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REVIEW

Structure/function relationships underlying regulation of FOXO transcription factors

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The FOXO subgroup of forkhead transcription factors plays a central role in cell-cycle control, differentiation, metabolism control, stress response and apoptosis. Therefore, the function of these important molecules is tightly controlled by a wide range of protein–protein interactions and posttranslational modifications including phosphorylation, acetylation and ubiquitination. The mechanisms by which these processes regulate FOXO activity are mostly elusive. This review focuses on recent advances in structural studies of forkhead transcription factors and the insights they provide into the mechanism of DNA recognition. On the basis of these data, we discuss structural aspects of protein–protein interactions and posttranslational modifications that target the forkhead domain and the nuclear localization signal of FOXO proteins.
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Introduction

The forkhead box (FOX) transcription factors contain approximately 110-amino-acid-long winged helix DNA-binding domain (DBD) known as the forkhead domain (Weigel and Jackle, 1990; Kaestner *et al*., 2000; Mazet *et al*., 2003). The FOX proteins display large functional diversity and play a wide range of roles in development, proliferation, differentiation, stress resistance, apoptosis and control of metabolism (recently reviewed by Carlsson and Mahlapuu, 2002; Lehmann *et al*., 2003; Greer and Brunet, 2005; and van der Horst and Burgering, 2007). Their name is derived from the *Drosophila melanogaster* forkhead gene, the first identified FOX transcription factor, which is important for the correct formation of the anterior and posterior gut of the embryo (Weigel *et al*., 1989). All FOX proteins show high degree of amino-acid sequence identity in their forkhead DBD and constitute a discrete family within the 'winged helix protein' superfamily that occurs in both eukaryote and prokaryote genomes (Clark *et al*., 1993; Gajiwala and Burley, 2000).

Among the forkhead box family of transcription factors (a group of more than 100 proteins identified so far), the 'O' subgroup consists of four members (FOXO1, FOXO3, FOXO4 and FOXO6). The FOXO proteins were initially identified in humans at chromosomal rearrangements in certain tumors (Galili *et al*., 1993; Davis *et al*., 1994; Parry *et al*., 1994; Borkhardt *et al*., 1997; Hillion *et al*., 1997; Anderson *et al*., 1998). They are the vertebrate orthologs of the *Caenorhabditis elegans* DAF-16 transcription factor and constitute key components of a highly conserved signaling pathway that connects growth and stress signals to transcriptional control (Lin *et al*., 1997; Ogg *et al*., 1997). Molecules of FOXO proteins consist of four domains: a highly conserved forkhead DBD, a nuclear localization signal (NLS) located just downstream of DBD, a nuclear export sequence (NES) and a C-terminal transactivation domain (Figure 1). FOXO1, FOXO3 and FOXO6 proteins have similar length of approximately 650 amino-acid residues, whereas FOXO4 sequence is shorter and contains about 500 amino-acid residues. Analysis of multiple sequence alignment shows that several regions of FOXO proteins are highly conserved (Figure 2). The regions showing the highest sequence conservation include the N-terminal region surrounding first AKT/protein kinase B (PKB) phosphorylation site, the forkhead DBD, the region containing NLS and the part of the C-terminal transactivation domain.

Transcriptional activity of FOXO proteins is regulated through insulin–phosphatidylinositol 3-kinase–AKT/PKB signaling pathway. Phosphorylation by AKT/PKB creates two binding sites for the 14-3-3 proteins and induces phosphorylation of additional sites by casein kinase-1 (CK1) and dual-specificity tyrosine-regulated kinase-1A. The AKT/PKB-mediated phosphorylation of FOXO induces binding of 14-3-3 proteins, and the resulting complex is then translocated to the cytosol where the bound 14-3-3 protein prevents reentry of FOXO into the nucleus likely by interfering with the function of their NLS (Brunet *et al*., 1999; Brownawell *et al*., 2001; Cahill *et al*., 2001; Zhao *et al*., 2004; Obsilova *et al*., 2005). In addition to

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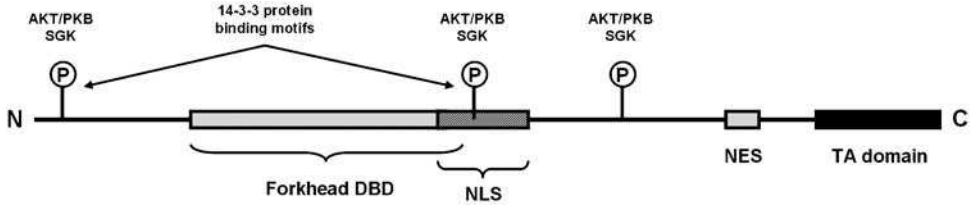

Figure 1 Schematic representation of primary structure of FOXO proteins. All FOXO proteins have the same domain organization and contain forkhead DNA-binding domain (DBD), nuclear localization signal (NLS), nuclear export sequence (NES) and transactivation domain (TA). Only the AKT/protein kinase B (PKB) phosphorylation sites are shown.

