Acta Crystallographica Section F
Structural Biology Communications
ISSN 2053-230X

Crystallization and preliminary X-ray analysis of a complex of the FOXO1 and Ets1 DNA-binding domains and DNA

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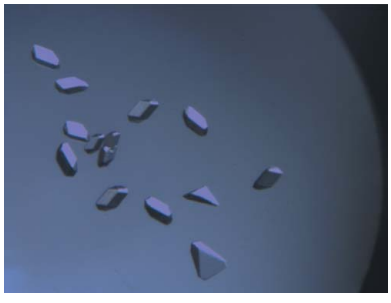
Received 1 July 2013
Accepted 5 September 2013

The Ets1 transcription factor is a member of the Ets protein family, a group of evolutionarily related DNA-binding transcriptional factors. Ets proteins activate or repress the expression of genes that are involved in various biological processes, including cellular proliferation, differentiation, development, transformation and apoptosis. FOXO1 is a member of the forkhead-box proteins (FOX proteins), which comprise a large family of functionally diverse transcription factors involved in cellular proliferation, transformation and differentiation. The FOXO subgroup of FOX proteins regulates the transcription of genes that control metabolism, cell survival, cellular proliferation, DNA damage responses, stress resistance and longevity. The DNA-binding domains (DBDs) of Ets1 and FOXO1 were crystallized in complex with DNA containing a composite sequence for a noncanonical forkhead binding site (AATAACA) and an ETS site (GGAA), FOX:ETS, by the sitting-drop vapor-diffusion method. The FOX:ETS motif has been shown to be a conserved *cis*-acting element in several endothelial cell-specific genes, including Vegfr2, Tie2, Mef2c and ve-cadherin. Crystals were grown at 291 K using 30% polyethylene glycol 400, 50 m*M* Tris pH 8.5, 100 m*M* KCl, 10 m*M* $MgCl_2$ as the reservoir solution. The crystals belonged to space group $C222_1$, with unit-cell parameters a = 68.7, b = 104.9, c = 136.3 Å. Diffraction data were collected to a resolution of 2.2 Å.

1. Introduction

The Ets (E26 transformation-specific) transcription-factor family is involved in cellular differentiation, proliferation, senescence, apoptosis and oncogenic transformation by regulating the transcription of various genes (Oikawa & Yamada, 2003; Sementchenko & Watson, 2000; Sharrocks, 2001). Approximately 30 members of the family have been identified in mammals and they contain a highly conserved 85 amino-acid winged helix–turn–helix DNA-binding domain termed the Ets domain. They recognize and bind to a consensus GGA(A/T) core sequence referred to as the Ets binding site (EBS). Additional flanking sequences on gene promoters or enhancer elements determine the binding specificity of each factor (Hollenhorst *et al.*, 2007). The conserved Ets domain consists of three α-helices on a small four-stranded antiparallel β-sheet scaffold. Ets transcription-factor family members can be classified into subfamilies (SP1, TEL, ESE, ELF, PEA3, SPDEF, TCF, ETS, ERF and ERG) based on the similarity of the Ets domain sequences and auxiliary domain compositions. Most Ets transcription factors are thought to function as monomers, unlike many other transcription factors which function as homodimers or heterodimers (Garvie & Wolberger, 2001). Several Ets proteins including Ets1 have been implicated in dimeric interactions on tandem or multiple ETS DNA motifs. Moreover, Ets-family members regulate gene transcription cooperatively with other transcription factors and cofactors through physical interactions (Li *et al.*, 2000) and through partnerships with transcription factors on adjacent DNA-binding sites (reviewed in Hollenhorst *et al.*, 2011; Verger & Duterque-Coquillaud, 2002).

