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Structure of the human FOXO4-DBD–DNA complex at 1.9 Å resolution reveals new details of FOXO binding to the DNA

Evzen Boura,[a] Lenka Rezabkova,[a] Jiri Brynda,[b] Veronika Obsilova[c] and Tomas Obsil[a,c]*

[a]Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, 128 43 Prague, Czech Republic, [b]Department of Recombinant Expression and Structural Biology, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, 166 37 Prague, Czech Republic, and [c]Department of Protein Structure, Institute of Physiology, Academy of Sciences of the Czech Republic, 142 20 Prague, Czech Republic

Correspondence e-mail: obsil@natur.cuni.cz

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FOXO4 is a member of the FOXO subgroup of forkhead transcription factors that constitute key components of a conserved signalling pathway that connects growth and stress signals to transcriptional control. Here, the 1.9 Å resolution crystal structure of the DNA-binding domain of human FOXO4 (FOXO4-DBD) bound to a 13 bp DNA duplex containing a FOXO consensus binding sequence is reported. The structure shows a similar recognition of the core sequence as has been shown for two other FOXO proteins. Helix H3 is docked into the major groove and provides all of the base-specific contacts, while the N-terminus and wing W1 make additional contacts with the phosphate groups of DNA. In contrast to other FOXO-DBD–DNA structures, the loop between helices H2 and H3 has a different conformation and participates in DNA binding. In addition, the structure of the FOXO4-DBD–DNA complex suggests that both direct water–DNA base contacts and the unique water-network interactions contribute to FOXO-DBD binding to the DNA in a sequence-specific manner.

1. Introduction

The forkhead transcription factors (FOXs) share a conserved winged-helix DNA-binding domain (DBD) known as the forkhead domain (Weigel & Jackle, 1990; Kaestner *et al.*, 2000). This domain 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) (Clark *et al.*, 1993). Topologically, the arrangement of the domain is H1-S1-H2-H3-S2-W1-S3-W2. Strand S1, which is inserted between helices H1 and H2, interacts with strands S2 and S3 to form a three-stranded antiparallel β-sheet. The main DNA-recognition element is helix H3, which binds to the major groove roughly perpendicular to the DNA axis and makes the majority of the base-specific contacts (Clark *et al.*, 1993; Gajiwala & Burley, 2000). The mechanism by which different classes of FOX proteins recognize diverse DNA sequences adjacent to the core sequence is still not fully understood (Gajiwala & Burley, 2000). It has been speculated that the less conserved regions of the forkhead domain, including the H2–H3 turn and both wings W1 and W2, provide fine-tuning of both the DNA-binding specificity and the stability of FOX–DNA complexes.

FOXO4 is a member of the 'O' subgroup of FOX factors that constitute a key component of a conserved signalling pathway that connects growth and stress signals to transcriptional control (Lin *et al.*, 1997; Ogg *et al.*, 1997). All FOXOs bind to the consensus sequence 5′-GTAAACAA-3′, known as the DAF-16 family member-binding element, which includes

the core sequence 5′-(A/C)AA(C/T)A-3′ recognized by all FOX-family members (Furuyama *et al.*, 2000; Biggs *et al.*, 2001; Overdier *et al.*, 1994; Kaufmann *et al.*, 1995; Weigelt *et al.*, 2001). FOXO proteins also recognize the insulin-responsive element present in the IGFBP-1 promoter region defined as 5′-(C/A)(A/C)AAA(C/T)AA-3′ (Biggs *et al.*, 1999; Guo *et al.*, 1999; Kops *et al.*, 1999; O'Brien & Granner, 1996). FOXO proteins can bind both sequences, but they bind to the DAF-16 family member-binding element with higher affinity (Furuyama *et al.*, 2000; Brent *et al.*, 2008). The crystal structures of FOXO3-DBD–DNA and FOXO1-DBD–DNA complexes showed the expected forkhead fold of FOXO-DBD and a similar mechanism of core-sequence recognition by helix H3 (Tsai *et al.*, 2007; Brent *et al.*, 2008). These structures also showed that wing 1, the N-terminus (FOXO1-DBD–DNA complex only) and the C-terminal wing W2 (FOXO3-DBD–DNA complex only) are involved in DNA binding. The activity of FOXOs is regulated by various post-translational modifications including phosphorylation, acetylation and ubiquitination (reviewed by van der Horst & Burgering, 2007). Many sites of post-translational modification are located within the forkhead domain, suggesting regulation through modulation of DNA-binding potential. Indeed, it has been demonstrated that modifications of these sites attenuate the DNA-binding affinity of FOXOs (Zhang *et al.*, 2002; van der Heide *et al.*, 2005; Matsuzaki *et al.*, 2005; Boura *et al.*, 2007; Tsai *et al.*, 2007; Brent *et al.*, 2008). In contrast, modification of wing W2 can also cause an opposite effect and promotes the transcriptional activity of FOXO proteins, as has been shown by Yuan *et al.* (2008).