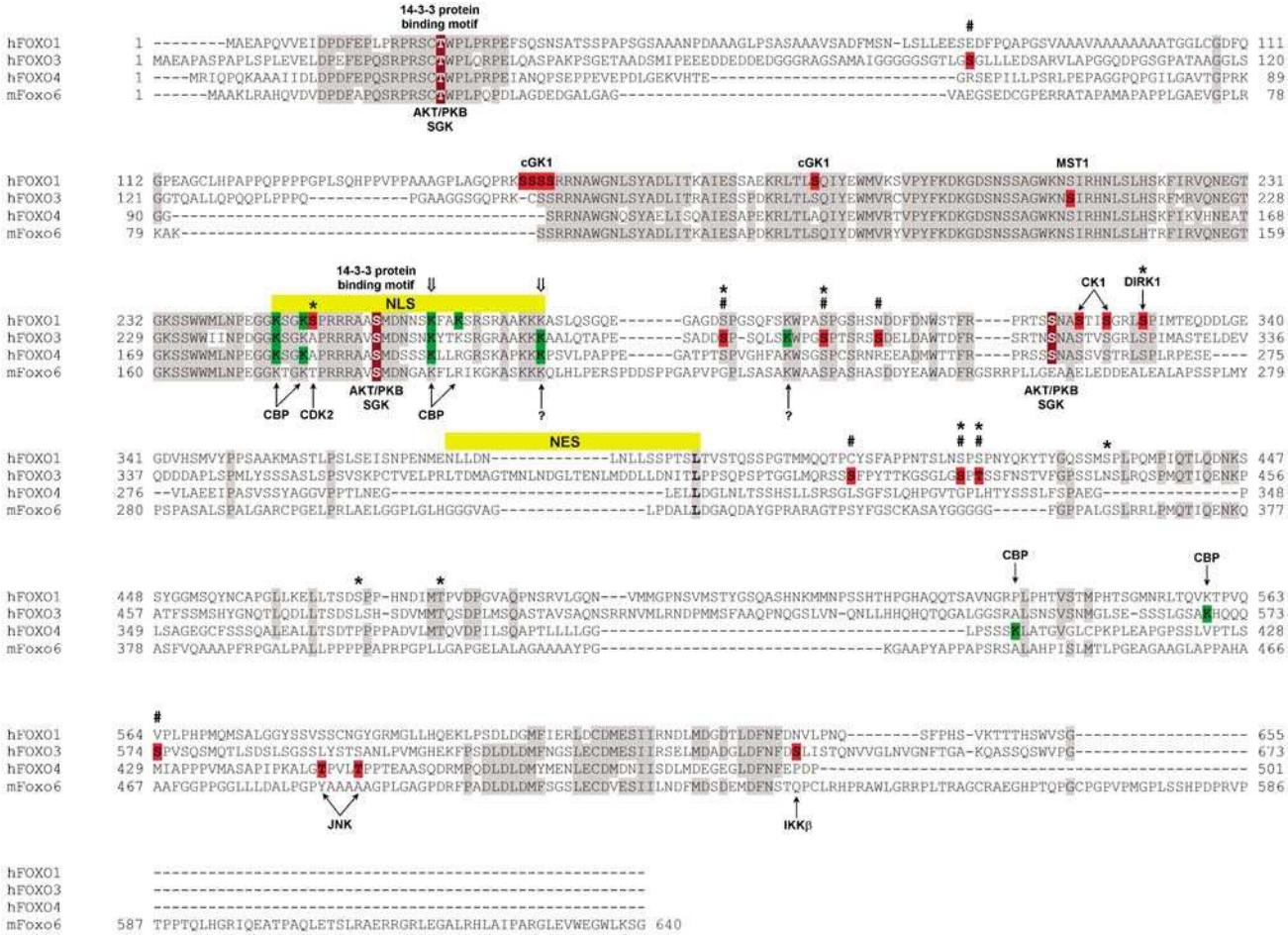

Figure 2 Sequence alignment and posttranslational modifications of FOXO subgroup members. Residues that are conserved at least in three sequences are shaded in gray. Residue color coding: brown, AKT/protein kinase B (PKB)/serum- and glucocorticoid-inducible kinase (SGK) phosphorylation sites; red, non-AKT/PKB phosphorylation sites; and green, acetylation sites. Symbol (⇓) denotes monoubiquitination sites. Regions of nuclear localization signal (NLS) and nuclear export sequence (NES) motifs are labeled by yellow boxes. Question marks indicate unknown acetylating enzymes. Symbol (#) denotes oxidative stress-induced phosphorylation events (Brunet *et al*., 2004). Symbol (*) denotes sites phosphorylated by mitogen-activated kinases ERK and p38 (Asada *et al*., 2007). CBP, cyclic-AMP responsive element binding (CREB)-binding protein; cGK1, cyclic GMP-dependent kinase-1; DIRK1, dual-specificity tyrosine-regulated kinase-1A; JNK, c-JUN N-terminal kinase.

AKT/PKB-mediated phosphorylation, the function of FOXO proteins is also controlled by other types of posttranslational modifications including non-AKT/PKB-mediated phosphorylation, acetylation and ubiquitination. Sites of these posttranslational modifications are often located within the conserved regions of FOXO molecule (Figure 2). The precise mechanisms by which posttranslational modifications regulate FOXO functions are mostly elusive, but in many cases they seem to affect DNA-binding potential of FOXO proteins, function of their NLS and NES, or interactions of FOXO with other proteins.

This review focuses on recent advances in structural studies of forkhead transcription factors and the insights they provide into the mechanism of DNA recognition. On the basis of these data, we discuss structural aspects of protein–protein interactions and posttranslational modifications that target the forkhead domain and the NLS of FOXO proteins.

Structure of forkhead DNA-binding domain

Structural studies of forkhead proteins began with the solution of co-crystal structure of the DBD of a FoxA3 (HNF-3γ)−DNA complex (Clark *et al*., 1993). FoxA3 is a liver-specific transcription factor that plays important roles in cell differentiation and tissue-specific gene expression (Lai *et al*., 1993). Its structure showed that the forkhead/winged helix motif is a compact structure containing about 110 amino-acid residues that fold into three α-helices (H1, H2 and H3), three β-strands (S1, S2 and S3) and two wing-like loops (W1 and W2) (Figure 3). Topologically, the arrangement of the domain is H1−S1−H2−H3−S2−W1−S3−W2. The strand S1, inserted between helices H1 and H2, interacts with strands S2 and S3 to form a three-stranded, twisted, antiparallel β-sheet. While the N-terminal part of the domain is formed by a cluster of three α-helices, the C-terminal half consists of β-strands S2 and S3 and two large loops (wings W1 and W2) that protrude from β-sheet.

More recently, several three-dimensional structures of forkhead domains have been determined using X-ray crystallography or nuclear magnetic resonance spectroscopy. These new data revealed that among various forkhead domains, there are small variations in the secondary structure content and topological arrangement (Figure 4). Forkhead domains of FOXO4 (human AFX) (Weigelt *et al*., 2001), FoxD3 (Genesis) (Marsden *et al*., 1998), FOXC2 (FREAC-11) (van Dongen *et al*., 2000) and FoxQ1 (HFH-1) (Sheng *et al*., 2002) contain an additional short 3_{10}-type helix located between α-helices H2 and H3. Structures of DNA complexes of FoxD3 DBD (Genesis) (Jin and Liao, 1999), FOXK1a DBD (ILF-1) (Tsai *et al*., 2006), FOXP2 DBD (Stroud *et al*., 2006) and an apo-form of FOXK1a DBD (ILF-1) (Liu *et al*., 2002) show that these forkhead domains contain an additional helix located at the C terminus downstream of β-strand S3. It seems that in the case of FoxD3, the formation of this C-terminal helix is induced by the binding to the target DNA (Marsden *et al*., 1998; Jin and Liao, 1999). On the other hand, the C terminus of FOXK1a DBD has helical fold both in the absence and presence of DNA, suggesting that the C-terminal helix can be a native part of the forkhead motif (Liu *et al*., 2002; Tsai *et al*., 2006).

Mechanism of DNA recognition by FOX proteins

Forkhead proteins share similar binding specificity to the core sequence 5′-(A/C)AA(C/T)A-3′ (Overdier *et al*., 1994; Kaufmann *et al*., 1995; Weigelt *et al*., 2001). However, different classes of forkhead proteins recognize diverse DNA sequences adjacent to the core sequence through still not fully understood mechanism. To date, six structures of forkhead domains bound to DNA have been solved: FoxA3−DNA (Clark *et al*., 1993), FoxD3−DNA (Jin and Liao, 1999), FOXP2−DNA (Stroud *et al*., 2006), NFAT−FOXP2−DNA (Wu *et al*., 2006), FOXK1a−DNA (Tsai *et al*., 2006) and FOXO3a−DNA (Tsai *et al*., 2007). Structure of FoxA3−DNA complex provided first details about interactions between forkhead DBD and the core sequence, showing that the helix H3 represents the main DNA recognition site (Clark *et al*., 1993). The forkhead domain of FoxA3 binds to the DNA duplex as a monomer with the helix H3 docked into the major groove roughly perpendicular to the DNA axis providing extensive interactions with the core sequence. Residues that are involved in these interactions (Asn165 and His169) are highly conserved among all forkhead proteins (Figure 3c). Contacts between forkhead domain and DNA involve direct hydrogen bonds between side chains and bases, water-mediated side chain−base interactions and van der Waals contacts with both the bases and the backbone of DNA (Figures 5a and 6a). Other regions of FoxA3 DBD that make important interactions with DNA, in addition to recognition helix H3, are both wings W1 and W2 flanking the helix H3. Mainly the wing W2 was shown to be an important part of DNA-binding interface. This loop, emerging from the C terminus of β-strand S3, makes both specific and unspecific contacts with the major and minor grooves of the DNA. The binding of FoxA3 DBD also induces a bend of 13° in the DNA duplex narrowing the major groove in which the helix H3 is located (Clark *et al*., 1993).

