Biochimica et Biophysica Acta 1813 (2011) 1946–1953

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

Structural basis for DNA recognition by FOXO proteins ☆

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ARTICLE INFO

Article history:
Received 1 November 2010
Accepted 30 November 2010
Available online 10 December 2011

Keywords:
Forkhead
FOXO
DNA
Structure
DNA recognition

ABSTRACT

The FOXO forkhead transcription factors are involved in metabolism control, cell survival, cellular proliferation, DNA damage repair response, and stress resistance. Their transcriptional activity is regulated through a number of posttranslational modifications, including phosphorylation, acetylation and ubiquitination. The recently determined three-dimensional structures of FOXO forkhead domains bound to DNA enable to explain the structural basis for DNA recognition by FOXO proteins and its regulation. The aim of this review is to summarize the recent structural characterization of FOXO proteins, the mechanisms of DNA recognition and the role of posttranslational modifications in the regulation of FOXO DNA-binding properties. This article is part of a Special Issue entitled: PI3K-AKT-FOXO axis in cancer and aging.

1. Introduction

Forkhead transcription factors (FOX) share a highly conserved 110-amino-acid DNA-binding domain, also known as forkhead box or winged–helix domain [1]. The members of the forkhead protein family, found in species ranging from yeast to human, play a central role in cellular proliferation, differentiation, tumorigenesis and longevity (reviewed in Refs. [2,3]). The FOX family comprises more than 100 proteins that fall into 17 subclasses designated A–Q. The "O" subclass consists of four members (FOXO1, FOXO3, FOXO4 and FOXO6) involved in metabolism control, cell survival, cellular proliferation, DNA damage repair response, and stress resistance (reviewed in Ref. [4]). The transcriptional activity of FOXO proteins is regulated through a number of posttranslational modifications, including phosphorylation, acetylation and ubiquitination (reviewed in Refs. [5,6]). The insulin/IGF-1 signaling pathway regulates FOXO proteins through the phosphorylation of three conserved Ser/Thr residues by protein kinase B (PKB or Akt). This phosphorylation causes FOXO binding to the 14-3-3 protein that both inhibits FOXO binding to the target DNA and induces rapid nuclear exit of FOXO proteins [7–9]. Besides this mode of regulation, stress signaling pathways also regulate FOXO activity through various proteins including c-Jun N-terminal kinase, acetyl transferases CBP and p300, deacetylase SIRT1, or enzymes involved in ubiquitination Skp2, MDM2, and USP7 (reviewed in Refs. [5,6,10,11]). Several sites for posttranslational modifications are located within or near the FOXO DNA-binding domain, thus enabling the regulation of FOXO interaction with the DNA either directly or through protein–protein interactions.

To date, high-resolution structures of a number of forkhead domains bound to DNA, including three FOXO proteins, have been determined [12–14]. This enabled the understanding of the structural basis for DNA recognition by FOXO proteins and its regulation. The aim of this review is to summarize recent structural characterization of FOXO proteins, the mechanisms of DNA recognition and the role of posttranslational modifications in the regulation of FOXO DNA-binding properties.

2. Overall structure of the forkhead domain

The crystal structure of the FoxA3/HNF-3γ forkhead domain bound to its DNA consensus sequence, the first reported structure of forkhead (winged–helix) domain, revealed a compact α/β fold consisting of three α helices (H1–H3), three β strands (S1–S3) and two wings (W1 and W2) [1] (Fig. 1). FoxA3/HNF-3γ interacts with the DNA as a monomer through a combination of direct and water-mediated side chain–base contacts. The helix H3 is positioned into the major groove roughly perpendicular to the DNA axis and provides the principal contact surface with the sequence 5′-TAAGTCA-3′ (strong transthyretin promoter binding site). Residues involved in these interactions are conserved among all forkhead proteins and make a combination of direct and water-mediated side chain–base contacts. Additional contacts with the DNA are made by both wings W1 and W2 and several residues from α-helices H1 and H3 and β-strand S3. The binding of FoxA3/HNF-3γ also narrows the major groove in which the helix H3 is located and induces a 13° bend of the DNA.

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

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doi:10.1016/j.bbamcr.2010.11.025

During the last decade, numerous X-ray and solution NMR structures of forkhead proteins have been reported. These structures revealed a similar mode of DNA recognition, as has been observed for the FoxA3–DNA complex [1]. The highly conserved residues of helix H3 (motif N–X–X–R–H–X–X–S/T), which is the main DNA recognition element that binds into the major groove, make majority of the direct base-specific contacts. On the other hand, the patterns of interactions between the wing regions (W1 and W2) and the DNA show higher variability. In FoxA3–DNA and FOXK1a–DNA structure residues from wing regions make additional direct and water-mediated side chain–base contacts [1,15], while in the other complexes (FOXP2–DNA, FOXO3–DNA, FOXO1–DNA, FOXO4–DNA) they are only involved in unspecific interactions with the DNA backbone [12–14,16].