In order to further investigate the mechanism of DNA recognition by FOXO proteins, we solved the 1.9 Å resolution crystal structure of human FOXO4-DBD bound to a 13 bp DNA duplex containing a FOXO consensus binding sequence. The structure shows a similar recognition of the core sequence as has been shown for two other FOXO proteins. Helix H3 is docked into the major groove and provides all of the base-specific contacts, while the N-terminus and wing W1 make additional contacts with the phosphate groups of DNA. In contrast to other FOXO-DBD–DNA structures, the loop between helices H2 and H3 has a different conformation and participates in DNA binding. In addition, the structure of the FOXO4-DBD–DNA complex suggests that both direct water–DNA base contacts and the unique water-network interactions contribute to FOXO-DBD binding to the DNA in a sequence-specific manner.

2. Materials and methods

2.1. Protein expression and purification

The forkhead domain of human FOXO4 (both the full-length domain consisting of residues 82–207 and a C-terminally truncated domain consisting of residues 82–183) was produced as an N-terminal 6×His-tag fusion protein using the pET-15b vector in *Escherichia coli* BL21 (DE3) cells (Boura *et al.*, 2007). Protein expression was induced by IPTG for 12 h at 303 K and the protein was purified using Chelating Sepharose Fast Flow (Amersham Pharmacia Biotech) according to a standard protocol. Proteins were then dialyzed against buffer containing 50 m*M* sodium citrate pH 6.3, 2 m*M* EDTA and 1 m*M* DTT. The affinity tag was removed by thrombin cleavage overnight at 277 K (10 U per milligram of recombinant protein). Proteins were further purified using cation-exchange (HiTrap SP column, Amersham Pharmacia Biotech) and gel-permeation chromatography (Superdex 75, Amersham Pharmacia Biotech).

2.2. Crystallization

Complementary oligonucleotides (5′-CTATGTAAACA-ATGTAAACAAC-3′ and 5′-GTTGTTTACATAG-3′) containing the FOXO consensus binding sequence 5′-GTAAACAA-3′ (Furuyama *et al.*, 2000; Biggs *et al.*, 2001;

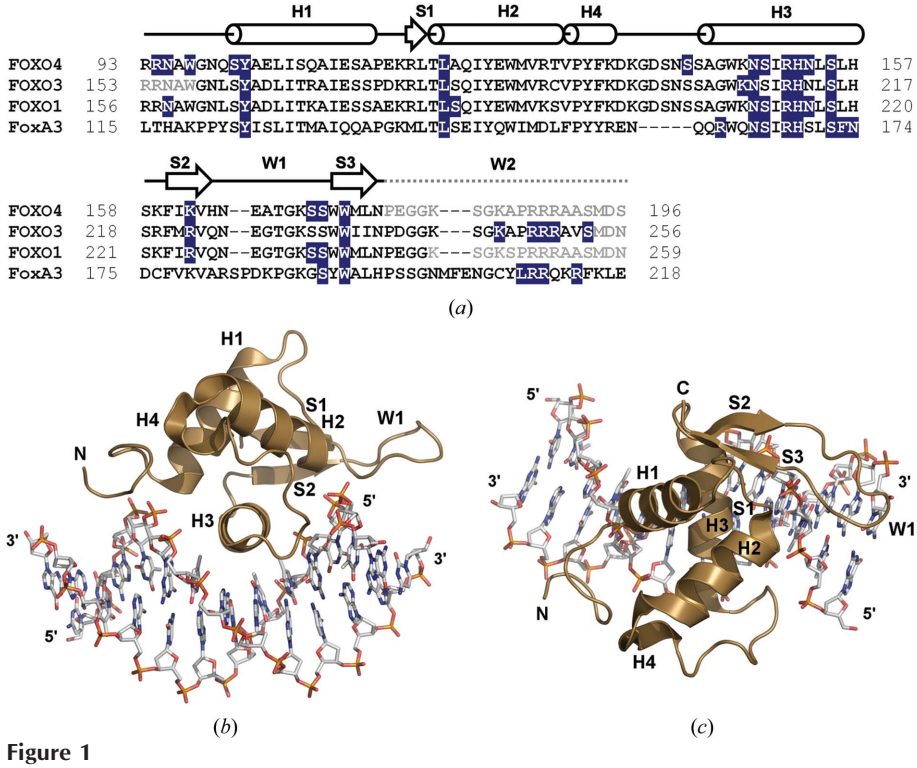


Figure 1
Overall structure of the FOXO4-DBD–DNA complex. (*a*) Sequence alignment of FOXO forkhead domains. Secondary-structure elements (based on the FOXO4-DBD–DNA complex) are indicated at the top. Residues involved in protein–DNA contacts are labelled in blue. Residues shown in grey are missing in the corresponding FOXO-DBD–DNA structures (Tsai *et al.*, 2007; Brent *et al.*, 2008). (*b*) Structure of the FOXO4-DBD–DNA complex. The FOXO-DBD is shown in ribbon representation and DNA is shown as sticks. Secondary-structure elements are labelled according to the nomenclature typical for the winged-helix motif (Clark *et al.*, 1993). (*c*) The complex rotated 90° towards the viewer around the horizontal axis relative to Fig. 1(*b*).