Forkhead-box (FOX) transcription factors display large functional diversity and play a wide range of roles in development, proliferation, differentiation, stress resistance, apoptosis and control of metabolism



(Carlsson & Mahlapuu, 2002; Greer & Brunet, 2005; Huang & Tindall, 2006; Lehmann *et al.*, 2003; Wijchers *et al.*, 2006). FOXO transcription factors are a conserved subfamily of forkheads consisting of FOXO1, FOXO3a, FOXO4 and FOXO6 (Kops & Burgering, 1999; Tran *et al.*, 2003). Structural studies of forkheads began with the FOXA3 (HNF3γ)–DNA complex (Clark *et al.*, 1993) showing the forkhead domain to be a compact structure containing ~110 amino acids that folds into three α-helices (H1–H3), three β-strands (S1–S3) and two wing-like loops (W1, W2). Although forkheads share a conserved fold and high sequence homologies in the helices, they use versatile modes for DNA recognition, such that their DNA-binding properties differ substantially and they recognize diverse DNA sequences extending beyond the shared core 5′-(T/C)(A/C)AA(C/T)A-3′ sequence through mechanisms which remain unclear (reviewed in Obsil & Obsilova, 2008). Binding specificity for the core sequence is due to highly conserved amino acids in the DNA-recognition helix H3.

Transcriptional activation of eukaryotic genes during development or in response to extracellular signals involves the regulated assembly of multiprotein complexes on enhancers and promoters. The complex nature of these processes provides virtually unlimited possibilities for regulation and elaborate mechanisms for controlling gene expression. Recently, a 44 bp transcriptional enhancer that is sufficient to direct expression specifically and exclusively to the developing vascular endothelium has been identified (De Val *et al.*, 2004, 2008). This enhancer is regulated by a composite *cis*-acting element, the FOX:ETS motif, which is bound and synergistically activated by forkhead and Ets transcription factors. FOX:ETS motifs have been identified in many known endothelial-specific gene enhancers. Ets1 and FOXO1 have been shown to bind selectively to the FOX:ETS sequence (De Val *et al.*, 2008). Activity by Ets1 and forkhead factors at a FOX:ETS composite motif in the vascular endothelial growth factor receptor 2 (Vegfr2) gene has been shown to play a role in the expression of genes important for adult neovascularization and vascular integrity (Murakami *et al.*, 2011).

To understand the molecular basis for a FOX and ETS transcription-factor partnership on a composite enhancer motif that is sufficient to direct expression specifically and exclusively to the developing vascular endothelium, we have purified and crystallized the DNA-binding domains (DBDs) of human Ets1 and FOXO1 proteins in complex with the FOX:ETS DNA sequence (bold) from the human ve-cadherin (CDH5) gene (5′-ac**aataacaggaa**accgtg-3′). X-ray data for the ternary complex were collected to 2.2 Å resolution and structure analysis is currently in progress. The FOXO1-DBD–Ets1-DBD–vecFOXETS DNA complex structure will provide an understanding of how FOX and ETS synergistically mediate endothelial-specific transcriptional activity on an enhancer directing expression specifically and exclusively to the developing vasculature. The structure will also provide insights into the cooperative regulation of gene expression in adult neovascularization and vascular homeostasis.

2. Experimental procedures

2.1. Protein expression and purification

A DNA fragment encoding the DBD of human Ets1 (NCBI NP_001137292.1), residues 331–440, was amplified by polymerase chain reaction (PCR) from a full-length Ets1 cDNA template and cloned into pET19 vector (Novagen). The Ets1-DBD protein carrying four additional N-terminal residues from cloning (MGGS) was expressed in pET19-Ets1-DBD plasmid-transformed *Escherichia coli* BL21 (DE3) cells grown in Luria–Bertani broth at 310 K until they reached an absorbance of 0.5 at 600 nm, followed by induction with 1.0 m*M* IPTG for 4 h at 310 K. Cells from induced cultures were harvested by centrifugation, resuspended in Tris-buffered saline with 500 m*M* NaCl and lysed by sonication. Cell lysate containing soluble recombinant Ets1-DBD protein was exchanged into citrate-buffered saline with 100 m*M* NaCl and purified by fast protein liquid chromatography (FPLC) using a FastFlow SP column (GE Healthcare) employing a linear gradient of NaCl for elution. Following buffer exchange into Tris-buffered saline with 500 m*M* NaCl, soluble recombinant Ets1-DBD protein was further purified using a Superdex S75 column (GE Healthcare). Fractions of Ets1-DBD in 50 m*M* Tris pH 8.0, 500 m*M* NaCl, 5 m*M* DTT were concentrated to ~20 mg ml^{-1} in centrifugation devices with an MWCO of 3000 (Millipore) and stored at 193 K.