The comparison of available forkhead protein structures also reveals significant variations in the secondary structure content and topological arrangement of the forkhead domain. For example, several forkhead proteins (FOXO4, FOXO1, FoxD3, FOXC2 and FoxQ1) contain an additional short 3_{10}-type helix between α-helices H2 and H3 [12,17–20]. Another structural difference, an additional α-helix at the C-terminus of forkhead domain, was observed in the structures of the FoxD3–DNA, FOXK1a–DNA and FOXP2–DNA complexes [15,16,21]. The formation of this C-terminal α-helix can be induced by the interaction with the DNA, as has been shown in the case of FoxD3 [18,21]. In addition, Forkhead domains of all FOXO proteins differ in the length of the loop between helices H2 and H3 as they contain a five amino acid insertion in this region.

3. DNA recognition by FOXO DNA-binding domain

3.1. Major groove recognition by helix H3

All FOXO proteins recognize two consensus sequences: 5′-GTAAA (T/C)AA-3′, known as the Daf-16 family member-binding element (DBE) [22,23]; and 5′-(C/A)(A/C)AAA(C/T)AA-3′, present in the IGFBP-1 promoter region and known as the insulin-responsive sequence (IRE) [24–27]. Although both sequences are closely related and include the core sequence 5′-(A/C)AA(C/T)A-3′, recognized by all forkhead proteins [17], FOXO proteins bind the DBE sequence with higher affinity. The crystal structure of FOXO3 bound to the DNA containing the DBE consensus sequence solved at 2.7 Å resolution provided the first structural glimpse of FOXO–DNA complex [13]. As expected, the structure revealed the recognition helix H3 docked perpendicular to the major groove making extensive contacts with the DNA (Fig. 2A). The conserved residues of helix H3 interact with the DBE consensus sequence through both direct hydrogen bonds and van der Waals contacts. Based on this structure and DNA substitution experiments, the authors suggest that van der Waals contacts between the methyl groups of thymine bases and side chains of the helix H3 are crucial for the recognition of the DBE consensus sequence by FOXO3. The structural basis for the high-affinity binding of DBE sequence were provided by Brent et al. [12], who solved the crystal structures of FOXO1 bound to both the DBE and IRE consensus sequences. These structures revealed different networks of hydrogen-

