Supporting Information

Molecular mechanism and structure of the *S. cerevisiae* iron regulator Aft2

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Materials and Methods

1. Aft2 expression and purification

Aft2(1-204) was cloned between the NdeI and XhoI sites in the pET30a plasmid system and the Aft2(1-204)-pET30a plasmid was transformed into BL21star(DE3). The different mutations were generated using the QuikChange Kit (Stratagene) following the provided protocol. A 10 mL overnight pre-culture was grown from a single colony and inoculated into 1 L of autoclaved LB medium containing 50 mg kanamycin. The cells were grown at 37 °C, 250 rpm to OD_{600} = 0.6 and then the temperature was reduced to 16 °C before the protein expression was induced with 0.5 mM IPTG and cells were grown overnight. Cells were harvested at 4 °C by centrifugation at 6000 rpm for 8 minutes and all subsequent protein purification steps were performed at 4 °C. The pellet was suspended in 20 ml buffer A (10 mM Tris-HCl [pH 7.4], 300 mM NaCl, 1 mM DTT) and 1 mM PMSF. The cells were lysed by sonication and centrifuged at 12000 rpm for 25 minutes. The supernatant was filtered through a 0.45 μm filter and applied to the column.

Purification of Aft2 would be done with two alternative columns. 1) Purification using HiTrap Heparin column: The clarified lysate was loaded to a HiTrap Heparin column (5 ml, CV) twice which had previously been equilibrated with buffer B (10 mM Tris-HCl [pH 7.4], 1 mM DTT). The column was washed with 25 % buffer C (10 mM Tris-HCl [pH 7.4], 1 M NaCl, 1 mM DTT) until the base line reading for the UV-VIS was flat and the protein was eluted with a linear gradient from 25% to 100% buffer C over 40 ml where the protein came off starting at 55 % Buffer C. 2) Purification using SFF column: The lysate was loaded to a SFF column (20 ml, CV) twice using a pump and the column was washed with buffer B until the UV-VIS signal was back to baseline levels. The protein was eluted over a linear gradient from 0 to 100 % buffer C over 200 ml. The protein eluted at around 60 % buffer C. Peak fractions from either purification were pooled and applied to a Superdex 200 column, which had been preequilibrated with buffer A, to obtain the purified protein. A truncated form of Aft2 including residues 38-193 was used for protein crystallization. Aft2(38-193) was purified by HiTrap Heparin column followed by cation exchange chromatography using a Mono S column and gel filtration chromatography using a Superdex 200 column. The protein purity was confirmed on SDS-PAGE gel (Fig. S1).

Aft2 was expressed in M9 medium by harvesting the cells from a 10 mL overnight pre-culture in LB by centrifugation and resuspending the cells in 1 L autoclaved M9 medium. The cells were grown at 250 rpm to OD_{600}= 0.5 at 37 °C and the temperature was reduced to

16 °C. The protein expression was induced with 0.5 mM IPTG and either 10 μM $CoCl_2$ or 200 μM $ZnCl_2$ were added. All subsequent steps were done as for the protein expressed in LB; however, the purification for Co^{2+}-Aft2 was only successful with the HiTrap Heparin column purification scheme.

Protein concentrations were determined by either UV-visible absorption ($\varepsilon_{280\ nm}$ = 16500 $M^{-1}cm^{-1}$) or the Bradford assay (Bio-Rad) using BSA as the standard. The Zn^{2+} concentration was determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) or Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The Co^{2+} concentration was determined using PAR, a colorimetric dye for divalent metal ions(1).

2. Crystallization and structure solution

Aft2 was further truncated for crystallization experiments, and this truncation included residues 38-193. A 12-bp oligonucleotide with single nucleotide 5' overhangs (adenine on the coding strand and thymine on the non-coding strand) crystallized with Aft2 (in buffer A) at room temperature using the hanging drop vapor diffusion method and a reservoir solution of 0.1 M BIS-TRIS [pH 6.6], 23% PEG 2000 monomethyl ether, and 0.2 M $MgCl_2$. Plate-shaped crystals grew within 5 days and were flash frozen in liquid nitrogen following cryoprotection with the reservoir solution and 16% glycerol. The data were collected at GM/CA@APS beamline 23-ID-B at the APS at Argonne National Laboratory. Data were indexed and integrated with XDS(2) and scaled with SCALA(3). The structure was phased with SAD data and a partial model was built using PHENIX(4). Manual model building was performed in Coot(5), and the structure was refined with PHENIX. Figures were prepared with PyMOL(6).