A DNA fragment encoding a region encompassing the DBD of human FOXO1 (NCBI NP_002006.2), residues 143–270, was amplified by PCR from a full-length FOXO1 cDNA template and cloned into pET50 vector (Novagen). The FOXO1-DBD protein carrying five additional N-terminal residues from cloning (GPGYP) was expressed in pET50-FOXO1-DBD plasmid-transformed *E. coli* BL21 (DE3) cells grown in Luria–Bertani broth at 310 K until they reached an absorbance at 600 nm of 0.5, followed by induction with 0.5 m*M* IPTG for 8 h at 298 K to produce the extended FOXO1-DBD fragment as a C-terminal fusion protein to NusA protein carrying an N-terminal hexahistidine tag and C-terminal hexahistidine tag with a cleavage site for human rhinovirus 3C (HRV3C) protease. Cells from induced cultures were harvested by centrifugation, resuspended in phosphate-buffered saline with 150 m*M* NaCl, supplemented with protease-inhibitor cocktail tablets (Roche Applied Science), lysed by sonication and clarified by centrifugation. Soluble His-NusA-His-FOXO1-DBD fusion protein in the lysate was purified on nickel–nitrilotriacetic acid–agarose (Qiagen). The recombinant FOXO1-DBD protein was released by digestion of the NusA fusion protein with HRV3C protease. FOXO1-DBD protein was further purified by reverse nickel-affinity chromatography followed by FPLC using a Superdex S75 column (GE Healthcare). Fractions of FOXO1-DBD in 25 m*M* sodium phosphate, 100 m*M* NaCl, 2 m*M* DTT were concentrated to ~25 mg ml^{-1} in a centrifugation device with an MWCO of 3000 (Millipore) and stored at 193 K.

2.2. Preparation of duplex vecFOXETS DNA

Synthetic DNA oligonucleotides with the sequences 5′-AAAC-AATAACAGGAAACCGTG-3′ and 5′-TGTTATTGTCCTTTGG-CACTT-3′, purified by HPLC, were purchased from Integrated DNA Technologies. Lyophilized oligonucleotides were resuspended in water and complementary strands were mixed at an equimolar ratio in 20 m*M* Tris pH 7.5, 100 m*M* NaCl, 1 m*M* EDTA and annealed by heating to 368 K for 10 min and slow-cooling to 277 K. The annealed DNA, called vecFOXETS, was precipitated with sodium acetate and ethanol, washed thoroughly in 70% ethanol and resuspended in water to a final concentration of 20–30 mg ml^{-1} as measured by the absorbance at 260 nm.

2.3. Cocrystallization of the FOXO1-DBD–Ets1-DBD–DNA ternary complex

Equimolar amounts of FOXO1-DBD and Ets1-DBD proteins were mixed with 1.3 molar equivalents of annealed vecFOXETS DNA at 277 K for 2 h. The resulting 40.7 kDa ternary complex was crystallized in 30% polyethylene glycol 400, 50 m*M* Tris pH 8.5, 100 m*M* KCl, 10 m*M* $MgCl_2$ at 291 K by the sitting-drop vapor-

diffusion method by mixing 2 µl complex solution with an equal volume of precipitant solution and equilibrating against 500 µl reservoir solution. Rectangular-shaped crystals appeared at 291 K in 6–10 d and grew to dimensions of 0.2 × 0.15 × 0.1 mm. Crystals were harvested and flash-cooled in liquid nitrogen directly from the mother liquor.