Table 1
Crystallographic data-set and refinement statistics.

Values in parentheses are for the highest resolution shell.

Space group	$C222_1$
Unit-cell parameters	
a (Å)	40.8
b (Å)	71.7
c (Å)	131.9
Asymmetric unit contents	FOXO4-DBD monomer bound to a 13 bp DNA duplex
Resolution range (Å)	44–1.9 (1.96–1.87)
Unique reflections	16135
Data multiplicity	7.0 (6.5)
Completeness (%)	96.6 (81.6)
$\langle I/\sigma(I)\rangle$	26.7 (7.1)
$R_{\rm merge}$†	0.044 (0.251)
$R_{\rm work}$	0.19
$R_{\rm free}$‡	0.23
No. of protein atoms	697
No. of nucleic acid atoms	527
No. of waters	163
No. of magnesium ions	2
Average *B* factors (Å^2)	
Protein	20.9
DNA	20.0
Water	31.6
R.m.s. deviations from ideal values	
Bonds (Å)	0.013
Angles (°)	1.92

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the statistically weighted average intensity of multiple observations of symmetry-related reflections. ‡ The free *R* value ($R_{\rm free}$) was calculated using 5% of the reflections, which were omitted from the refinement.

Kaufmann *et al.*, 1995; Weigelt *et al.*, 2001; Tsai *et al.*, 2007) were purchased from VBC-Biotech Services GmbH (Austria). The DNA was annealed in 25 m*M* HEPES pH 7.2 and 150 m*M* NaCl. FOXO4 (sequence 82–183) in buffer containing 20 m*M* HEPES pH 7.2, 100 m*M* NaCl, 1 m*M* EDTA was mixed with DNA in a 1:1 molar ratio. The final protein concentration was 3 mg ml^{-1}. Crystals grew in 6–7 d at 291 K in hanging drops consisting of a 1:1 mixture of the complex and a well solution consisting of 100 m*M* HEPES pH 7.3, 37–39% PEG MME 550 and 50 m*M* $MgCl_2$. Crystals were frozen by rapid immersion in liquid nitrogen.

2.3. Data collection and structure determination

A complete data set was collected at 150 K on a MAR345 image-plate detector (X-ray Research, Germany) mounted on a Nonius FR591 rotating-anode source operated at 50 kV and 80 mA (Nonius, The Netherlands). The crystal belonged to space group $C222_1$, with unit-cell parameters a = 40.81, b = 71.70, c = 131.86 Å, and had a solvent content of 55%. Data were processed using *MOSFLM* (Leslie, 2006) and were scaled using *SCALA* (Evans, 2006). Initial phases were obtained by molecular replacement using the coordinates of the FOXO3-DBD–DNA complex (Tsai *et al.*, 2007). Molecular replacement was performed with *MOLREP* (Vagin & Teplyakov, 2010) and refinement was carried out with *REFMAC* 5.2 (Murshudov *et al.*, 1997). In the current model, 94.8% of all residues are in the most favoured regions of the Ramachandran plot and none are in the disallowed region (calculated using *PROCHECK* v.3.5; Laskowski *et al.*, 1993). In the final stages of the refinement, 163 solvent molecules were included. The helical parameters of DNA were analyzed using the *CURVES* program (Lavery & Sklenar, 1988). The atomic coordinates and structure factors for the FOXO4-DBD–DNA complex have been deposited in the RCSB PDB with accession code 3l2c. All structural figures were prepared using *PyMOL* (http://www.pymol.org).

