a000945.01).

4.1. Stress-activated kinases and other phosphorylation events

FoxO proteins are phosphorylated on multiple sites besides the discussed AKT phosphorylation sites. These include, S207, S295/345/426, S413/588/626, and S644 in FoxO3 as well as S249, S322/325 and S329 in FoxO1 and T447/451 in FoxO4 (Fig. 2). The phosphorylation at S207 on FoxO3 is mediated by MST1 and is induced by oxidative stress [107]. This phosphorylation reduces FoxO3 binding with 14-3-3 and results in increased FoxO activity and nuclear localization. Similar results were obtained with DAF-16 and FoxO1 [107–109]. The phosphorylation cluster at FoxO3 S295/345/426 is targeted by ERK-1/2 and mediates MDM2-dependent ubiquitination and protein degradation [110,111]. S413/588/626 are targeted by AMPK in response to nutrient deprivation and this phosphorylation positively regulates FoxO transcriptional activity, without affecting localization or DNA binding directly [112]. S644 is targeted by IKKb and this phosphorylation inhibits FoxO3 function by increasing its nuclear exclusion and degradation [113,114]. S249 of FoxO1, which is located within a nuclear localization sequence (NLS)

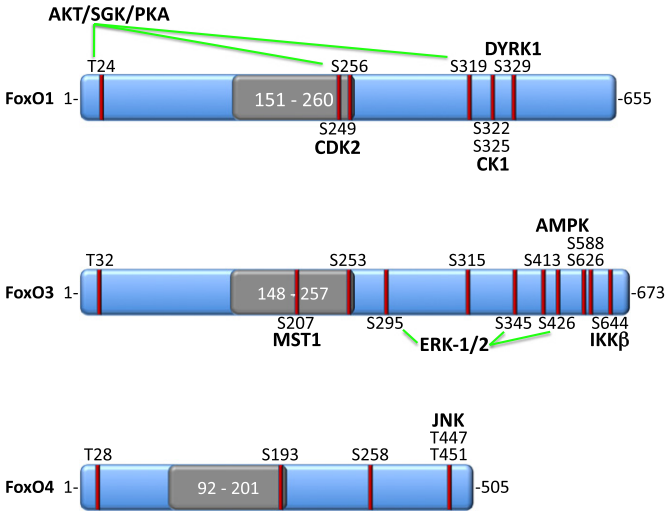

Fig. 2. FoxO proteins are regulated by multiple Ser/Thr kinases. Depiction of the reported FoxO phosphorylation sites and the kinases that can phosphorylate these sites. See text for more detail.

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is targeted by CDK2 and its phosphorylation induces the nuclear exclusion of FoxO1, possibly through interfering with the function of the NLS [115]. S322/325 of FoxO1 are targeted by CK1 and facilitate FoxO1 nuclear export [116,117]. S329 of FoxO1 is targeted by DYRK1 and this phosphorylation increases FoxO1 cytoplasmic localization [118]. T447/451 of FoxO4 were identified as JNK phosphorylation sites and their phosphorylation in response to oxidative stress or TNF results in FoxO4 translocation to the nucleus and increased transcriptional activity in a manner that seems independent of 14-3-3 binding or phosphorylation by AKT [119,120].

4.2. FoxO regulation by reversible acetylation

Several studies described the acetylation of FoxO proteins at sites corresponding to K242, K245 and K262 of FoxO1 [121,122]. These acetylations are mediated by CBP/P300 and PCAF and modulate FoxO activity (for an update review see the article in this section by Daitoku et al [123]). There is some controversy however, regarding the exact effect of acetylation on FoxO activity: some of the result point to increased FoxO activity while other to decreased activity. This controversy if further complicated by the fact that while FoxO acetylation itself may be inhibitory, recruitment of CBP/P300 to promoter regions by FoxO induces histone acetylation, which serves as a positive signal for transcription initiation. Furthermore, FoxO acetylation was suggested to reduce DNA binding and to increase its phosphorylation at the S256 site by AKT, again, providing negative regulation [124]. FoxO deacetylation involves both class-I histone deacetylases and the class-III, NAD-dependent histone deacetylases designated sirtuins. Sirt1, 2 and 3 all have been shown to bind FoxO proteins and induce their deacetylation. The role of sirtuins and FoxO deacetylation in FoxO function, however, is also controversial, with results suggesting both negative and positive effects [121–123,125,126]. It is also suggested that some FoxO target genes, especially genes related to cell cycle control and senescence, are up-regulated while pro-apoptotic genes are down-regulated [122].