2.4. Data collection and processing

Native diffraction data were collected on beamline X29 at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (Upton, New York, USA) at 100 K using a crystal which diffracted to a maximum resolution of 2.2 Å. All data were processed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The crystals belonged to space group $C222_1$, with unit-cell parameters a = 68.7, b = 104.9, c = 136.3 Å. With one complex molecule in the asymmetric unit comprised of one FOXO1-DBD chain, one Ets1-DBD chain and one molecule of double-stranded vecFOXETS DNA, the Matthews coefficient V_M is 3.30 Å^3 Da^{-1} and the solvent content is 66.8%. With two complex molecules in the asymmetric unit cell, the Matthews coefficient V_M is 1.65 Å^3 Da^{-1} and the solvent content is 33.6% (Matthews, 1968).

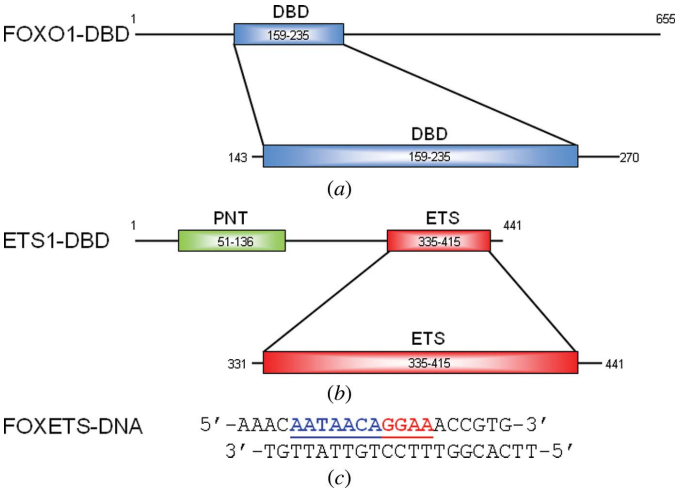


Figure 1
Protein and DNA constructs used in crystallization. (*a*) Human FOXO1-DBD protein region, residues 143–270. (*b*) Human Ets1-DBD protein region, residues 331–441. (*c*) The composite FOX:ETS enhancer sequence (De Val *et al.*, 2008) used in crystallization from the human ve-cadherin gene (CDH5) containing an ETS binding site (red) and a noncanonical forkhead binding site (blue). Complementary strands of the vecFOXETS DNA contain 19 paired bases and two overhanging bases on each of the 5′ ends.

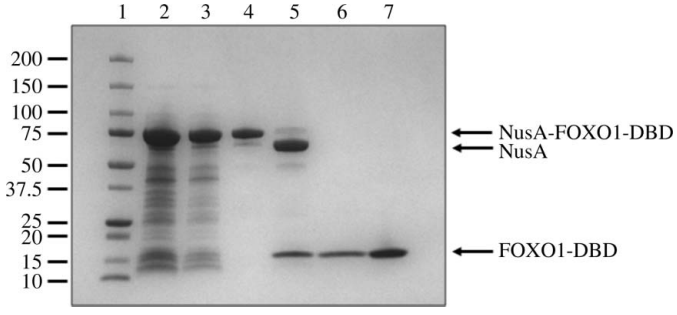


Figure 2
SDS–PAGE analysis of expressed and purified FOXO1-DBD protein; lane 1, molecular-weight marker proteins (Bio-Rad); lane 2, total *E. coli* cell lysate fraction after sonication; lane 3, soluble *E. coli* cell lysate fraction after centrifugation; lane 4, nickel-column elution fraction; lane 5, protein products following HRV3C protease cleavage; lane 6, column runthrough fraction from nickel-column reapplication post HRV3C cleavage; lane 7, pooled fractions of pure FOXO1-DBD protein following elution from size-exclusion medium and concentration. FOXO1-DBD is >95% pure and is homogeneous and appears to be a monomer in solution.