3. Results and discussion

3.1. FOXO4-DBD preparation and characterization

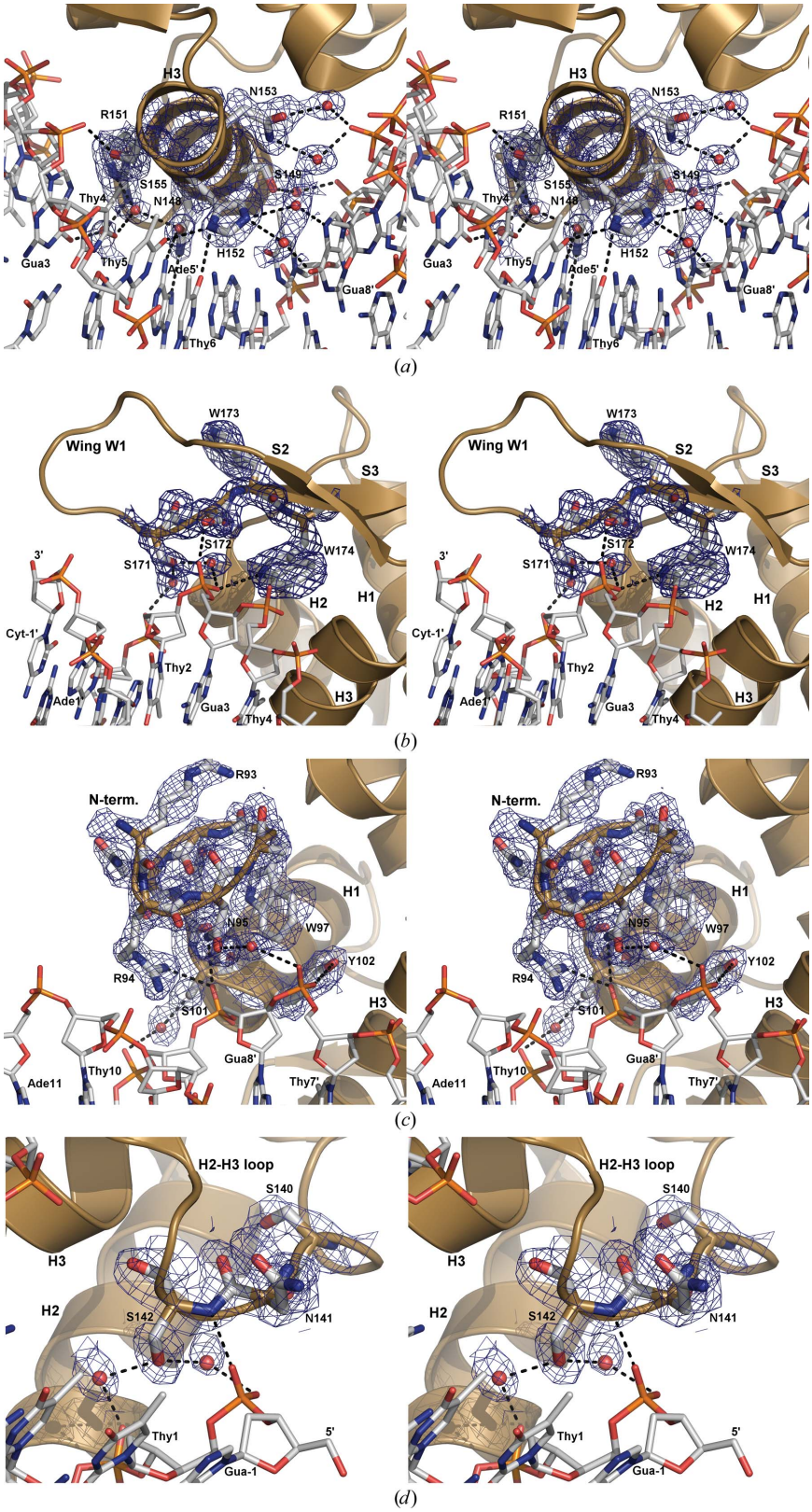
Our first attempts to crystallize the FOXO4-DBD–DNA complex were performed using the FOXO4-DBD construct consisting of residues 82–207 and containing the complete forkhead domain. However, we were unable to obtain well diffracting crystals using this construct. Our previous work suggested that the flexible C-terminal wing W2 of FOXO4-DBD, although playing an important regulatory role, is not essential for FOXO4-DBD binding to DNA (Boura *et al.*, 2007). Therefore, we decided to use C-terminally truncated FOXO4-DBD (sequence 82–183, in which part of wing W2 is missing) for crystallization trials (Fig. 1). This construct binds dsDNA containing the FOXO consensus binding sequence with a $K_{\rm d}$ of ∼360 n*M* (Boura *et al.*, 2007). The FOXO4-DBD bound to a 13 bp DNA duplex containing a FOXO consensus binding sequence (GTAAACA) crystallized in space group $C222_1$ and the structure was solved at 1.9 Å resolution by molecular replacement using the FOXO3-DBD–DNA structure (Tsai *et al.*, 2007) as a search model. The structure was refined to $R_{\rm work}$ = 19% and $R_{\rm free}$ = 23% (Table 1). The current model consists of one FOXO4-DBD–DNA complex, two Mg^{2+} ions and 163 solvent molecules.