4.3. FoxO regulation by methylation and ubiquitination

FoxO protein levels are mediated among others by ubiquitin-dependent protein degradation [105] (for recent review see also the article by Huang and Tindall in this issue [127]). Some of the signals that induce FoxO ubiquitination and degradation include phosphorylation by AKT, ERK-1/2 and IKK. The identified E3 ligases for FoxO proteins include SKP2 [128], which binds AKT-phosphorylated FoxO1 at Ser 256 and MDM2, which binds ERK-phosphorylated FoxOs [110,111,129]. Interestingly, MDM2 can both induce FoxO mono-ubiquitination as well as its poly-ubiquitination. Mono-ubiquitination in contrast to poly-ubiquitination, which targets FoxO for degradation, results in FoxO translocation to the nucleus and increased transcriptional activity [127]. Our recent finding showing FoxO3 stabilization by 14-3-3 offers a cross talk between 14-3-3 binding to AKT-phosphorylated FoxO and its degradation [51]. It remains to be examined whether 14-3-3 binding interferes with FoxO association with SKP2 or other degradation mechanisms.

Another FoxO post-translational modification that was shown to cross talk with the AKT/14-3-3 FoxO regulatory mechanism is arginine methylation [106]. Yamagata et al reported FoxO1 methylation at Arg 248 and 250, within the AKT phosphorylation motif, demonstrating that this methylation interfered with the ability of AKT to phosphorylate S253 (these sites correspond to R250/252/S256 in human FoxO1). This study also showed that the arginine methyl-transferase PRMT1 mediated the observed FoxO1 methylation and that PRMT1 knockdown resulted in decreased FoxO1 function through its increased exclusion from the nucleus and protein degradation.

5. Conclusions and future perspectives

FoxO proteins represent an evolutionary conserved pathway that serves to coordinate cellular responses to changing environmental conditions. Through transcriptional regulation of a large list of target genes and interactions with a vast array of transcriptional regulators

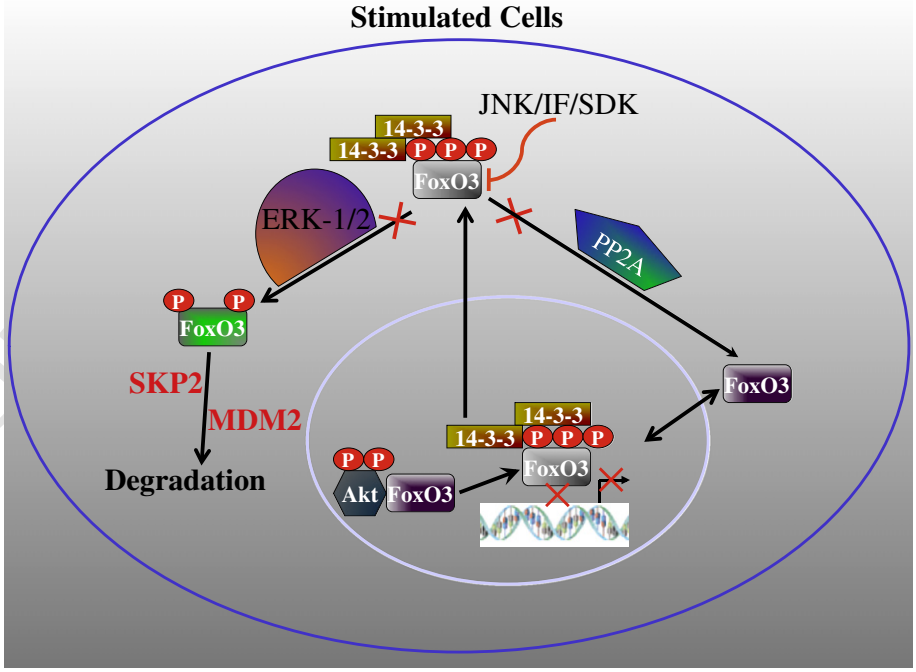

Fig. 3. Regulation of FoxO proteins by AKT and 14-3-3. In growth factor-stimulated cells AKT phosphorylation of FoxO proteins generates binding points for 14-3-3 proteins. 14-3-3 binding blocks FoxO DNA binding and accelerates its nuclear export while inhibiting import. In the cytoplasm, 14-3-3 binding attenuates FoxO dephosphorylation and degradation. Stress-activated kinases such as JNK and sphingosine-dependent kinase (SDK) can phosphorylate 14-3-3 proteins and prevent its binding to target proteins. Also, intermediate filaments (IF), such as vimentin and keratins can sequester 14-3-3 proteins and limit their availability to other target proteins. See text for more details.

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they affect multiple cellular functions such as cell cycle regulation, apoptosis and cellular metabolism. They can integrate signals coming from the PI3K-AKT pathway with various stress signals mediated through JNK, MST1 or IKK (Fig. 3). Our understanding of this complex network and tight regulation is probably at its beginning and will require much more work to fully unfold this pathway. Some of the remaining questions include the identification of the full spectra of direct FoxO target genes, comprehensive determination of FoxO interacting proteins and elucidation of isoform specific functions of the four FoxO family members.

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