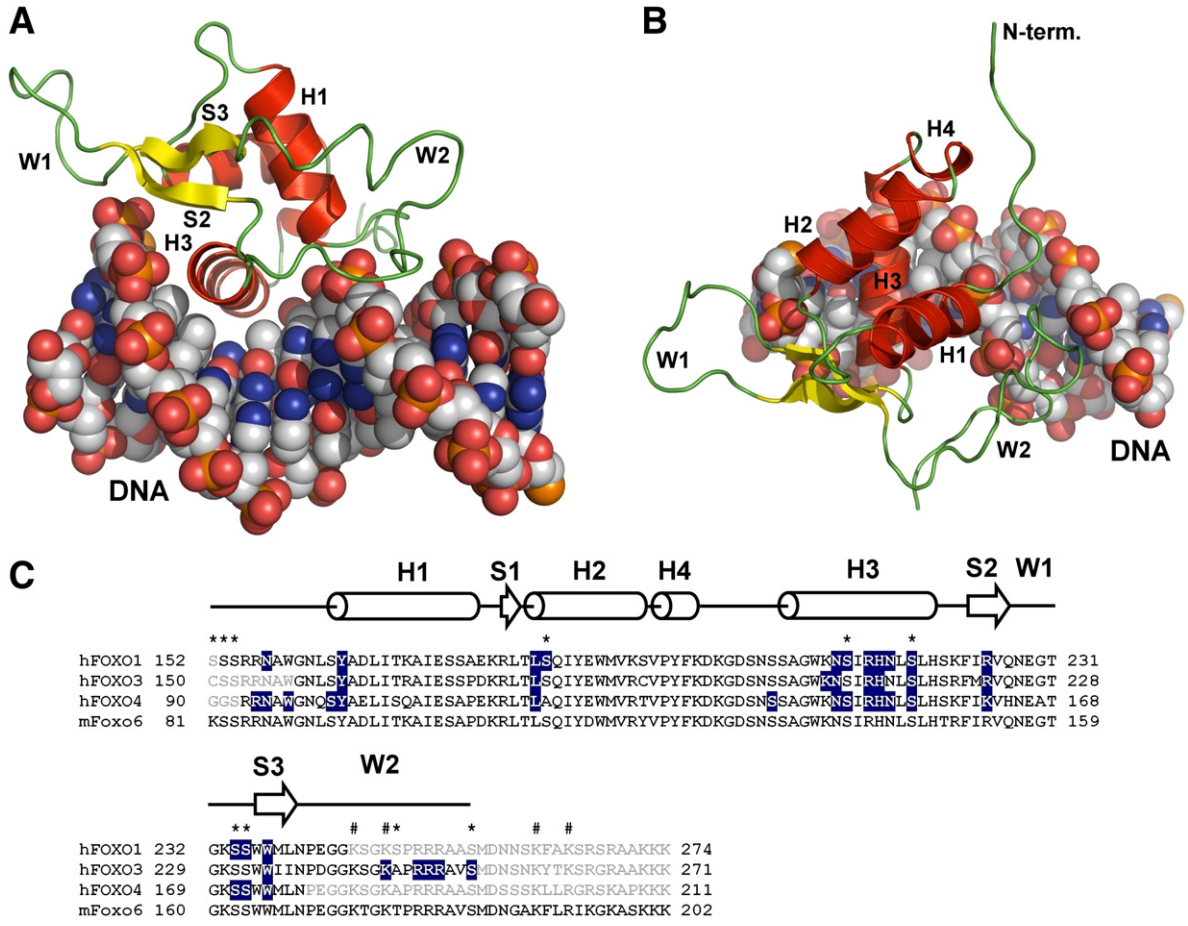
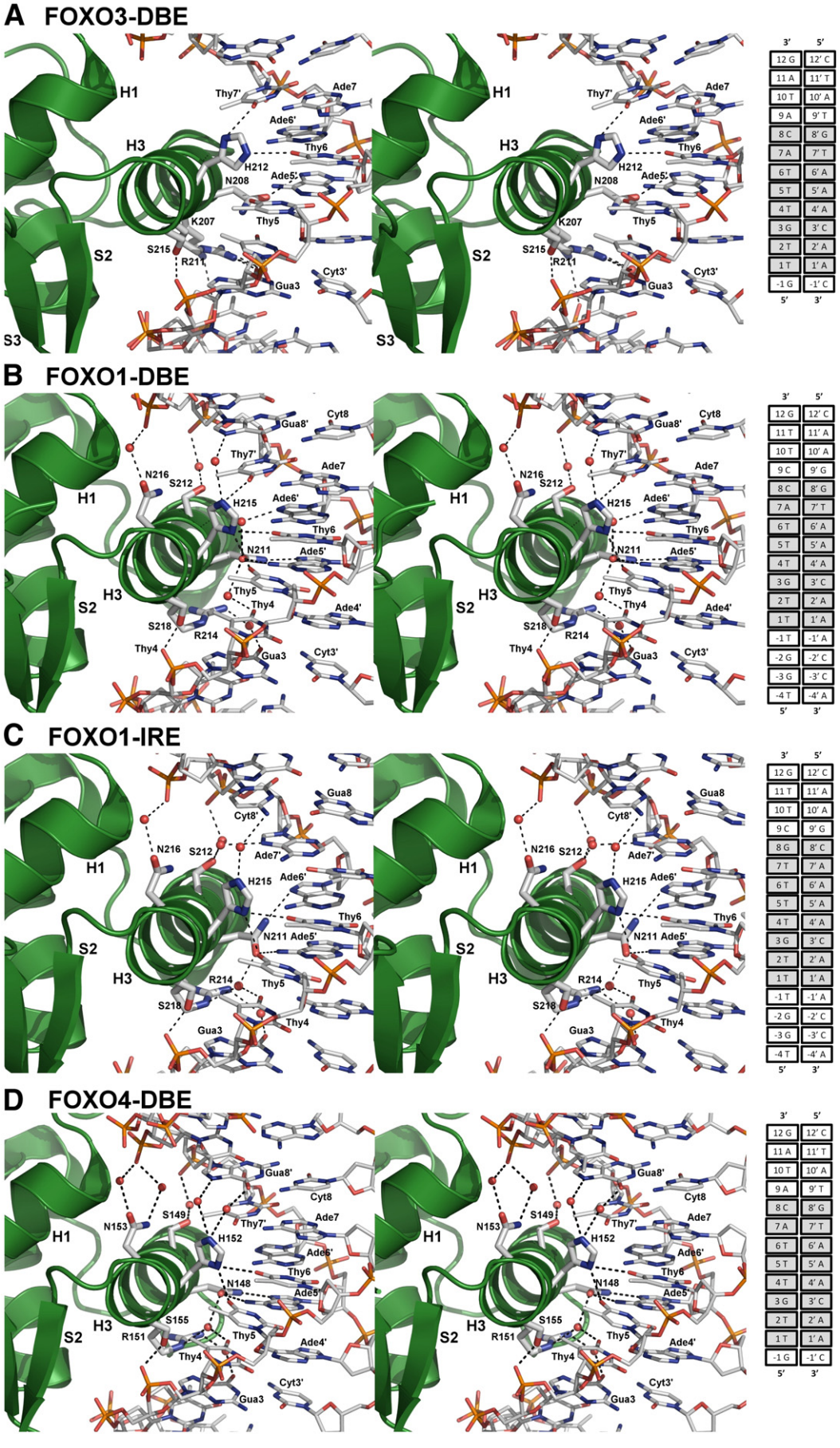


Fig. 1. The crystal structure of the FoxA3/HNF-3γ–DNA complex [1]. (A) The forkhead domain of FoxA3 is shown in ribbon representation and the DNA is shown as spheres. Secondary-structure elements are labeled according to the nomenclature typical for the winged–helix motif. (B) The complex rotated 90° towards the viewer around the horizontal axis relative to A. (C) Sequence alignment of FOXO forkhead domains. Secondary-structure elements are indicated at the top. Residues involved in protein–DNA contacts are labeled in blue. Residues shown in grey are disordered or missing in the corresponding FOXO–DNA structures [12–14]. Symbol (*) denotes phosphorylation sites. Symbol (#) denotes acetylation and/or monoubiquitination sites.