3.2. Overall structure of the FOXO4-DBD–DNA complex

The FOXO4-DBD (sequence 82–183) adopts the expected forkhead winged-helix fold and contains three α-helices (H1–H3), a fourth short 3_{10}-helix H4 located between H2 and H3, and a short twisted three-stranded antiparallel β-sheet comprising of strands S2 and S3 and residues Leu118 and Thr119 acting as a third strand S1 (secondary-structure elements are labelled according to the nomenclature typical for the winged-helix motif; Fig. 1; Clark *et al.*, 1993). The FOXO-DBD binds to the DNA duplex in a similar manner as observed in other FOX-DBD–DNA structures, with the α-helix H3 docked into the major groove roughly perpendicular to the DNA axis (Clark *et al.*, 1993; Tsai *et al.*, 2007; Brent *et al.*, 2008; Gajiwala & Burley, 2000). Other parts of FOXO4-DBD that also interact with DNA include the flexible wing W1 located between strands S2 and S3, the turn region between helices H4 and H3 and the N-terminal loop preceding the first helix H1 (Fig. 1). All of these regions have the highest sequence variability relative to the rest of the forkhead DBD among the FOX classes and presumably play a role in the modulation of the DNA-binding specificity. The DNA forms pseudo-continuous helices through stacking interactions of symmetry-related DNA molecules. Analysis of the DNA

structure revealed small deviations from the canonical B-form DNA. The average helical twist is 32.7° and the mean rise per base pair is 3.1 Å. The DNA molecule is bent about 13° toward FOXO4-DBD, with the major groove being slightly wider in the core-sequence region. These deviations are similar to those observed in other FOX-DBD–DNA structures (Clark *et al.*, 1993; Tsai *et al.*, 2007; Brent *et al.*, 2008).

The structure of the FOXO4-DBD–DNA complex also revealed a metal-binding site at the C-terminus of helix H3. Considering the octahedral coordination (by the main-chain carbonyl O atoms of residues Leu154, Ser155, His157, Phe160 and two water molecules with bond distances ranging from 2.4 to 3.9 Å) and the presence of magnesium chloride in the crystallization solution, we assume that it contains a magnesium ion (Black *et al.*, 1994). Similar Mg^{2+}-binding sites were observed in DNA complexes of FOXO1, FoxA3, FOXP2 and FOXK1a (Clark *et al.*, 1993; Stroud *et al.*, 2006; Tsai *et al.*, 2006; Brent *et al.*, 2008). The function of this metal ion is probably the stabilization of the turn between helix H3 and strand S2 by neutralizing the helix dipole and providing a water-mediated contact to the phosphate group of the DNA backbone.



3.3. DNA recognition by FOXO4-DBD

The conserved motif of helix H3 (Asn148-*X*-*X*-Arg-His-*X*-*X*-Ser/Thr), which is present in all FOX factors, makes extensive contacts with the bases of the core sequence *via* both direct and water-mediated hydrogen bonds as well as van der Waals contacts (Figs. 2*a* and 3). The pattern of these interactions is similar to those of the FOXO3-DBD–DNA and FOXO1-DBD–DNA complexes (Brent *et al.*, 2008; Tsai *et al.*, 2007). Residues His152 and Asn148 make all of the direct base-specific contacts. The side chain of His152 forms direct hydrogen bonds to the bases of Thy5 and Thy6 (the imidazole side chain is protonated, with a delocalized positive charge between the ND1 and NE2 atoms) and water-mediated hydrogen bonds with the base of Gua8′. Residue Asn148 forms bidentate hydrogen bonds with Ade5′ and

Figure 2
Stereoview of FOXO4-DBD interactions with DNA. (*a*) Interactions between helix H3 of FOXO4-DBD and the 5′-TAAACA-3′ core sequence. (*b*) Interactions between wing W1 and DNA. (*c*) Interactions between the N-terminal segment and DNA. (*d*) Interactions between the H2–H3 loop and the DNA backbone. Water molecules are represented as red spheres. Hydrogen bonds important for recognition and complex stability are represented by dashed black lines. The electron density shown is from an OMIT map calculated using *SFCHECK* v7.03.16 (Vaguine *et al.*, 1999) and contoured at 1.0σ around selected residues and water molecules.

water-mediated hydrogen bonds with Thy4 (Figs. 2*a* and 3). In addition, Arg151 interacts with the bases of Gua3 and Thy4 through water-mediated hydrogen bonds.

Wing W1 of FOXO4 interacts with DNA in a similar manner as observed in other FOXO-DBD–DNA structures (Fig. 2*b*; Tsai *et al.*, 2007; Brent *et al.*, 2008). The lack of specific protein–base interactions suggests that this region does not play a significant role in DNA recognition, but rather stabilizes the FOXO-DBD–DNA complex.

We have previously shown that the N-terminal region of FOXO4-DBD is an important part of the FOXO DNA-binding interface and have suggested that the boundaries of FOXO-DBD should include the positively charged N-terminus when the isolated forkhead domain is used in experiments addressing the effects of various factors on DNA-binding affinity (Boura *et al.*, 2007). The FOXO4-DBD–DNA structure shows that residues from the N-terminus of DBD are involved in DNA binding (Fig. 2*c*). The side chains of Arg94, Asn95 and Ser101 form direct and water-mediated hydrogen bonds with the phosphate groups of DNA. In addition, these contacts are further supported by the hydrogen bond between the hydroxyl group of Tyr102 from helix H1 and the phosphate group of Thy7′. A similar interaction was also observed in the FOXO1-DBD–DNA complex (Brent *et al.*, 2008).