3. Fe^{2+} and [2Fe-2S] cluster loading and content analysis

All loading experiments were performed in an anaerobic chamber (O_2< 5ppm) (Coy Laboratory Products, Inc.).

Method for Fe^{2+} loading: Buffer D (50 mM Tris-HCl [pH 7.4], 150 mM NaCl) was degassed by placing it under vacuum for 30 min and leaving it inside the anaerobic chamber for at least 24 hours. Other chemicals were brought in as solids and diluted to appropriate stock concentrations inside the chamber. Purified proteins were dialyzed against the degassed buffer D with 1 mM DTT for 2 hours before it was used in loading experiments. For loading of Aft2(1-204) with Fe^{2+} the protein was first dialyzed against 100 μM $Fe(NH_4)_2SO_4$ in buffer D with 1 mM DTT and then the excess iron was dialyzed away against buffer D with 1 mM DTT.

Method A for [2Fe-2S] cluster loading: Aft2 was dialyzed against buffer D with 1 mM DTT under anaerobic conditions as described for the Fe^{2+} loading. Loading an Fe-S cluster involved adding 1.5 equivalents $Fe(NH_4)_2(SO_4)_2$, 1.5 equivalents Na_2S, and 2 equivalents GSH to the Aft2 solution. After incubation for 1h, the excess iron and sulfur species were removed by exchanging it into buffer D with 1 mM DTT using a spin column.

*Method B for [2Fe-2S] cluster loading***:** Aft2 was buffer exchanged inside the anaerobic chamber into buffer E (50 mM Tris-HCl [pH 7.4], 300 mM NaCl). Then Aft2 was added to the reaction mixture included 5 mM DTT, 16 mM ferrous ammonium sulfate (FAS), 16 mM L-cysteine and catalytic amounts of *E. coli* IscS (6.5 μM), and was incubated at 4 °C for 2 h. After loading the sample onto a 1-ml HiTrap heparin HP column equilibrated with buffer E the Fe-S cluster-bound form of Aft2 was purified through one-step elution using 50 mM Tris-HCl [pH 7.4], 1 M NaCl and concentrated for further studies.

Iron concentrations were determined using the colorimetric ferrozine assay(7). Acid-labile sulfur concentrations were determined using published methods(8, 9).

4. Analytical ultracentrifugation and small-angle X-ray scattering

For ultracentrifugation experiments Aft2 or Aft2 C187A at OD_{280}= 0.5, which corresponds to ~ 30 μM protein, were used and Fe^{2+} or [2Fe-2S] cluster (method A) were loaded freshly before the experiment and loaded to the ultracentrifugation chambers and sealed inside the anaerobic chamber. The samples were centrifuged at 60,000 rpm for 15 hours for complete sedimentation. Sedimentation profiles were fit to the Lamm equation using the SEDFIT program where the fits have rmsd < 0.009.

Aft2 at a concentration of ~100 μM was used for SAXS experiments. Fe^{2+}-Aft2 and [2Fe-2S]-Aft2 (method A) were prepared as noted above. For nickel(II) or cadmium(II) loading, Aft2 in buffer D with 5 mM TCEP was incubated with 100 μM $CdCl_2$ or $NiSO_4$ for 30 min at room temperature. All samples were filtered with 0.22 μm filters immediately before analysis. Solution SAXS measurements were carried out at BIO-CAT beamline 18-ID-D at the APS at Argonne National Laboratory. Data were reduced using the program Igor Pro (WaveMetrics, Inc., Lake Oswego, OR, USA). Electron pair distribution curves and radii of gyration were generated and calculated by GNOM(10).

5. Electrophoretic mobility shift assays (EMSA)

Initial EMSA were performed with a 31 bp DNA probe derived from the *FET3* promoter (5'-ATCTTCAAAAGTGCACCCATTTGCAGGTGC-3' and its reverse

complement). To test the effect of various biological and biotoxic metals on the formation of the Aft2-DNA complex, 200 nM Aft2 and 50 nM DNA were mixed in a buffer containing 20 mM Tris [pH 6.8], 20% glycerol, 100 mM KCl, and 3 mM $MgCl_2$. Then, appropriate volumes of metal ions were added directly to the Aft2p-DNA complexes to yield a final volume of 40 µL. Fe^{2+}, Mn^{2+}, Cu^{2+}, Hg^{2+} and Sn^{2+} were used up to 2.5 µM, and Cd^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Fe^{3+} were used up to 250 µM. The samples were incubated for 30 minutes and 15 µL from each reaction were loaded into the wells of an 11% polyacrylamide gel. Gels were run with 0.5x TBE buffer at 120 V for 60 min at room temperature to resolve the DNA and Aft2-DNA complex. DNA was stained with SYBR Gold (Invitrogen). EMSA assays to determine protein:DNA stoichiometry were run as above, except without metals added to the reaction and the DNA sequences are as noted in the legend to Fig. S2.