A FOXO3-DBE
H1
H3
S2
S3
Thy7'
Ade7
Ade6'
Thy6
H212
N208
Ade5'
K207
Thy5
S215
R211
Gua3
Cyt3'
B FOXO1-DBE
Gua8'
Cyt8
N216
S212
H215
N211
Thy4
S218
R214
Thy4
C FOXO1-IRE
Cyt8'
D FOXO4-DBE
N153
S149
H152
N148
S155
R151
3' 5'
12 G 12' C
11 A 11' T
10 T 10' A
9 A 9' T
8 C 8' G
7 A 7' T
6 T 6' A
5 T 5' A
4 T 4' A
3 G 3' C
2 T 2' A
1 T 1' A
-1 G -1' C
5' 3'
11 T 11' A
9 C 9' G
-1 T -1' A
-2 G -2' C
-3 G -3' C
-4 T -4' A

bonds and water-mediated interactions in the major groove of the DNA around bases 7′ and 8′ (Figs. 2B and C). In both structures the side chains of N211 and H215 make all of the direct base-specific contacts. In the case of FOXO1–DBE structure (Fig. 2B), the imidazole ring of H215 mediates direct interactions with Thy7′, Thy5, Thy6, and forms a water-mediated hydrogen bond to Gua8′. The side chain of N211 makes bidentate hydrogen bonds with Ade5′ and water-mediated hydrogen bonds with Ade6′ and Thy4. In the FOXO1–IRE structure, the side chain of N211 is hydrogen bonded with Ade5′ and Ade6′. Residue H215 still interacts with Thy5, Thy6, and Cyt8′ (in place of Gua8′ in the DBE sequence) but has no interaction with Ade7′, which replaces Thy7′ of the DBE sequence. These differences are likely the reason of decreased binding affinity of FOXO1 for the IRE sequence compared to the DBE sequence. The recently reported crystal structure of FOXO4 bound to the DNA containing the DBE consensus sequence revealed a very similar network of interactions at the helix H3/DNA interface as has been observed in the FOXO1–DBE structure (Fig. 2D) [14].

The forkhead domain of FOXO1 shows a 5-fold increase in the binding affinity for the DBE sequence with thymine rich 3′ flanking bases [12]. The DNA in the corresponding crystal structure exhibits bent and distortion in the 3′ flanking region, where the interactions with the wing W2 should take place. It is well known that the DNA sequences containing stretches of A·T base pairs including ApA (TpT) and ApT steps frequently exhibit significant bending [28,29]. This suggests that the 3′ flanking sequence and its physical properties (e.g. flexibility) may be an additional factor modulating the FOXO binding to the target DNA.

3.2. Role of loop regions in DNA binding and recognition

The crystal structure of FoxA3 bound to DNA revealed that both loops (wings) W1 and W2 participate in DNA binding [1]. The loop W1 connects strands S2 and S3 (Fig. 1) and in all FOXO–DNA structures interacts only with the phosphate backbone of the DNA suggesting that this region helps to stabilize the protein–DNA complex but without significant contribution to the DNA recognition (Fig. 3A) [12–14]. The wing W2, located at the C-terminus of the forkhead domain, is also important for the stability of the FOXO–DNA complexes. The FOXO3–DNA structure showed it adopts a coiled structure and its basic residues (R248, R249, and R250) make ionic contacts with the phosphate groups in the major groove without any base-specific contacts (Fig. 3B) [13]. Both the truncation of the C-terminal wing W2 and the substitution of these three basic residues with alanine result in the significant reduction of FOXO DNA-binding affinity [12,13,30]. The solution structure of apo-FOXO4 [17] and the molecular dynamics simulation of modeled FOXO4–DNA complex [30] suggested the high flexibility of wing W2. Structural studies confirmed this, as the wing W2 is completely disordered in all three reported FOXO1–DNA structures [12]. In the FOXO3–DNA structure the majority of wing W2 residues show high B-factors, also indicating its high flexibility. The construct used to crystallize the FOXO4–DNA complex was, in order to get well diffracting crystals, truncated at the C-terminus and contained only a part of the wing W2. Taken together, the wing W2, although being very flexible, is important for the FOXO binding to the target DNA.