All FOXO proteins contain a five-amino-acid insertion (Lys-Gly-Asp-Ser-Asn) between helices H2 and H3 compared with other FOX proteins (Figs. 1*a* and 2*d*). The function of these residues is unknown and there are no protein–DNA contacts in the FOXO3-DBD–DNA and FOXO1-DBD–DNA structures (Tsai *et al.*, 2007; Brent *et al.*, 2008). However, the FOXO4-DBD–DNA structure suggests that this region also participates in stabilization of the complex by adopting a different conformation within the major groove of the DNA. The amide N atom and the side chain of Ser142, one of the inserted residues, interact with the phosphate groups of the DNA backbone *via* both a direct hydrogen bond and two water-mediated hydrogen bonds (Fig. 2*d*). It has been shown that residues from both the H2–H3 loop and the N-terminal border of helix H3 affect the DNA-binding specificity of FOX proteins, probably through repositioning of the recognition helix H3 (Pierrou *et al.*, 1994; Overdier *et al.*, 1994). In the case of FOXO proteins, this could be the result of contacts between the H2–H3 loop, the N-terminal part and the N-terminal border of helix H3. In the FOXO4-DBD–DNA structure these regions interact through the cluster of hydrophobic residues consisting of Trp97 and Tyr102 from the N-terminus, Tyr133 and Phe134 from the H2–H3 loop, and Trp146 from helix H3 (Fig. 4).

DNA sequences containing stretches of A·T base pairs including ApA (TpT) and ApT steps frequently exhibit significant bending (Hizver *et al.*, 2001; Nelson *et al.*, 1987). Thus, in addition to direct readout of bases (through hydrogen bonds, water-mediated contacts and hydrophobic contacts within the major groove), indirect (shape) readout could also play a role in DNA recognition by FOXO proteins (Koudelka *et al.*, 2006).

3.4. Water molecules at the protein–DNA interface

The data obtained from the resolution of our structure suggest that unique water-network interactions significantly contribute to FOXO4-DBD binding to the DNA. 30 well ordered water molecules (with an average temperature factor of 25.4 Å^2) are located at the FOXO4-DBD–DNA interface at distances of less than 3.0 Å from atoms of both the protein and the DNA (Fig. 5). Four water molecules are engaged in side

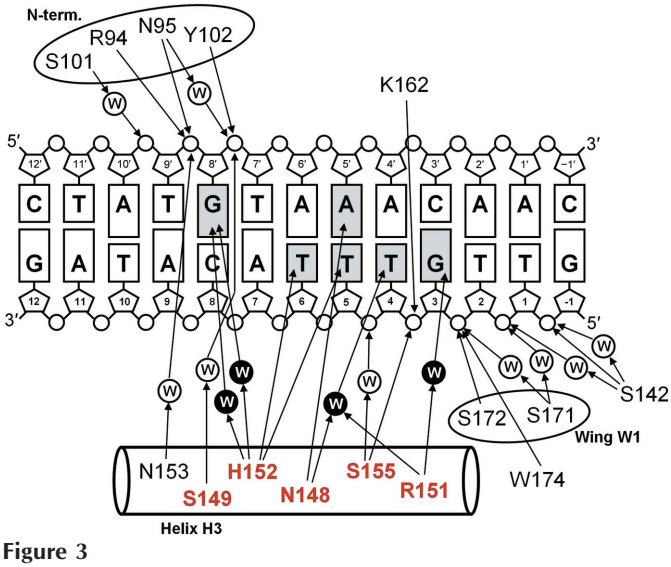


Figure 3
Schematic diagram of FOXO4-DBD–DNA contacts. The residues and bases that participate in specific polar contacts between FOXO4-DBD and DNA are shown in red and grey, respectively. Polar interactions are indicated by solid arrows. Water molecules that are involved in specific interactions between FOXO4-DBD and bases are shown as filled black circles.