3. Results and discussion

Recombinant FOXO1-DBD protein, amino acids 143–270 (Fig. 1*a*), was expressed in *E. coli* as a fusion to NusA, liberated by protease cleavage and purified to homogeneity by immobilized metal-ion and size-exclusion chromatography. Final fractions of purified FOXO1-DBD protein were shown to be >95% pure by SDS–PAGE analysis (Fig. 2). FOXO1-DBD binds to forkhead family consensus DNA sequence (5′-...GCAAAACAA...-3′) with high affinity in electrophoretic mobility shift assays (data not shown). Recombinant Ets1-DBD, amino acids 331–441 (Fig. 1*b*), was directly expressed in *E. coli*, purified to homogeneity and shown to be >95% pure by SDS–PAGE analysis (Fig. 3). Size-exclusion analysis using a Superdex S75 GL 10/300 column (GE Healthcare) showed that both the purified FOXO1-DBD (14.7 kDa) and Ets1-DBD (13.2 kDa) proteins are monomers in solution (Fig. 4) when their elution profile is compared to a set of molecular-weight standards (GE Healthcare). Ets1-DBD binds to the ETS site DNA sequence (5′-....CCGGAAGT...-3′)

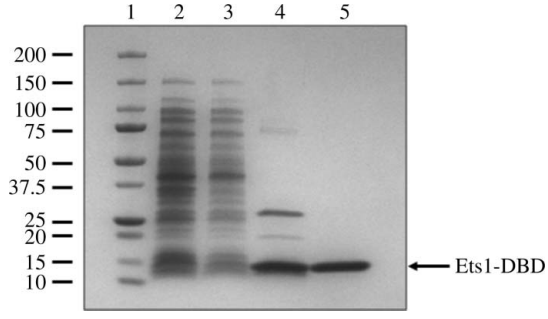


Figure 3
SDS–PAGE analysis of expressed and purified Ets1-DBD protein; lane 1, molecular-weight marker proteins (BioRad); lane 2, total *E. coli* cell lysate fraction after sonication; lane 3, soluble *E. coli* cell lysate fraction after centrifugation; lane 4, elution fraction from Fast Flow SP ion-exchange chromatography; lane 5, pooled fractions of pure Ets1-DBD protein following elution from size-exclusion medium and concentration. Ets1-DBD is >95% pure and is homogeneous and appears to be a monomer in solution.

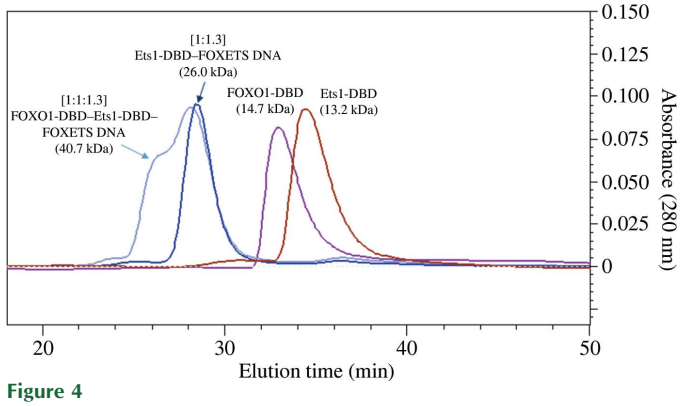


Figure 4
Elution profile of the purified FOXO1-DBD (magenta) and Ets1-DBD (red) proteins, as well as the binary complex of Ets1-DBD–vecFOXETS DNA (dark blue) and a mixture of the binary complex Ets1-DBD–vecFOXETS DNA and the ternary complex of FOXO1-DBD–Ets1-DBD–vecFOXETS DNA (light blue) from an analytical size-exclusion column (Superdex 75 5/150 GL column; GE Healthcare).

with high affinity in electrophoretic mobility shift assays (data not shown). Purified Ets1-DBD forms a single complex (26.0 kDa) with vecFOXETS DNA (Fig. 1*c*), and the ternary complex (40.7 kDa) of FOXO1-DBD–Ets1-DBD–vecFOXETS DNA forms when mixtures are prepared in a 1:1:1.3 molar ratio, respectively (Fig. 4). FOXO1-DBD binds weakly to the noncanonical FOX site in vecFOXETS DNA alone in electrophoretic mobility shift assays, but binds well in the presence of bound Ets1-DBD to form the ternary complex (data not shown). These results suggest that the binding of Ets1-DBD to vecFOXETS might alter the DNA in such a way as to facilitate high-affinity binding of FOXO1-DBD.