All FOXO proteins contain a five-amino-acid insertion (sequence KGDSN) between helices H2 and H3 (Fig. 1C). The function of inserted residues is unclear as they do not contact DNA in FOXO1–DNA and FOXO3–DNA structures [12,13]. On the other hand, in the FOXO4–DNA structure this region adopts a different conformation within the major groove and the amide nitrogen and the side-chain of Ser142, one of the inserted residues, interact with the phosphate groups of DNA backbone (Fig. 3C) [14]. Thus, it seems this region could also help to stabilize the FOXO–DNA complex. It has been shown that the residues adjacent to the N-terminus of the recognition helix H3 affect the DNA-binding specificity of the FOX proteins, probably through the repositioning of the helix H3 [31,32]. In FOXO proteins, the turn between helices H2 and H3 interacts with the N-terminus of the forkhead domain and the N-terminal part of the helix H3 through the cluster of hydrophobic residues (Fig. 4A). These interactions might fine-tune the FOXO DNA-binding affinity.

Another region involved in the stabilization of the FOXO–DNA complex is the N-terminal segment. Deletion analysis and molecular modeling suggested that the N-terminal region of FOXO4 forkhead domain is an important part of the FOXO DNA-binding interface [30]. This was confirmed by the crystal structures of FOXO1–DNA and FOXO4–DNA complexes [12,14]. These structures showed that several residues from the N-terminus of the forkhead domain participate in DNA binding. For example, the side-chains of FOXO4 residues Arg94, Asn95 and Ser101 form direct and water-mediated hydrogen bonds with the phosphate groups of DNA (Fig. 3D) [14]. In addition, these contacts are further supported by the hydrogen bonds between the hydroxyl group of Tyr102 from the helix H1 and the phosphate group of Thy7′. The N-terminal loop of the forkhead domain is therefore an integral part of the DNA-binding interface and should be present when the isolated forkhead domain is used in experiments addressing the effects of various factors on DNA-binding affinity.

3.3. Water molecules at FOXO/DNA interface

Data obtained from the resolution of FOXO1 and FOXO4 structures suggest that in the case of the FOXO proteins ordered water molecules are an integral part of the sequence-specific DNA contacts [12,14]. For example, thirty well-ordered water molecules are located at the FOXO4–DNA interface at a distance of less than 3 Å from the atoms of both the protein and the DNA. Four of them are involved in base-specific interactions, nine water molecules participate in the phosphate interactions (Figs. 2D and 4B). The rest of the water molecules contact either polar amino-acid or polar DNA atoms and often stabilize nearby waters at the interface between the FOXO and the DNA. The presence of such an extensive network of the ordered water molecules at the binding interface suggests that hydration enthalpy might play a role in the FOXO–DNA complex stabilization. The highly ordered water molecules could also help to mediate the specific readout of bases by the FOXO proteins, as has been observed, for example, in the trp repressor–operator complex [33], the restriction enzyme BamH1–DNA complex [34] or the RXR-retinoid acid receptor–DNA complex [35].

3.4. Comparison of available FOXO–DBD structures

Two solution NMR structures of apo-FOXO4 and apo-FOXO3 forkhead domains have been reported [17,36]. The comparison of DNA-bound and apo- structures shows that they differ mainly in regions involved in DNA binding: the loop between helices H2 and H3, the N-terminal segment and both wings W1 and W2 (Fig. 5A). In both NMR solution structures the loop between helices H2 and H3 adopts the helical structure while in the FOXO–DNA complexes is mostly

Fig. 2. Stereoview of the interactions between the recognition helix H3 and the DNA containing consensus sequences. (A) Interactions between the helix H3 of FOXO3 and the 5′-GTAAACA-3′ core sequence of the DBE motif [13]. (B) Interactions between the helix H3 of FOXO1 and the 5′-GTAAACA-3′ core sequence of the DBE motif [12]. (C) Interactions between the helix H3 of FOXO1 and the 5′-CAAAACA-3′ core sequence of the IRE motif [12]. (D) Interactions between the helix H3 of FOXO4 and the 5′-GTAAACA-3′ core sequence of the DBE motif [14]. The sequence of the DNA used for co-crystallization is shown on the right. Water molecules are represented as red spheres. Polar contacts important for the recognition and the FOXO–DNA complex stability are represented by dashed black lines.