Transcription factor FoxD3 is a transcriptional repressor, whose expression is restricted to embryonic stem cells and certain tumor cell lines (Sutton *et al*., 1996). Solution nuclear magnetic resonance structure of its DNA complex demonstrated that FoxD3 uses similar mechanism of DNA recognition employing the same residues from helix H3 and wing W2 (Jin and Liao, 1999). However, the question is how to explain that FoxA3 and FoxD3 recognize significantly different DNA sequences while possessing almost identical recognition helix H3? It has been suggested that these differences arise from less conserved regions outside of the main DNA-binding interface. Pierrou *et al*. (1994) showed that amino-acid residues around the N-terminal border of helix H3 and in the wing W2 determine, at least in part, the DNA-binding specificity. In addition, studies on chimeras of HFH proteins have shown that residues from the loop between helices H2 and H3 modulate binding specificity probably through the repositioning of the recognition helix H3 (Overdier *et al*., 1994). The loop-linking helices H2 and H3 and the wing W2 can adopt different conformations in different forkhead domains. For example, these two regions adopt a helical structure in the FoxD3−DNA complex, but a coiled structure in the FoxA3−DNA complex (Clark *et al*., 1993; Jin and Liao, 1999). The role of

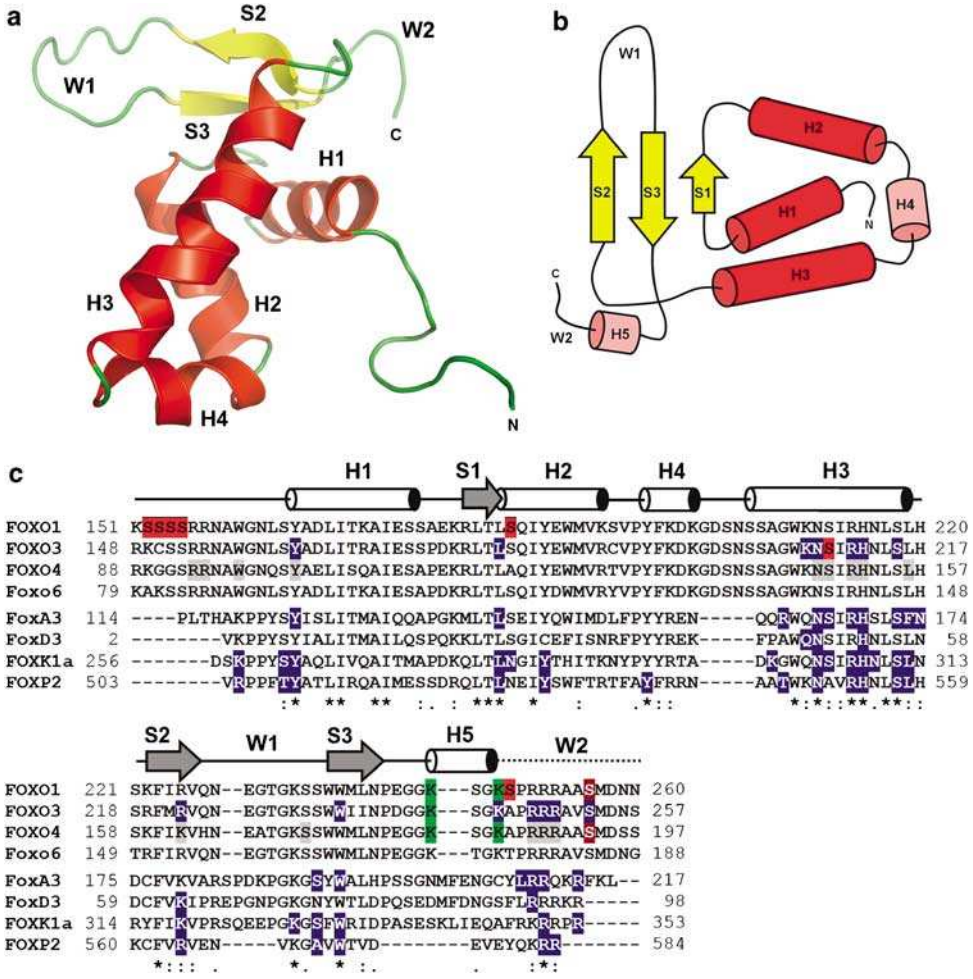

Figure 3 Three-dimensional structure of the forkhead domain. (**a**) Ribbon representation of the solution structure of FOXO4 forkhead domain sequence Ser92-Gly181 (Weigelt *et al*., 2001). (**b**) Topology of the forkhead domain. Helices H4 and H5 are present only in certain forkhead domains. (**c**) Sequence alignment of FOXO forkhead domains and forkhead domains from available structures of FOX–DNA complexes. Secondary structure elements are indicated at the top. Residue color coding: blue, residues involved in protein–DNA contacts (Clark *et al*., 1993; Jin and Liao, 1999; Stroud *et al*., 2006; Tsai *et al*., 2006, 2007); gray, residues of FOXO4 that were predicted to be involved in DNA binding based on homology modeling (Boura *et al*., 2007); brown, AKT/protein kinase B (PKB)/serum- and glucocorticoid-inducible kinase (SGK) phosphorylation sites; red, non-AKT/PKB phosphorylation sites; and green, acetylation sites. Symbol (*) means that the residues in that column are identical in all sequences in the alignment. Symbol (:) means that conserved substitutions have been observed. Symbol (.) means that semiconserved substitutions are observed.

highly flexible wings W1 and W2 in the specificity and tightness of DNA binding was further investigated by Shiyanova and Liao (1999), who showed that substitutions of non-DNA contact residues in wing sequences can dramatically change the dissociation rate of FoxD3–DNA complex. Comparison and analysis of the FOXC2 and FoxD3 structures also suggested that the positions of wings W1 and W2, which are strongly coupled to the position of the β-sheet, are correlated to the position of the DNA-recognition helix H3 (van Dongen *et al*., 2000).

Recently, four new crystal structures of forkhead DBD bound to the target DNA have been solved: FOXP2–DNA (Stroud *et al*., 2006), NFAT–FOXP2–DNA (Wu *et al*., 2006), FOXK1a–DNA (Tsai *et al*., 2006) and FOXO3a–DNA (Tsai *et al*., 2007). Although the overall recognition of core sequence by helix H3 is in all four cases similar to that seen in FoxA3–DNA structure, the recognition patterns are different (Figures 5 and 6). In FOXK1a–DNA complex, residues from the recognition helix H3 interact with eight bases of the DNA duplex through direct hydrogen bonds, water-mediated interactions and van der Waals contacts (Figures 5b and 6b). In addition to the contacts with the major groove, both the wing W1 and the C-terminal segment of FOXK1a interact with the minor groove of DNA and appear to be important for DNA recognition. The wing W1 recognizes the TA sequence 1 bp downstream from the 5′-TAAACA-3′ core sequence and makes several other contacts with phosphate groups. Cluster of basic residues following helix H4 in the C terminus of FOXK1a DBD interacts with nucleotides upstream from the core sequence through hydrogen bonds and bridging water molecules. In the periphery of