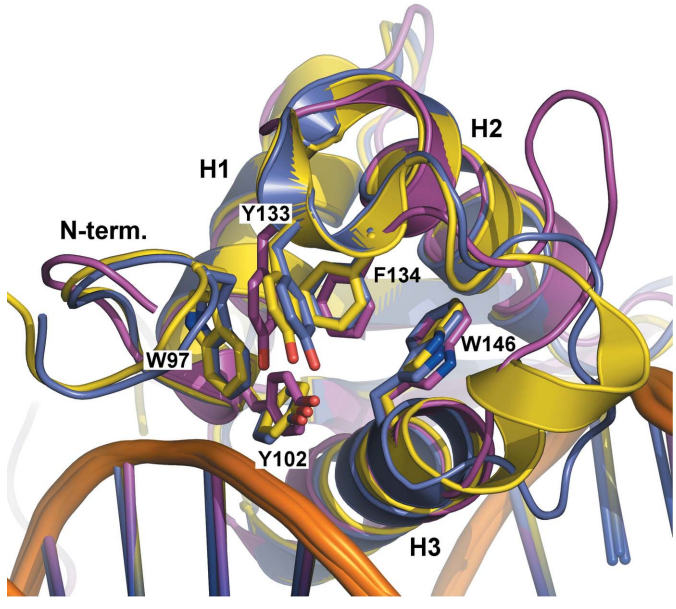


Figure 4
Cluster of hydrophobic residues on the interface between the N-terminal segment, the H2–H3 loop and helix H3. FOXO4-DBD is shown in blue, FOXO3-DBD in violet and FOXO1-DBD in yellow. Residues are labelled according to the FOXO4 sequence.

chain–water–base interactions (between the side chains of Asn148, Arg151 and His152 and the bases of Ade5′, Thy4, Gua3 and Gua8′), nine water molecules participate in protein–water–phosphate group interactions and the rest contact either polar amino-acid or polar DNA atoms and often stabilize nearby waters interacting in the protein–DNA interface (Figs. 2 and 3). A similar network of water molecules at the FOXO-DBD–DNA interface was observed in the FOXO1-DBD–DNA structure (solved at 2.1 Å resolution; PDB code 3co6) but not in the FOXO3-DBD–DNA structure, which is likely to be a consquence of its lower resolution (2.7 Å; PDB code 2uzk) (Brent *et al.*, 2008; Tsai *et al.*, 2007). The presence of an extensive network of ordered water molecules at the binding interface linking FOXO-DBD and DNA through numerous water-mediated hydrogen bonds suggests that, besides protein–DNA interactions, hydration enthalpy might be an important factor in complex stabilization. In addition, the hydrophobic contribution to binding could be restricted by the retention of bound water molecules (Bhat *et al.*, 1994). The network of highly ordered water molecules can also help to mediate the specific readout of bases by FOXO-DBD, similarly to as has been observed, for example, in the RXR–retinoid acid receptor–DNA complex (Rastinejad *et al.*, 2000). It therefore seems that both direct DNA base contacts and the unique water-network interactions significantly contribute to FOXO-DBD binding to DNA in a sequence-specific manner.

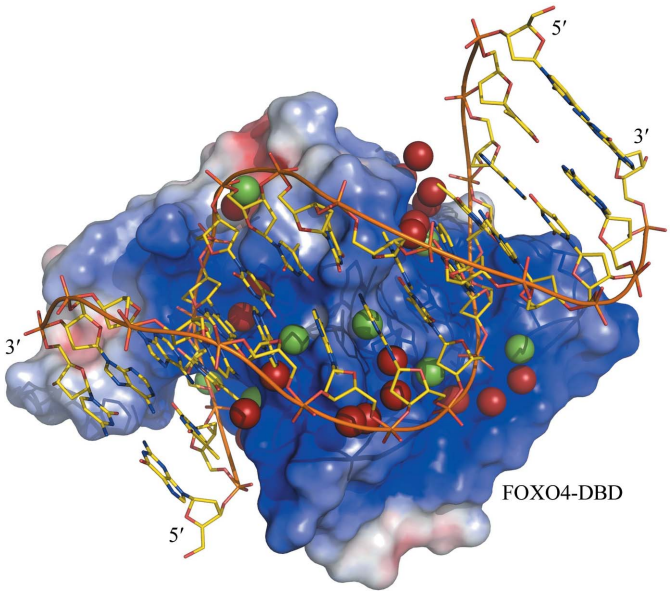


Figure 5
Water molecules at the FOXO4-DBD–DNA interface. The molecular surface of FOXO4-DBD is coloured according to its electrostatic potential (as calculated using the *APBS* software package; Baker *et al.*, 2001): blue, positive; red, negative. Spheres represent water molecules located at a distance of less than 3.0 Å from atoms of both FOXO4-DBD and DNA. Green spheres represent water molecules that are engaged in protein–water–DNA interactions. Red spheres represent water molecules that contact either polar amino acids or polar DNA atoms.

3.5. Structural comparison with previous FOXO structures

The structure can be superimposed with the solution structure of apo FOXO4-DBD (Weigelt *et al.*, 2001) with a root-mean-square deviation (r.m.s.d.) of 2.48 Å over 85 C^{α} atoms. The most significant differences can be observed in flexible regions that participate in DNA binding: the turn region between helices H2 and H3, the N-terminal loop and wing W1 (Fig. 6*a*). While the positions of the three major helices (H1, H2 and H3) are almost identical, the recognition helix H3 is shortened at the N-terminus by approximately one turn and the fourth short helix H4 is shifted closer to the C-terminus of helix H2.