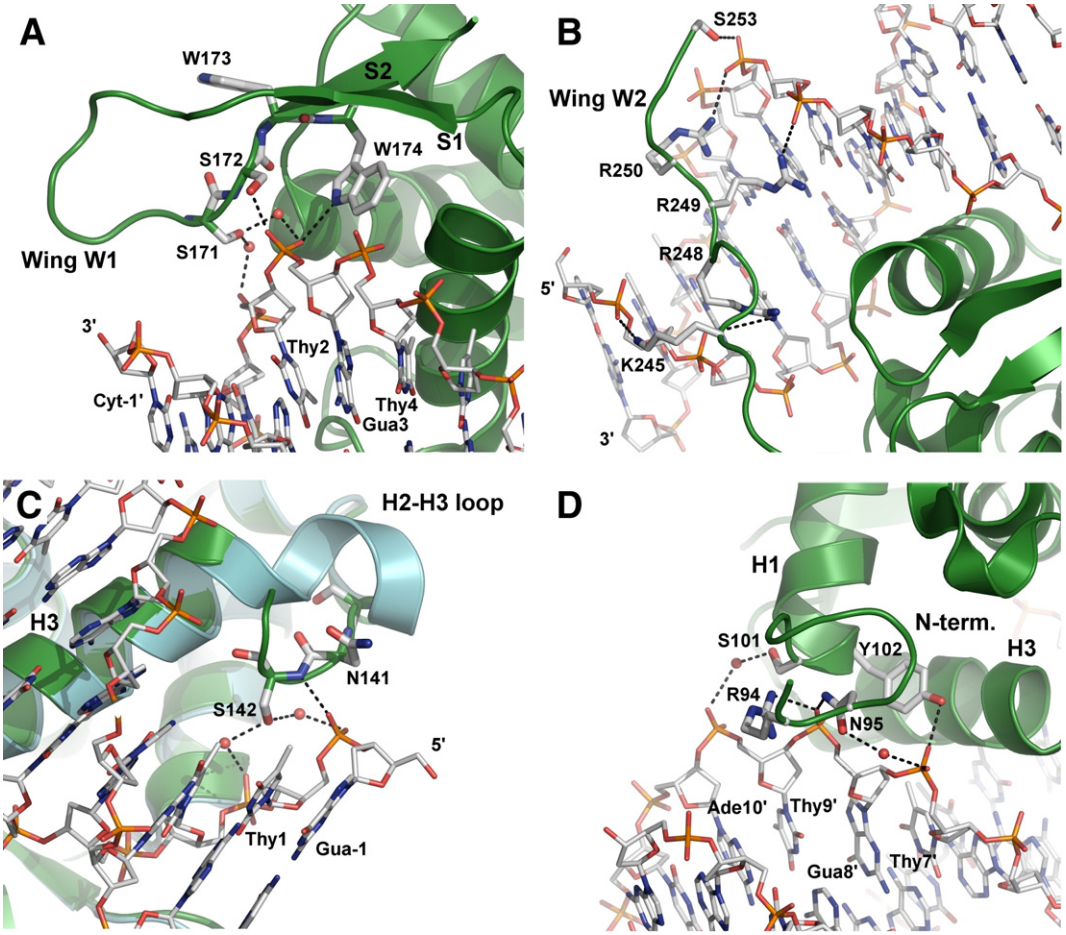


Fig. 3. The role of loop regions in DNA binding. (A) Interactions between the wing W1of FOXO4 and the DNA [14]. (B) Interactions between the wing W2 of FOXO3 and the DNA [13]. (C) Superimposition of forkhead domains of FOXO1 (shown in blue; [12]) and FOXO4 (shown in green; [14]). Residues from the H2–H3 loop of FOXO4 that participates in DNA binding are shown as sticks. For clarity, only the DNA in the FOXO4–DNA complex is shown. (D) Interactions between the N-terminal segment of FOXO4 and the DNA [14]. Water molecules are represented as red spheres. Polar contacts important for the FOXO–DNA complex stability are represented by dashed black lines.

unstructured. Differences in H2–H3 loop conformation are likely the result of not only the interaction with the DNA but also the crystal-packing interactions in the FOXO–DNA crystals [13,14].

The FOXO–DNA structures are very similar and differ mainly in the conformation of the H2–H3 loop and the wing W1 (Fig. 5B). Residues V194–K198 (in FOXO1 numbering) from the H2–H3 loop of FOXO1 and FOXO4 forkhead domains form a short 3_{10}-helix (H4) which is absent from the FOXO3–DNA complex. Since the sequence of the H2–H3 loop is conserved among the FOXO proteins, the observed differences are likely the result of both the crystal-packing differences and, possibly, the interaction with the N-terminal segment, which is shorter in the FOXO3–DNA structure. Similarly, variations in the wing W1 conformation likely reflect the crystal-packing differences and have no biological relevance.

4. Modulation of FOXO–DNA interactions through posttranslational modifications

The activity of FOXO proteins is regulated through posttranslational modifications including phosphorylation, acetylation and ubiquitination (reviewed in [5,6,10,11]). Several sites of these modifications are located within the forkhead domain and map to regions directly involved in the DNA binding (Fig. 1C).