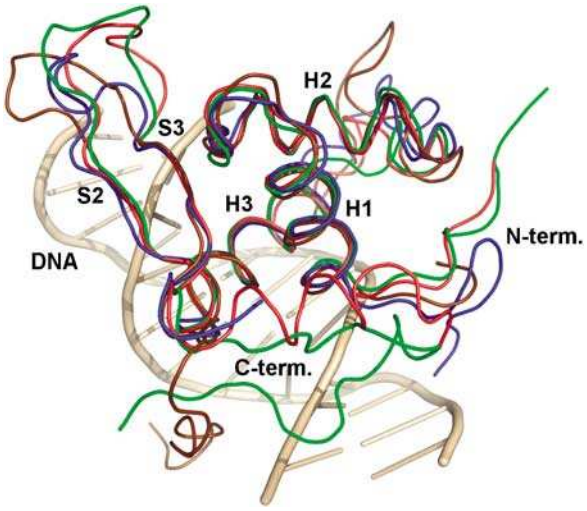

Figure 4 Superimposition of the FOXO4 DBD with FOXO3a DBD (Tsai *et al*., 2007), FOXK1a DBD (Tsai *et al*., 2006) and FoxA3 DBD (Clark *et al*., 1993). For clarity, only the DNA in FOXO3a DBD–DNA complex is shown. The FOXO4 is shown in blue, FOXO3a in brown, FOXK1a in red and FoxA3 in green. This figure was created using PyMOL (http://www.pymol.org).

the FOXK1a–DNA interface, two residues from the N-terminal segment preceding helix H1 make contacts with the DNA backbone, thus providing additional stability to the complex (Tsai *et al*., 2006). The participation of both the N and C termini of forkhead domain in DNA binding was also observed in FOXP2–DNA structure, where residues from these two regions make hydrogen bonds, van der Waals contacts and electrostatic interactions with the DNA backbone (Figures 5d and 6d) (Stroud *et al*., 2006). The C terminus of FOXP2 DBD forms helix H5 running atop of the helix H1 and terminating at the DNA backbone. Forkhead domain of FOXP2 displays additional differences from other FOX proteins as it contains simple type I turn between β-strands S1 and S2 instead of wing W1. The structural variability of the wing regions relative to the rest of forkhead domain across various FOX subgroups further supports the hypothesis that these loops may have specialized functions within each subgroup.

DNA recognition by FOXO proteins

The forkhead domain of FOXO proteins binds as a monomer to the consensus sequence 5′-GTAAACAA-3′ known as the DAF-16 family member-binding element (Furuyama *et al*., 2000; Biggs *et al*., 2001). This sequence includes the core sequence 5′-(A/C)AA(C/T)A-3′ recognized by all forkhead family members. FOXO proteins also recognize insulin-responsive sequence present in the IGFBP-1 promoter region (Biggs *et al*., 1999; Guo *et al*., 1999; Kops *et al*., 1999; Nakae *et al*., 1999; Rena *et al*., 1999; Tang *et al*., 1999). The sequence of insulin-responsive sequence is defined as 5′-(C/A)(A/C)AAA(C/T)AA-3′ and differs from the DAF-16 family member-binding element in the first two bases (O'Brien and Granner, 1996; Streeper *et al*., 1997). FOXO proteins can bind to both sequences, but they bind to the DAF-16 family member-binding element with higher affinity (Furuyama *et al*., 2000). The solution nuclear magnetic resonance structure of FOXO4 DBD (Weigelt *et al*., 2001) and the crystal structure of FOXO3a–DNA complex (Tsai *et al*., 2007) showed that forkhead domains of FOXO factors have very similar structure to other known forkhead domains. All FOXO members contain a 5-amino-acid insertion between helices H2 and H3 that creates a small extra loop, but has a little effect on the overall structure of the forkhead domain (Weigelt *et al*., 2001; Tsai *et al*., 2007). In the FOXO3a DBD, this H2–H3 loop is a coil structure, but in the FOXO4 DBD, it forms a short helix. Similarly to other structures, highly conserved residues in the recognition helix H3 of the FOXO3a DBD make several hydrogen bonds as well as van der Waals contacts with bases of the FOXO consensus sequence in the major groove of DNA duplex (Figures 5c and 6c). It has been suggested that among these interactions, the van der Waals contacts between the methyl groups of thymine bases from the FOXO consensus sequence and the side chains of Arg211, His212 and Ser215 are essential for FOXO3a promoter recognition (Tsai *et al*., 2007). In addition, both the wing W1 and the C-terminal segment of FOXO3a interact with the DNA and are important for DNA recognition. The C-terminal region of FOXO3a adopts a coil structure and its basic residues (Lys245, Arg248, Arg249 and Arg250) make direct contacts with phosphate groups of DNA (Figure 5c). Mutagenesis analysis revealed that either the removal of the C-terminal region of FOXO3a DBD or substitutions of basic residues in this region with alanines or the insertion of negatively charged residue to the end of this segment (mutation Ser253Asp) reduces DNA binding (Tsai *et al*., 2007). Molecular modeling and deletion analysis suggested that the N-terminal region of FOXO forkhead domain (a region located upstream of the first helix H1) also participates in DNA binding (Boura *et al*., 2007). Similar involvement of the N-terminal region of forkhead domain in DNA binding was observed in FOXK1a–DNA and FOXP2–DNA complexes (Stroud *et al*., 2006; Tsai *et al*., 2006).

In conclusion, highly conserved helix H3 of forkhead DBD serves as the main DNA recognition site interacting with the core sequence within the major groove of DNA, whereas more variable regions including N-terminal sequence preceding helix H1, region between helices H2 and H3, wing W1 and C-terminal segment provide fine-tuning of both the DNA-binding specificity and the stability of forkhead DBD–DNA complexes.

Posttranslational modifications of FOXO forkhead domain and NLS

FOXO proteins are subject to several posttranslational modifications including phosphorylation, acetylation