The structure can be superimposed with those of other FOXO-DBD–DNA complexes (Tsai *et al.*, 2007; Brent *et al.*, 2008) with r.m.s.d.s of 1.68 Å (for FOXO4 and FOXO1) and 2.85 Å (for FOXO4 and FOXO3) over 80 C^{α} atoms. The FOXO4-DBD–DNA complex differs mainly in the conformation of the H2–H3 turn and wing W1 (Fig. 6*b*). The H2–H3 turns of these structures (FOXO4 residues 131–145) can be superimposed with r.m.s.d.s of 2.99 Å (for FOXO4 and FOXO1) and 3.99 Å (for FOXO4 and FOXO3) between corresponding C^{α} atoms. Residues Val131–Lys135 from the H2–H3 turn of the FOXO4-DBD–DNA complex form a short 3_{10}-helix H4 which is also present in the apo form of FOXO4-DBD and the FOXO1-DBD–DNA complex but is absent from the FOXO3-DBD–DNA complex (Weigelt *et al.*, 2001; Tsai *et al.*, 2007; Brent *et al.*, 2008). The different conformations of the H2–H3 turn, which is conserved among FOXO proteins, are likely to be

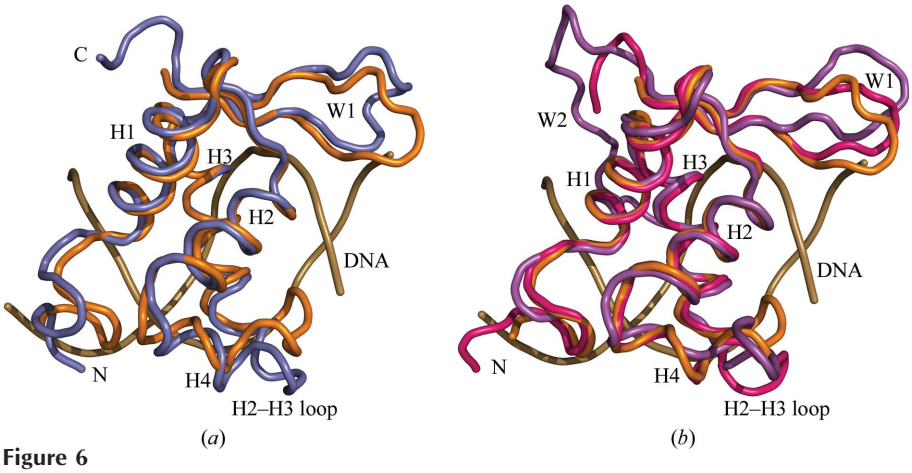


Figure 6
Comparison of FOXO4-DBD with previously reported structures of FOXO-DBD. For clarity, only the DNA in the FOXO4-DBD–DNA complex is shown. (*a*) Superimposition of the FOXO4-DBD–DNA structure (shown in orange) with the solution structure of apo FOXO4-DBD (shown in blue; Weigelt *et al.*, 2001). (*b*) Superimposition of the FOXO4-DBD–DNA complex (orange) with the crystal structures of the FOXO3-DBD–DNA (violet; Tsai *et al.*, 2007) and FOXO1-DBD–DNA (magenta; Brent *et al.*, 2008) complexes.

the result of both crystal-packing differences and the interaction with the N-terminal segment (Fig. 4), which is shorter in the FOXO3-DBD–DNA structure.

While the contacts between wing W1 and DNA are similar in all three complexes, its conformations differ, with wing W1 of FOXO4-DBD being located closer to the DNA. These loops (FOXO4 residues 164–172) can be superimposed with r.m.s.d.s of 0.85 Å (for FOXO4 and FOXO1) and 1.65 Å (for FOXO4 and FOXO3) between corresponding C^{α} atoms. The variations in wing W1 conformation are likely to be the result of crystal-packing differences and have no biological relevance.

Other differences between these three FOXO-DBD–DNA structures include the N-terminal loop that is missing in the FOXO3-DBD–DNA structure and wing W2 that is missing in the FOXO4-DBD–DNA and FOXO1-DBD–DNA structures (Figs. 1*a*, 4 and 6*b*).

4. Conclusions

In summary, we report the high-resolution structure of the FOXO4-DBD bound to a 13 bp DNA duplex containing a FOXO consensus binding sequence. The structure shows a similar recognition of the core sequence as has been shown for the other two FOXO proteins. The helix H3 is docked into the major groove and provides all of the base-specific contacts, while the N-terminus and wing W1 make additional contacts with the phosphate groups of DNA. In contrast to other FOXO-DBD–DNA structures, the loop between helices H2 and H3 has a different conformation and participates in DNA binding. In addition, the structure of the FOXO4-DBD–DNA complex suggests that the network of highly ordered water molecules at the binding interface contribute to FOXO-DBD binding to the DNA in a sequence-specific manner.

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