Protein kinase B (PKB, also known as Akt) phosphorylates FOXO proteins at three sites [7,8,24,27,37,38]. One of them (S256 in FOXO1 numbering) is located in the wing W2 of the forkhead domain close to the cluster of the basic residues that are involved in DNA binding. The PKB/Akt-induced phosphorylation itself has a small effect on the FOXO DNA-binding affinity [12,30]. However, two of the three sites phosphorylated by PKB/Akt kinase are the 14-3-3 protein binding motifs [7,39]. The first one is located at the N-terminus of the FOXO molecule and the second one is in the wing W2. The 14-3-3 proteins are a family of the acidic and highly conserved molecules that bind to other proteins, mostly in a phosphorylation-dependent manner, and regulate their functions [40–42]. Both 14-3-3-binding motifs that border the forkhead domain are necessary for optimal FOXO binding to the 14-3-3 protein [7,39,43,44]. The 14-3-3 protein binding completely inhibits the DNA-binding affinity of the phosphorylated FOXO proteins and causes the cytoplasmic sequestration of the resulting complex, probably by interfering with the function of FOXO nuclear localization signal (NLS) [7,24,26,38,39,45]. Our group has recently showed that the 14-3-3 protein physically interacts with the DNA-binding interface of the FOXO4 forkhead domain [46]. Such interactions probably mask the DNA binding interface, thus blocking the FOXO binding to the target DNA.

The cyclic GMP-dependent kinase-1 (cGK1) phosphorylates FOXO1 and abolishes its DNA binding activity during the muscle cell fusion [47]. The cGK1 kinase phosphorylates cluster of serine residues upstream of the first helix H1 (S152–S155) and S184 at the N-terminus of helix H2 (Fig. 1C). This modification seems to be specific for FOXO1 as the cluster of the serine residues preceding the helix H1 is unique to FOXO1 and S184 is absent from the FOXO4 sequence. The cluster of the serine residues is located close to the DNA backbone

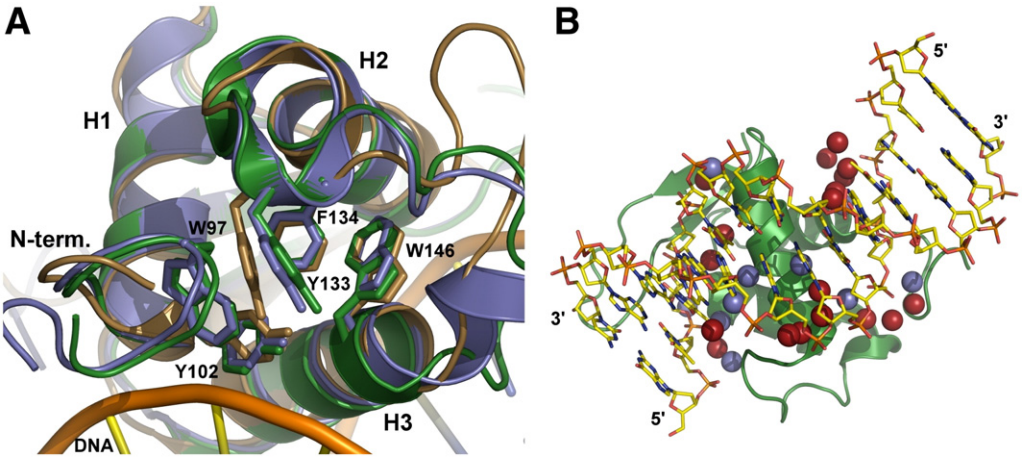


Fig. 4. Details of the interactions between the FOXO forkhead domain and the DNA. (A) Cluster of hydrophobic residues on the interface between the N-terminal segment, the H2–H3 loop and the helix H3. FOXO1 is shown in blue, FOXO3 in brown and FOXO4 in green. Residues are labeled according to the FOXO4 sequence. (B) Water molecules at the FOXO4–DNA interface [14]. Spheres represent water molecules located at a distance of less than 3.0 Å from atoms of both FOXO4 and the DNA. Blue spheres represent water molecules that are engaged in FOXO–water–DNA interactions. Red spheres represent water molecules that contact either polar amino acids or polar DNA atoms.

just upstream of the residues involved in DNA binding (N158 and Y165) [12]. Residue S184 at the N-terminus of helix H2 is also located close to the DNA backbone. Therefore, the phosphorylation of these residues can easily interfere with the FOXO1 binding to the DNA.

Another kinase known to phosphorylate the forkhead domain of FOXO proteins is the oxidative stress-regulated mammalian Ste-20 like kinase-1 (MST1) [48]. MST1-induced phosphorylation causes the disruption of the FOXO–14-3-3 complex and promotes the FOXO nuclear translocation. MST1 phosphorylates four highly conserved serine residues (S209, S215, S231, and S232 in FOXO3 numbering, Fig. 1C). Residues S209 and S215 are located within the helix H3 while S231 and S232 lie at the C-terminus of wing W1 [13]. In all available structures of the FOXO–DNA complexes these residues make either direct or water-mediated contacts with the phosphates of the DNA backbone suggesting that their phosphorylation should efficiently inhibit the FOXO DNA-binding affinity. Indeed, this was confirmed by Brent et al. [12] who showed that MST1 phosphorylation completely inhibits the DNA-binding affinity of the FOXO1 forkhead domain. Therefore, it is likely that an additional process, e.g. the dephosphorylation, takes place during the MST1-related activation of the FOXO proteins.