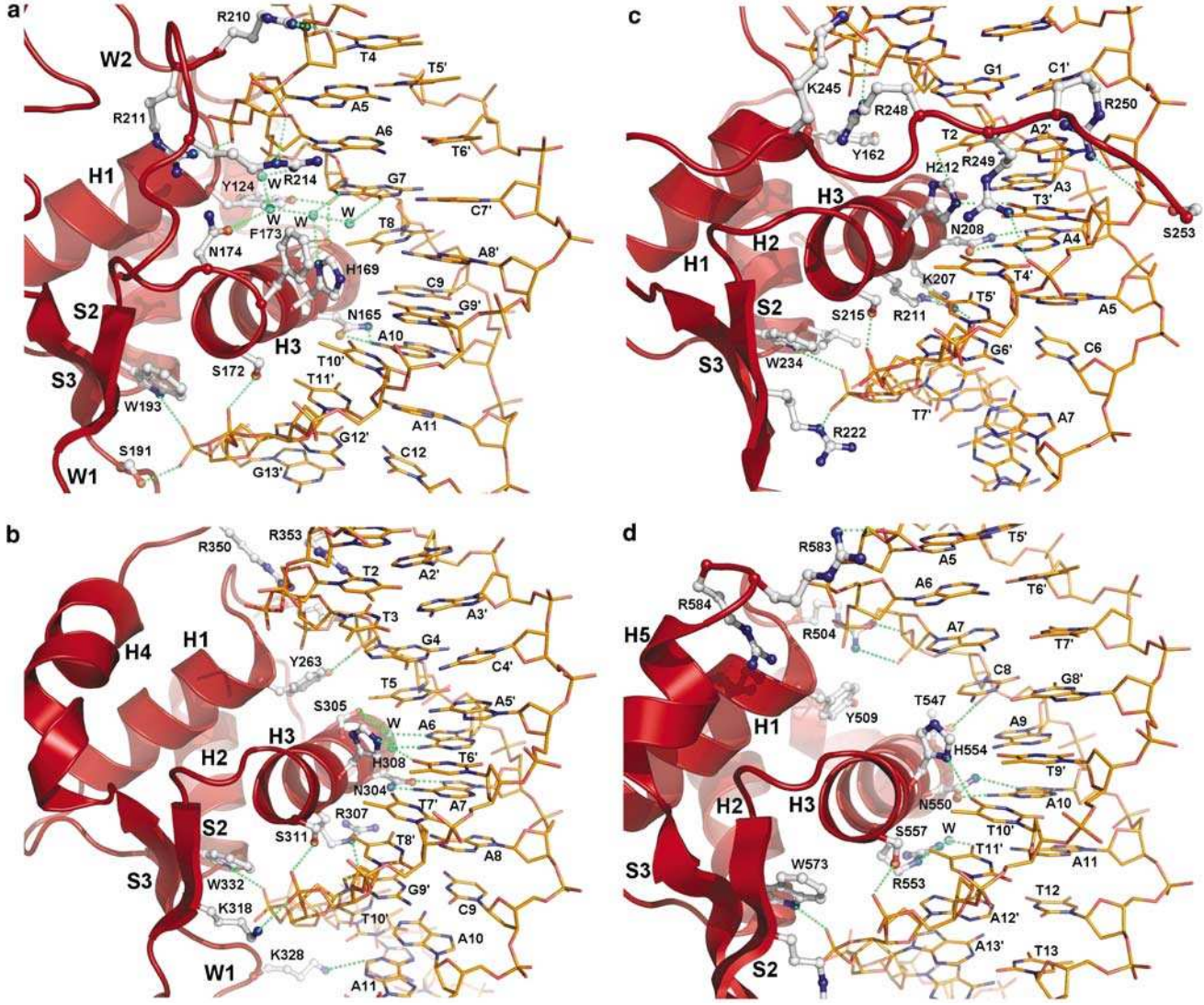

Figure 5 Close-up view of key interactions between forkhead domains and DNA. (**a**) Recognition of the 5′-GTCAACC-3′ core sequence by forkhead domain of FoxA3 (HNF-3γ) (Clark *et al*., 1993). (**b**) Recognition of the 5′-TAAACA-3′ core sequence by forkhead domain of FOXK1a (ILF-1, PDB code '2c6y') (Tsai *et al*., 2006). (**c**) Recognition of the 5′-TAAACA-3′ core sequence by forkhead domain of FOXO3a (FKHR-L1, PDB code '2uzk') (Tsai *et al*., 2007). (**d**) Recognition of the 5′-CAAAT-3′ core sequence by forkhead domain of FOXP2 (PDB code '2a07') (Stroud *et al*., 2006). Contacts important for recognition are represented by dashed green lines. Water molecules are represented as green balls. This figure was created using PyMOL (http://www.pymol.org).

and ubiquitination (extensively reviewed in Greer and Brunet, 2005; Vogt *et al*., 2005; and van der Horst and Burgering, 2007). The precise mechanisms of these modifications in FOXO regulation are still unclear, but in several cases they have been shown to modify DNA-binding potential of FOXO proteins. On the basis of available structural data, several sites of FOXO posttranslational modifications map to regions that are directly involved in DNA binding (Figures 3c and 7).

Phosphorylation of the FOXO DBD

Phosphorylation by AKT/PKB

In response to growth signals, the phosphatidylinositol 3-kinase activates AKT/PKB, and related serum- and glucocorticoid-inducible kinase that then phosphorylate FOXO proteins at three sites (Biggs *et al*., 1999; Brunet *et al*., 1999; Kops *et al*., 1999; Takaishi *et al*., 1999; Tang *et al*., 1999; Brownawell *et al*., 2001). The first site is located at the N terminus, the second one in the forkhead domain and the last one between NLS and NES (Figures 1 and 2). The second AKT/PKB phosphorylation site is located in the wing W2 of FOXO DBD close to the cluster of basic residues that are known to participate in DNA binding (Figures 3c and 6) (Clark *et al*., 1993; Jin and Liao, 1999; Stroud *et al*., 2006; Tsai *et al*., 2006, 2007). It has therefore been suggested that phosphorylation in this basic region might inhibit FOXO binding to DNA (Zhang *et al*., 2002). Indeed, the introduction of negative charge to this region either by phosphorylation or by mutagenesis