EMSA also were performed via an alternate protocol to measure the DNA binding affinity of as-purified vs. Fe-S cluster loaded Aft2. A 30-bp DNA probe with the *FET3* promoter Aft1/2 binding site was labeled with IRDye700 (Integrated DNA Technologies). Binding reactions were prepared in the dark, and consisted of hybridization buffer (20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 100 mM KCl, 3 mM $MgCl_2$, 5% glycerol, and 4 ng/µl sonicated salmon sperm DNA), 200 pM *FET3* DNA, and purified recombinant protein. Monomeric as-purified Aft2 protein contained 5 mM DTT in the purification buffer. [2Fe-2S] Aft2 was prepared by mixing Aft2 monomer with [2Fe-2S] Grx3-Fra2 and incubating on ice for 30 min. This mixture was then applied to desalting and Heparin columns to separate Aft2 from Grx3-Fra2. The amount of cluster loaded to Aft2 was determined by Bradford assay (Bio-Rad) and UV-visible absorption spectroscopy. [2Fe-2S] cluster-loading on Aft2 from Grx3-Fra2 was determined to be ~50-60%. Once Aft2 protein was added, DNA binding reactions were incubated for 20 minutes in the dark. A 6% polyacrylamide non-denaturing gel containing Tris-borate was pre-electrophoresed in 0.5x Tris-borate buffer, pH 8 until the current was stable. The binding reactions were then applied to the equilibrated gel and electrophoresed for 1 h at 80 V. All sample preparation, reaction incubations, and electrophoresis were carried out in a Coy anaerobic glovebox (O_2 < 5 ppm). Gels were imaged and quantified using an Odyssey Infrared Imaging System (LI-COR).

6. Zn(II) EXAFS of Aft2

As-purified Aft2 was desalted and exchanged into 50 mM Tris/MES, pH 8, 300 mM NaBr, 30% glyercol. Aft2 (1.4 mM Aft2 monomer, 1 mM Zn) was loaded into a Lucite

sample cell, wrapped in Kapton tape, and flash frozen in liquid nitrogen. Spectra were collected at the Stanford Synchrotron Radiation Lightsource (SSRL), beamline 9-3. The SSRL storage ring was operated at 3 GeV with a maximum current of 200 mA. Beamline 9-3 is equipped with a focusing mirror, Si(220) monochromator crystals, and a 13-keV energy-cutoff mirror to reject harmonics. Zinc fluorescence was detected with a Canberra 100-element germanium detector. Sample temperature was maintained at 10K using an Oxford continuous flow cryostat. A 6 ml Copper filter with a Soller-slit assembly was placed between the cryostat window and the detector to reduce scatter. Data analysis procedures were described previously(11-13).

7. Interaction studies on Grx3, Fra2, and Aft2

Grx3, Fra2, [2Fe-2S]-Grx3 homodimer and [2Fe-2S]-Fra2-Grx3 heterodimer were prepared as previously described(11, 14). To examine the interaction among the different apo-proteins, Grx3 + Fra2, Grx3 + Aft2, Fra2 + Aft2, Grx3 + Fra2 + Aft2, were mixed at 300 μM final concentration for each protein in 75-μl reactions and pure Grx3, Fra2 and Aft2 were used as controls. In the interaction study between Aft2 and [2Fe-2S]-Grx3 homodimer or [2Fe-2S]-Fra2-Grx3 heterodimer, the ratio of Aft2 to [2Fe-2S] cluster was 2.0 with the cluster concentration at 40 μM and Aft2 monomer, [2Fe-2S]-Grx3 homodimer, and [2Fe-2S]-Fra2-Grx3 heterodimer were used as internal controls. All the samples were prepared in Buffer F (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 1 mM GSH) under anaerobic conditions (O_2 < 5 ppm), incubated at room temperature for 15 minutes, and analyzed by gel filtration chromatography and reducing SDS-PAGE. For cluster-containing samples, UV-visible CD spectroscopy was also used to investigate the cluster environment. Analytical gel filtration analyses were performed on a Superdex 75 or 200 10/300 GL column (GE Healthcare) pre-equilibrated with 50 mM Tris-MES [pH 8.0], 500 mM NaCl. In particular, 5 mM GSH was included in the running buffer for [2Fe-2S]-Grx3 homodimer containing samples to stabilize the cluster. UV-visible absorption, circular dichroism (CD), and resonance Raman were recorded as previously described (11, 14).