In the crystallization of the ternary FOXO1-DBD–Ets1-DBD–vecFOXETS DNA complex, a variety of protein concentrations, temperatures, molar ratios of FOXO1-DBD and Ets1-DBD to vecFOXETS DNA and variations in the double-stranded vecFOXETS DNA (length, blunt ends, single and double-base 5′ overhangs) were screened. Initial crystallization screening included sitting-drop vapor-diffusion experiments with the Nucleix (Qiagen) and Protein–Nucleic Acid Complex Crystal Screen (Kerafast) screening kits. Based on conditions that produced single crystals, the pH and the concentrations of salts, metal ions and precipitants were modified for optimization of crystal growth. Crystallization optimizations resulted in single crystals that grew in 30% polyethylene glycol 400, 50 m*M* Tris pH 8.5, 100 m*M* KCl, 10 m*M* $MgCl_2$ within 6–10 d at 291 K (Fig. 5) and continued to grow until they reached average dimensions of 0.2 × 0.15 × 0.1 mm. The presence of FOXO1-DBD and Ets1-DBD proteins in the crystals was confirmed by SDS–PAGE analysis (Fig. 6).

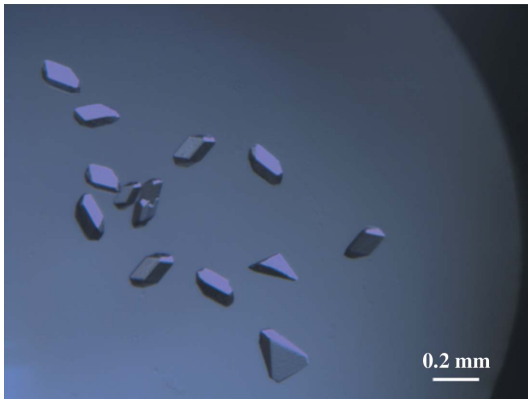


Figure 5
Crystals of the FOXO1-DBD–Ets1-DBD–vecFOXETS DNA ternary complex after 14 d of crystallization by sitting-drop vapor-diffusion methods at 291 K. The crystals have average dimensions of approximately 0.2 × 0.15 × 0.1 mm.

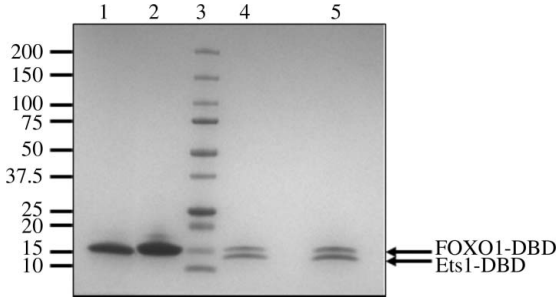


Figure 6
SDS–PAGE analysis of the ternary complex crystallization mixture and crystals; lane 1, fraction of pure Ets1-DBD protein following elution from size-exclusion medium and concentration; lane 2, fraction of pure FOXO1-DBD protein following elution from size-exclusion medium and concentration; lane 3, molecular-weight marker proteins (Bio-Rad); lane 4, the 1:1:1.3 molar ratio mixture of FOXO1-DBD, Ets1-DBD and vecFOXETS DNA used in crystallization experiments; lane 5, redissolved crystals of FOXO1-DBD, Ets1-DBD and vecFOXETS DNA.

All crystals were mounted in CryoLoops (Hampton Research) and flash-cooled in liquid nitrogen directly from the mother liquor. Native diffraction data were collected to 2.2 Å resolution on beamline X29 at Brookhaven NSLS at 100 K (Fig. 7). All data were processed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The FOXO1-DBD–Ets1-DBD–vecFOXETS DNA ternary complex crystals belonged to space group $C222_1$, with unit-cell parameters $a = 68.7$, $b = 104.9$, $c = 136.3$ Å. Assuming the presence of one ternary complex per asymmetric unit, the V_M value is 3.30 Å^3 Da^{-1} and the calculated solvent content is 66.8% (Matthews, 1968). Assuming the presence of two ternary complexes per asymmetric unit, the V_M value is 1.65 Å^3 Da^{-1} and the calculated solvent content is 33.6%. The

Table 1
Data-collection statistics for the FOXO1-DBD–Ets1-DBD–DNA ternary complex.