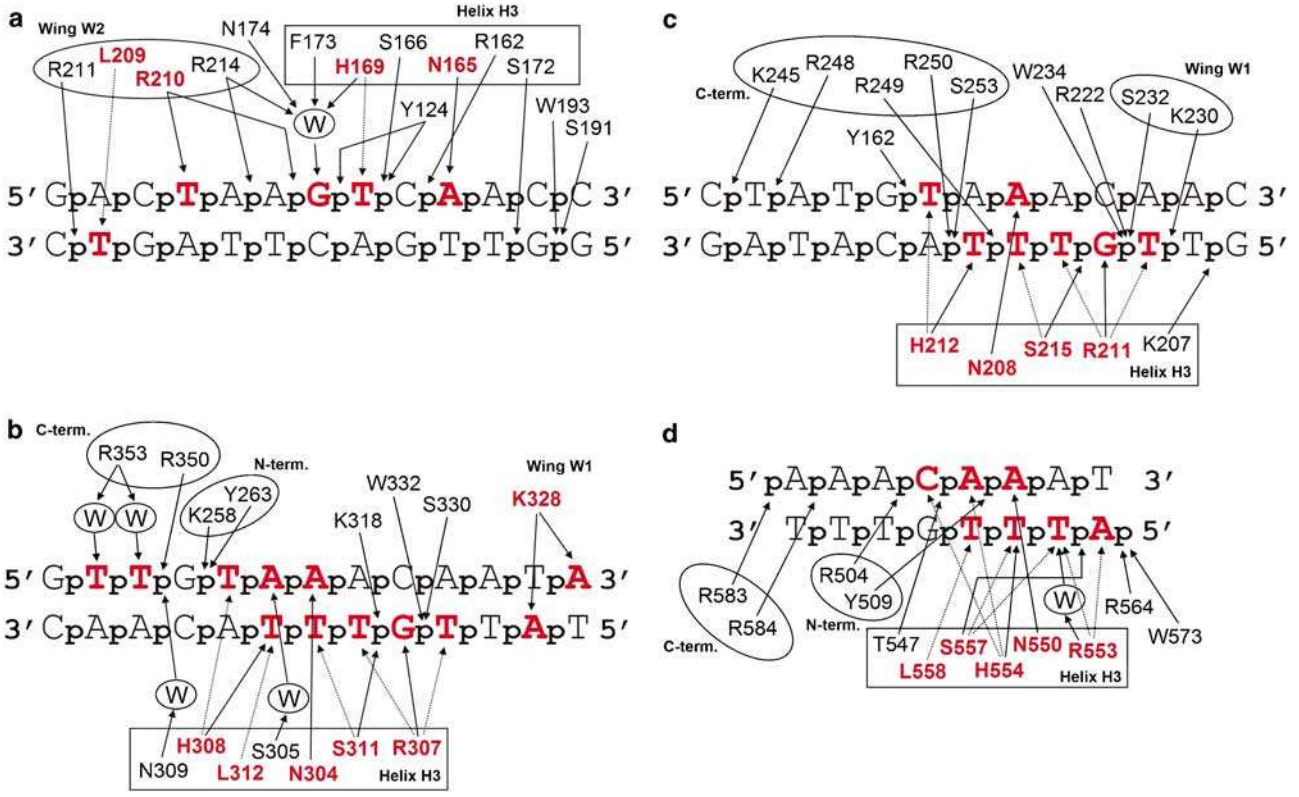

Figure 6 Schematic diagram of protein–DNA contacts. (**a**) Complex FoxA3–DNA (Clark *et al*., 1993). (**b**) Complex FOXK1a–DNA (Tsai *et al*., 2006). (**c**) Complex FOXO3a–DNA (Tsai *et al*., 2007). (**d**) Complex FOXP2–DNA (Stroud *et al*., 2006). The residues and bases that participate in specific contacts between protein and DNA are shown in red. Polar interactions are indicated with arrows. Van der Waals contacts are shown as dashed arrows.

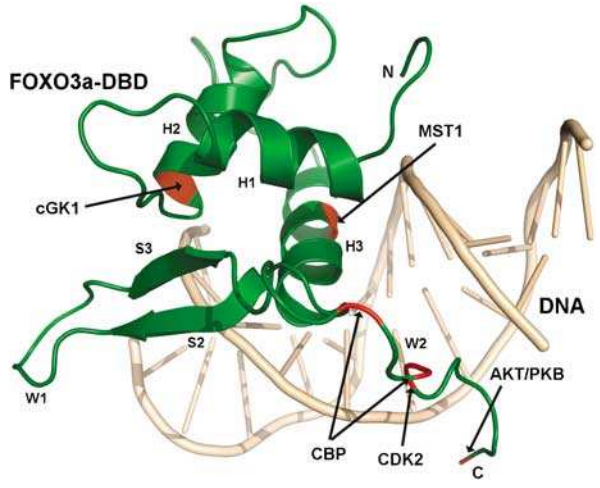

Figure 7 Posttranslational modifications that target the forkhead domain of FOXO proteins. Crystal structure of FOXO3a DBD–DNA complex is used to show sites of these posttranslational modifications (in red) (Tsai *et al*., 2007). This figure was created using PyMOL (http://www.pymol.org). CBP, cyclic-AMP responsive element binding (CREB)-binding protein; CDK2, cyclin-dependent kinase-2; cGK1, cyclic GMP-dependent kinase-1; MST1, mammalian Ste-20 like kinase-1.

reduces DNA-binding affinity of FOXO proteins (Zhang *et al*., 2002; van der Heide *et al*., 2005; Boura *et al*., 2007; Tsai *et al*., 2007). The observed effect, however, varied from complete reduction of DNA-binding affinity to a mild inhibition depending on the construct used for DNA-binding studies.

The AKT/PKB phosphorylates substrates that carry an RXRXX(S/T) motif, which is close to the consensus 14-3-3 binding motifs (Alessi *et al*., 1996; Yaffe *et al*., 1997; Rittinger *et al*., 1999). The 14-3-3 proteins are regulatory molecules that bind to other proteins in a phosphorylation-dependent manner by recognizing a motif containing either a phosphorylated serine or a phosphorylated threonine residue (Muslin *et al*., 1996; Fu *et al*., 2000; Aitken, 2006). The AKT/PKB-dependent phosphorylation of sites at the N terminus and in the forkhead domain of FOXO proteins creates two 14-3-3 binding motifs and induces FOXO binding to nuclear 14-3-3 proteins (Brunet *et al*., 1999, 2002). It has been shown that simultaneous use of both AKT/14-3-3 motifs is necessary for optimal FOXO binding to the 14-3-3 proteins (Brunet *et al*., 1999, 2002; Obsil *et al*., 2003; Zhao *et al*., 2004). These two motifs border the DBD, raising the possibility that the 14-3-3 proteins could participate in the disruption of FOXO binding to DNA. Such 14-3-3 protein-dependent inhibition of DNA binding has been observed for DAF-16 and FOXO4 (Cahill *et al*., 2001; Obsil *et al*., 2003). However, the exact mechanism of this 14-3-3-dependent inhibition of DNA binding is still unclear. Since the second AKT/PKB motif is embedded in the C-terminal part of forkhead domain, the 14-3-3 protein could affect the

binding of this region to the DNA, mask other parts of FOXO DNA-binding interface or change the conformation of forkhead domain core.

The association of FOXO factors with 14-3-3 proteins has another important consequence. Upon the 14-3-3 protein binding, the resulting FOXO–14-3-3 complexes are rapidly transported out of the nucleus and retained within the cytoplasm (Biggs *et al*., 1999; Brunet *et al*., 1999, 2002; Nakae *et al*., 1999; Tang *et al*., 1999). The exact mechanism of this process is unknown, but 14-3-3 proteins have been shown to bind to phosphorylated FOXO in the nucleus where, along with the inhibition of DNA binding, it could induce a conformational change in FOXO molecules and expose their NES for interaction with Exportin/Crm1 (Brunet *et al*., 2002). The NESs of FOXO proteins consist of leucine-rich sequence positioned downstream of the third AKT/PKB phosphorylation site (for example, sequence MEN**LL**DN**L**N**LL** of FOXO1). Although both 14-3-3 binding motifs are located in the N-terminal half of FOXO molecule and relatively far from the NES position, the 14-3-3 proteins can affect conformation of their binding partners in regions that are remote from segments containing 14-3-3 binding motifs (Obsil *et al*., 2001; Yaffe, 2002). In addition, all FOXO proteins contain a sequence that represents a nonclassical bipartite NLS. In general, NLSs do not conform to a specific consensus sequence but form two distinct classes termed monopartite NLS, consisting of a single cluster of basic amino-acid residues, and bipartite NLS, consisting of two basic clusters separated by a variable spacer. These two basic clusters are independent and usually both are required for nuclear targeting (Dingwall and Laskey, 1991). NLS found in FOXO proteins consists of two clusters containing in total 12 arginine and lysine residues positioned on both sides of the second AKT/14-3-3 binding motif in the C terminus of forkhead domain (Figure 2). Therefore, it has been suggested that the 14-3-3 proteins may prevent nuclear reimport of FOXO proteins by masking their NLS (Brunet *et al*., 1999; Brownawell *et al*., 2001; Rena *et al*., 2001). Regulation of subcellular localization of the binding partner is one of the main 'modes of function' of the 14-3-3 proteins (Muslin and Xing, 2000; Tzivion *et al*., 2001). Examples of this mode of regulation include protein phosphatase Cdc25C (Dalal *et al*., 1999), histone deacetylase (Grozinger and Schreiber, 2000), telomerase (Seimiya *et al*., 2000) and protein kinase U-α (Zhang *et al*., 1999). In agreement with this hypothesis, the 14-3-3 protein has been shown to physically interact with both parts of bipartite NLS of phosphorylated FOXO4 (fragment 11-213) (Obsilova *et al*., 2005).