The forkhead domain of the FOXO1 protein is also phosphorylated by cyclin-dependent kinase-2 (CDK2) in the wing W2 at S249 [49]. CDK2-induced phosphorylation results in cytoplasmic sequestration and inhibition of FOXO1 function. However, the FOXO1 DNA-binding affinity was found to be unchanged for the S249E mutant mimicking the phosphorylation at this site [12]. Therefore, different mechanisms, e.g. the interference with the function of the adjacent nuclear localization sequence or the disruption of the FOXO1–14-3-3 complex, is likely responsible for the CDK2-mediated inhibition of the FOXO1 function [49,50].

The C-terminus of the FOXO forkhead domain (wing W2) contains four sites (K245, K248, K262, K265 in FOXO1 numbering) that are acetylated by histone acetyltransferases such as p300 and cAMP-response element-binding protein-binding protein (CBP) [51–54]. Several studies have shown that the acetylation of these sites or the removal of their positive charge by mutagenesis causes a moderate reduction in the FOXO DNA-binding affinity [12,13,55].

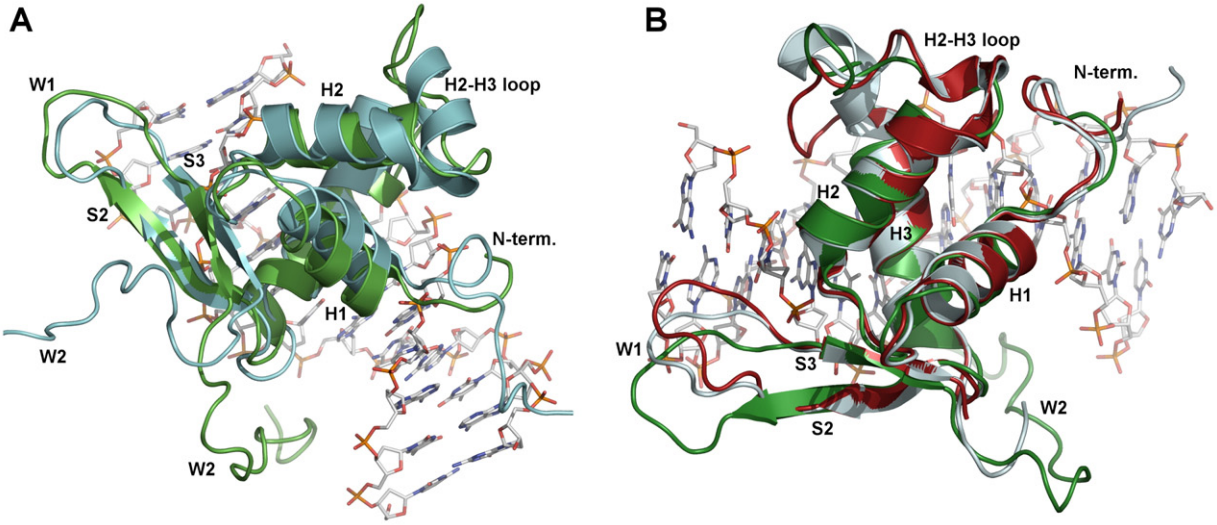


Fig. 5. Comparison of FOXO forkhead domains. (A) Superimposition of the FOXO3–DNA structure (shown in green) with the solution structure of apo FOXO3 (shown in blue; [36]). (B) The superimposition of FOXO1–DNA (blue; [12]), FOXO3–DNA (green; [13]) and FOXO4–DNA complexes (dark red; [14]). For clarity, only the DNA in the FOXO3–DNA complex is shown.

5. Conclusions

The three-dimensional structures determined for members of the FOXO subclass of forkhead transcription factors show very similar mode of DNA binding. The main DNA-recognition element of the FOXO forkhead domain is the helix H3, while the N-terminal segment, the loop between helices H2 and H3 and both flexible wings W1 and W2 provide additional stabilizing unspecific contacts. It appears that van der Waals contacts between the methyl groups of thymine bases and side chains of the helix H3 are essential for the recognition of the core sequence 5′-(A/C)AA(C/T)A-3′. The presence of well-ordered water molecules at the FOXO–DNA interface suggests that unique water-mediated interactions are important for DNA recognition by FOXO proteins. Differences in the network of the hydrogen-bonds and water-mediated interactions in the major groove of the DNA explain the higher binding affinity for the DBE consensus sequence over the IRE sequence. The additional factor that likely plays an important role in the FOXO binding to the DNA is the 3′ flanking sequence and its physical properties (e.g. flexibility). Several post-translational modifications that target sites at the DNA-binding interface significantly affect the FOXO DNA-binding affinity, either directly or indirectly through protein–protein interactions, suggesting that its regulation is an essential part of the FOXO function regulation.

Acknowledgments

The authors are supported by the Grant Agency of the Academy of Sciences of the Czech Republic (Grant IAA501110801); Czech Science Foundation (Projects P207/11/0455 and P305/11/0708); Ministry of Education, Youth, and Sports of the Czech Republic Research (Project MSM0021620857 and Center of Neurosciences LC554); and Academy of Sciences of the Czech Republic (Research Project AV0Z50110509).

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