Values in parentheses are for the outermost resolution shell.

Wavelength (Å)	1.075
Space group	$C222_1$
Resolution range (Å)	68.17–2.22 (2.30–2.22)
No. of observed reflections	327192
No. of unique reflections	27044
Completeness (%)	99.7 (97.5)
Redundancy	12.1 (6.4)
$\langle I/\sigma(I)\rangle$	37.5 (2.1)
R_{merge}† (%)	9.3 (84.4)
Unit-cell parameters (Å)	$a = 68.66$, $b = 104.94$, $c = 136.32$
V_M (Å^3 Da^{-1})	3.30‡/1.65§
Solvent content (%)	66.8‡/33.6§
No. of molecules in asymmetric unit	1 complex (1 Ets1-DBD, 1 FOXO1-DBD, 1 dsDNA)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed integrated intensity, $\langle I(hkl)\rangle$ is the average integrated intensity obtained from multiple measurements and the summation is over all observed reflections. ‡ Assuming the presence of one ternary complex per asymmetric unit. § Assuming the presence of two ternary complexes per asymmetric unit.

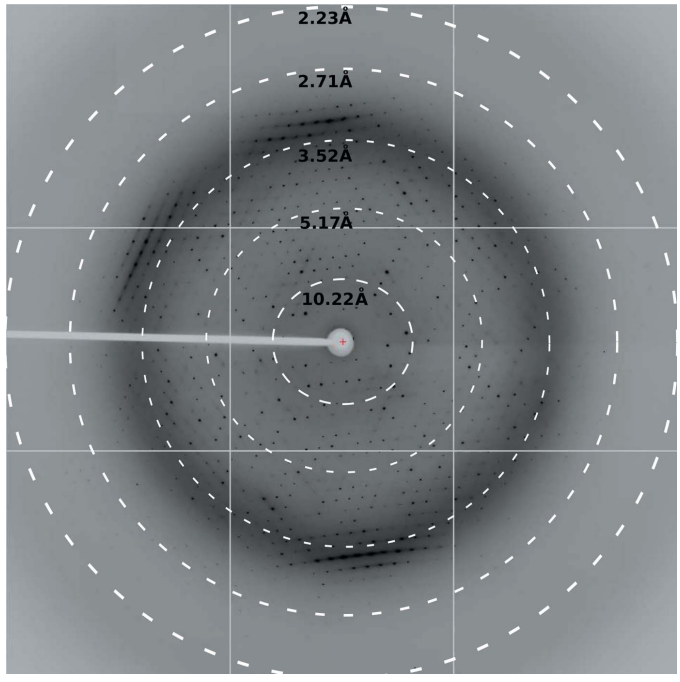


Figure 7
Typical X-ray diffraction pattern image of a FOXO1-DBD–Ets1-DBD–vecFOXETS DNA ternary-complex crystal (oscillation width of 1°). The edge of the diffraction frame corresponds to approximately 2.2 Å resolution.

data-collection statistics of the best data set are summarized in Table 1.

Molecular-replacement calculations were performed using *Phaser* (McCoy *et al.*, 2007) with models corresponding to the FOXO1 protein in complex with insulin response element (IRE) DNA (PDB entry 3coa; Brent *et al.*, 2008) and Ets1 protein in complex with Ets binding site (EBS) DNA (PDB entry 2stt; Werner *et al.*, 1997). No suitable solution was obtained using these models and variations thereof. Experiments are currently under way to express FOXO1-DBD and Ets1-DBD in *E. coli* B834(DE3) cells in minimal medium supplemented with selenomethionine (SeMet) for MAD (multi-wavelength anomalous dispersion) phasing experiments.

This work was supported by the National Institutes of Health grant No. HL086766A2 to MAG. We thank the staff at beamline X29, National Synchrotron Light Source (Brookhaven National Laboratory) for the opportunity for data collection.

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