Phosphorylation by other kinases

Recently, it has been shown that the cyclic GMP-dependent kinase-1 (cGK1) regulates FOXO1 activity during muscle cell fusion (Bois *et al*., 2005). FOXO1 directly activates cGK1 transcription and cGK1 then in turn harnesses FOXO1 activity by phosphorylating cluster of serine residue located upstream of the first helix H1 (Ser152-155 of FOXO1) and Ser184 in the N-terminal part of helix H2 (Figures 3c and 7). The cGK1-induced phosphorylation also promotes relocalization of FOXO1 into the cytoplasm. The cGK1-induced phosphorylation seems to be specific for FOXO1 because the cluster of serine residues preceding the helix H1 is unique to FOXO1, and the residue Ser184 is absent in FOXO4 sequence (Figure 3c). Phosphorylation of FOXO1 by cGK1 efficiently inhibits its DNA-binding activity, in agreement with the fact that both these regions make polar and van der Waals interactions with DNA backbone, and the introduction of negatively charged phosphate groups would be expected to disrupt these contacts (Bois *et al*., 2005; Tsai *et al*., 2006, 2007).

Another kinase that has been shown to interact with and phosphorylate forkhead domains of FOXO factors is the oxidative stress-regulated mammalian Ste-20 like kinase-1 (MST1) (Lehtinen *et al*., 2006). MST1-mediated phosphorylation causes disruption of FOXO interaction with 14-3-3 proteins in the cytoplasm, promotes FOXO nuclear translocation, and thereby induces cell death in neurons. However, the mechanism of this FOXO activation is unclear. MST1 phosphorylates highly conserved serine in recognition helix H3 (Figures 3c and 7). In available FOX–DNA structures, this residue lies close to the negatively charged DNA backbone and directly participates in DNA binding. For example, in FoxA3–DNA complex this serine (Ser166) makes hydrogen bond with phosphate group of DNA backbone (Figures 5a and 6a) (Clark *et al*., 1993), whereas in FOXK1a–DNA complex (Ser305), it interacts with base A6 using two water-mediated hydrogen bonds (Figures 5b and 6b) (Tsai *et al*., 2006). Therefore, it would be expected that phosphorylation of this residue reduces FOXO binding to the DNA.

Forkhead domain of FOXO proteins can also be phosphorylated by the cyclin-dependent kinase-2 (Huang *et al*., 2006). However, it seems that this modification is specific for FOXO1 only. Cyclin-dependent kinase-2-induced phosphorylation of Ser249 results in cytoplasmic localization and inhibition of FOXO1. Residue Ser249 together with residue Ser256, a second AKT/PKB phosphorylation site whose phosphorylation reduces FOXO1 DNA-binding potential, lie in the wing W2 and border the cluster of basic residues that participate in DNA binding (Figures 3c, 5 and 6). The insertion of Asp residue to corresponding position in FOXO3a DBD (to mimic a negative charge of the phosphate group) has been shown to substantially reduce FOXO3a binding to the target DNA (Tsai *et al*., 2007). Therefore, it is reasonable to speculate that the mechanism behind cyclin-dependent kinase-2-induced inhibition of FOXO1 is the suppression of its DNA-binding potential by phosphorylating Ser249.

Acetylation of the FOXO forkhead domain and NLS

The activation of the insulin–phosphatidylinositol 3-kinase–AKT/PKB signaling pathway causes

phosphorylation of FOXO proteins, their binding to the 14-3-3 proteins and sequestration into the cytoplasm (Biggs *et al*., 1999; Brunet *et al*., 1999; Nakae *et al*., 1999; Tang *et al*., 1999). On the other hand, the activation and nuclear localization of FOXO proteins can be induced by depletion of insulin or by stress (Tran *et al*., 2003; Brunet *et al*., 2004; Van Der Heide *et al*., 2004; van der Horst and Burgering, 2007). The FOXO nuclear translocation is accompanied by their acetylation by histone acetyltransferases such as p300, the cyclic-AMP responsive element binding-binding protein or cyclic-AMP responsive element binding-binding protein-associated factor (Fukuoka *et al*., 2003; Brunet *et al*., 2004; Daitoku *et al*., 2004; Motta *et al*., 2004; van der Horst *et al*., 2004). The consequences of FOXO acetylation are still the matter of debate. The majority of recently published studies indicate that FOXO acetylation paradoxically suppresses FOXO transcriptional activity, thus probably playing the role of a negative feedback signal in the process of FOXO activation (Daitoku *et al*., 2004; van der Horst *et al*., 2004; Frescas *et al*., 2005; Kobayashi *et al*., 2005). On the other hand, acetylation has also been suggested to enhance FOXO activity (Motta *et al*., 2004) or to act on FOXO in a target gene-specific context (Brunet *et al*., 2004). One of the possible reasons of these conflicting results can be the difficulty to separate the direct effects of acetylation on FOXO proteins from the changes in histone acetylation caused by the recruitment of histone acetyltransferases and deacetylase SIRT1 to regions where FOXO factors function.

The acetylation of FOXO proteins is reversible process and SIRT1, a mammalian homolog of the yeast histone deacetylase Sir2 (silent information regulator-2), was identified as specific FOXO deacetylase (Brunet *et al*., 2004; Daitoku *et al*., 2004; Motta *et al*., 2004; van der Horst *et al*., 2004). Interaction between FOXO and SIRT1 seems to require the presence of 14-3-3 proteins. It has been shown that sir-2.1 deacetylase (SIRT1 ortholog from *C. elegans*) binds nuclear DAF-16 in a 14-3-3 protein-dependent manner (Berdichevsky *et al*., 2006; Wang *et al*., 2006). Thus, 14-3-3 proteins seem to play two antagonistic roles in DAF-16 (FOXO) regulation: (i) they escort and retain phosphorylated DAF-16 (FOXO) proteins in the cytoplasm; and (ii) they mediate interaction between DAF-16 (FOXO) and sir-2.1 (SIRT1) in the nucleus to elicit expression of genes involved in stress resistance and extension of life span (Berdichevsky *et al*., 2006).

The 14-3-3 protein isoforms form very stable homo- and heterodimers, and thus are able to bind simultaneously two ligands (Liu *et al*., 1995; Xiao *et al*., 1995; Tzivion *et al*., 1998; Yaffe, 2002). This mode of 14-3-3 protein action has also been suggested for other signaling complexes including Raf-Bcr (Braselmann and McCormick, 1995), Raf-A20 (Vincenz and Dixit, 1996) and Raf-PKCζ (Van Der Hoeven *et al*., 2000). In addition, crystal structure of the 14-3-3ζ bound to serotonin *N*-acetyltransferase demonstrated that each monomer of the 14-3-3 dimer is able to bind one molecule of protein ligand (Obsil *et al*., 2001). Therefore, it is possible to speculate that 14-3-3 protein bridges FOXO and SIRT1 together in one multiprotein complex.

Several acetylation sites have been described up to date in FOXO proteins and three of them are located within the wing W2 of the forkhead domain (Figures 2 and 3c). The presence of acetylation sites at the C terminus of forkhead domain raises the possibility that acetylation of positively charged lysine residues affects the wing W2 binding to the DNA in a similar way as observed for phosphorylation of nearby located AKT/PKB site. Since the C-terminal part of forkhead domain directly participates in DNA recognition and/or stabilization of FOX−DNA complex, the acetylation of wing W2 might inhibit FOXO binding to the DNA changing the sensitivity of residues located therein to other posttranslational modifications. Indeed, an acetylation of three lysine residues in the wing W2 has been shown to reduce FOXO1 binding to the DNA and increase phosphorylation of the nearby located second AKT/PKB site (Matsuzaki *et al*., 2005). In addition, the simultaneous substitution of Lys242 and Lys245 of FOXO3a with alanines has been shown to significantly reduce the DNA-binding affinity of FOXO3a DBD (Tsai *et al*., 2007). Since the C terminus of FOXO forkhead domain overlaps with nuclear localization sequence, its function can also be affected by acetylation. Modification of lysine residues in the NLS may contribute to its masking and thus blocking FOXO interaction with nuclear import machinery and FOXO reentry to the nucleus (Brunet *et al*., 2004).

In conclusion, a majority of recent studies indicate that acetylation suppresses FOXO functions. However, the mechanism of FOXO regulation by acetylation is very complex and the elucidation of the exact role played by acetylation in the modification of FOXO DNA-binding potential and the function of their NLS would require further experiments.

Ubiquitination of FOXO forkhead domain

Recently, it has been shown that FOXO factors are also subject to poly- and monoubiquitination (Matsuzaki *et al*., 2003; Plas and Thompson, 2003; Huang *et al*., 2005; van der Horst *et al*., 2006). The F-box protein Skp2 has been identified as the putative E3 ubiquitin ligase responsible for polyubiquitination of FOXO proteins in response to growth-factor signaling (Huang *et al*., 2005). However, both polyubiquitination and subsequent proteasomal degradation of FOXO proteins are rather slow and their role in FOXO regulation is still unclear (Matsuzaki *et al*., 2003; Plas and Thompson, 2003). The activity of AKT/PKB and proper cellular localization are necessary for ubiquitin-mediated proteasomal degradation of FOXO (Plas and Thompson, 2003; Huang *et al*., 2005). The proteasomal degradation of FOXO proteins can also be induced by I kappaB kinase-β (Hu *et al*., 2004). This kinase phosphorylates residue Ser644 of FOXO3 at the C terminus of

FOXO molecule. This causes polyubiquitination and subsequent degradation of FOXO3 (Hu *et al*., 2004). However, it seems that this phosphorylation is specific for FOXO3 because Ser644 is not conserved in other FOXO proteins.

A monoubiquitination of FOXO proteins is induced by increased cellular oxidative stress and results in nuclear relocalization of FOXO proteins and an enhancement of their transcriptional activity (van der Horst *et al*., 2006). Monoubiquitination occurs, at least in part, at the same lysine residues (Lys199 and Lys211 of FOXO4) in the C terminus of forkhead domain as acetylation (Figures 2, 3c, 7). The mechanism of monoubiquitination-induced FOXO nuclear localization remains unknown. The potential increase in DNA-binding affinity is in this case rather unlikely because monoubiquitination targets residues whose acetylation reduces DNA binding. Ubiquitination of FOXO proteins is counteracted by deubiquitinating enzyme USP7, which binds to FOXO proteins and inhibits their activity. Both oxidative stress-induced ubiquitination and USP7-mediated deubiquitination have no significant effect on FOXO protein stability (van der Horst *et al*., 2006).

Posttranslational modifications of FOXO proteins in other regions

Phosphorylations by CK1 and DYRK1A kinases

The insulin–phosphatidylinositol 3-kinase–AKT/PKB signaling pathway triggers phosphorylation of several other sites located outside of the forkhead domain and NLS. The phosphorylation of the third AKT/PKB site in FOXO1 (Ser319) creates a consensus motif for phosphorylation by CK1, allowing it to modify Ser322, which in turn primes CK1 to phosphorylate Ser325 (Rena *et al*., 2002). These three phosphorylation sites are closely followed by the fourth one, which is targeted by the DYRK1A (dual tyrosine phosphorylated regulated kinase-1A) (Woods *et al*., 2001). Simultaneous phosphorylation of these four sites creates negatively charged patch that appears to accelerate FOXO relocalization to the cytoplasm by stimulating the interaction between FOXO proteins and the exporting machinery (Exportin/Crm1 and Ran) (Rena *et al*., 2002). Acidic patches created by repeats of phosphorylation sites can be found in many proteins that shuttle between the cytoplasm and nucleus. For example, the CK1-mediated phosphorylation of multiple sites in NFAT4 and mPER proteins induces their nuclear exclusion (Zhu *et al*., 1998; Vielhaber *et al*., 2000).

Stress-induced phosphorylation of the C-terminal part of FOXO proteins

A variety of stress stimuli including oxidative stress, UV radiation and heat shock induce phosphorylation of FOXO proteins at eight different sites within the C-terminal half of FOXO molecule (labeled by symbol '#' in Figure 2) (Brunet *et al*., 2004). Another kinase that phosphorylates sites at the C terminus is the c-JUN N-terminal kinase (JNK), a MAPK family member activated by stress stimuli (Essers *et al*., 2004; Oh *et al*., 2005; Wang *et al*., 2005). JNK targets residues located in the transactivation domain (Thr447 and Thr451), and their modification seems to increase transcriptional activity, rather than relieve the inhibition by AKT/PKB imposed via 14-3-3 protein binding (Essers *et al*., 2004). In addition, JNK can regulate FOXO function through phosphorylation and consequent inhibition of 14-3-3 proteins (Tsuruta *et al*., 2004; Sunayama *et al*., 2005). Since the activity of JNK is controlled by MST1 kinase, the MST1-induced disruption of FOXO interaction with 14-3-3 proteins (see above) can be mediated by JNK.

The 14-3-3 protein isoforms can be phosphorylated by a number of different kinases including SDK1, AKT/PKB, JNK, CK1α, Bcr and certain PKC isoforms (Aitken, 2006). JNK phosphorylates serine residue located in the loop between α-helices 7 and 8 (Ser184 in human 14-3-3ζ) (Tsuruta *et al*., 2004). Phosphorylation of this site has been shown to release the proapoptotic protein Bad and FOXO3 from the complex with 14-3-3 proteins (Sunayama *et al*., 2005). Mammalian brain tissue contains high levels of 14-3-3β and 14-3-3ζ isoforms phosphorylated at Ser184 and these phospho-forms were initially described as α and δ isoforms (Aitken *et al*., 1995). The mechanism by which the phosphorylation of Ser184 inhibits binding properties of 14-3-3 proteins is unknown. One possibility is the structural change of 14-3-3 ligand-binding groove, because Ser184 is located between α-helices 7 and 8 and helix 7 forms a part of the ligand-binding groove (Yaffe *et al*., 1997; Obsil *et al*., 2001).

Conclusions

The FOXO subgroup of forkhead transcription factors plays a central role in cell-cycle control, differentiation, metabolism control, stress response and apoptosis. Therefore, the function of these important molecules is tightly controlled by a wide range of protein–protein interactions and posttranslational modifications including phosphorylation, acetylation and ubiquitination. The exact mechanisms by which these factors regulate FOXO activity are often elusive. One of the reasons of this situation is that the only structural information available to date is for isolated forkhead domains and their DNA complexes. Most of the FOXO molecule, with the exception of the forkhead domain, is probably disordered and thus difficult to investigate by methods of structural biology. However, structural studies of multiprotein complexes involving FOXO proteins might provide missing clues on the mechanism of regulation in this important group of transcription factors. Thus, in the future, we can hopefully expect the better understanding of the structural bases of FOXO regulation by protein–protein interactions and posttranslational modifications.

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