8. Titration of [2Fe-2S] Fra2-Grx3 with Aft2

A sample with 40 μM [2Fe-2S] cluster from [2Fe-2S]-Fra2-Grx3 in buffer F was titrated under anaerobic conditions at room temperature with 0 to 8 equivalents Aft2 per [2Fe-2S] cluster. UV-visible CD spectra were recorded after equilibration for 15 minutes at room temperature.

9. Cluster transfer from [2Fe-2S] Fra2-Grx3 to Aft2

A 1-mL reaction mixture with 80 μM Aft2 (desalted prior to use) and 38 μM Fra2-Grx3 heterodimeric complex (1.05 [2Fe-2S] clusters per Fra2-Grx3 heterodimer, [2Fe-2S] cluster = 40 μM) in buffer F was incubated for 15 min at room temperature, then desalted into 50 mM Tris-HCl [pH 7.4], 250 mM NaCl with a 5-ml HiTrap desalting column. The reaction mixture was loaded onto a 1-ml HiTrap heparin HP column (GE Healthcare) equilibrated with 50 mM Tris-HCl [pH 7.4], 250 mM NaCl, 1 mM GSH and Aft2 was then eluted with 50 mM Tris-HCl [pH 7.4], 1 M NaCl, 1 mM GSH in one step. The fractions from the flow-through and the eluate were pooled, analyzed by UV-visible absorption and circular dichroism (CD) spectroscopy, and the iron and sulfur content were quantified. The Fe-S cluster transfer experiment was also performed in the presence of varying EDTA concentrations to assess cluster stability (see Fig. S9 for details). As a control for cluster disassembly/reassembly, 80 μM ferrous ammonium sulfate and 80 μM Na_2S were incubated with 80 μM apo-Aft2 in a 1-ml reaction for 15 minutes and then treated with the same procedure as described above.

Experiment notes

The crystallographic asymmetric unit contains two molecules of duplex DNA, each bound by two Aft2 monomers. Both monomers bind in the major groove of a single duplex DNA. Each monomer interacts with a short palindromic sequence of 6-bp, 5'-GTGCAC-3'; however, only one monomer interacts with the entirety of the Fe-RE (Fe-responsive element). To probe the protein:DNA stoichiometry under conditions other than the high concentrations of crystallization, electrophoretic mobility shift assays were performed with promoter sequences with mutations in the palindromic sequence. In all cases, only one Aft2-DNA band was observed, suggesting 1:1 stoichiometry (Fig. S2).

To identify the ligands of the proposed structural zinc site, the three histidine and four cysteine residues conserved among Aft1 and Aft2 were mutated to phenylalanine and alanine, respectively. Single mutant proteins C86A, C109A, H133F, and H135F were insoluble under the standard expression conditions used for wild type Aft2, which suggests that these amino acids are the likely ligands for the structural zinc(II) site, the disruption of which is detrimental to the proper folding of the protein. The Co^{2+}-loaded single mutants H44F, C187A, and C189A exhibited similar UV-visible absorption spectra to the wild type Co^{2+}-Aft2 (Fig. S4*C*), and the Co^{2+} loading of the purified mutant proteins, measured using the PAR dye, was found to be roughly one equivalent (Fig. S4*B*). Thus, the structural tetrahedral zinc(II) site uses Cys 86, Cys 109, His 133, and His 135 as ligands, and not His 44, Cys 187, or Cys 189.

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Table S1 Aft2-DNA Data Collection, Phasing, and Refinement Statistics

Data Collection and Crystal Parameters	
Space group	$P2_1$
Cell dimensions	
a, b, c (Å)	41.5, 127.5, 70.8
α, β, γ (°)	90, 90.04, 90
Wavelength (Å)	1.28162
Resolution (Å)	19.93-2.20 (2.32-2.20)[a]
R_{merge} (%)	7.8 (55.9)
I/σI	16.5 (3.6)
Completeness (%)	99.8 (100.0)
Multiplicity	7.5 (7.5)
SAD Phasing	
Resolution (Å)	19.93-2.20
Zn sites (#)	6
Figure of merit (before/after density modification)	0.28/0.62
Refinement	
Resolution (Å)	19.93-2.20 (2.26-2.20)
R_{work}/R_{free} (%)	18.91/23.61 (25.25/ 29.22)
Rmsd bond lengths (Å)	0.003
Rmsd bond angles (°)	0.682
Ramachandran plot statistics (%/#)	
Preferred regions	97.6/455
Allowed regions	2.4/11
Outliers	0/0
PDB ID	4LMG

[a]Values for the highest resolution shell are shown in parentheses.

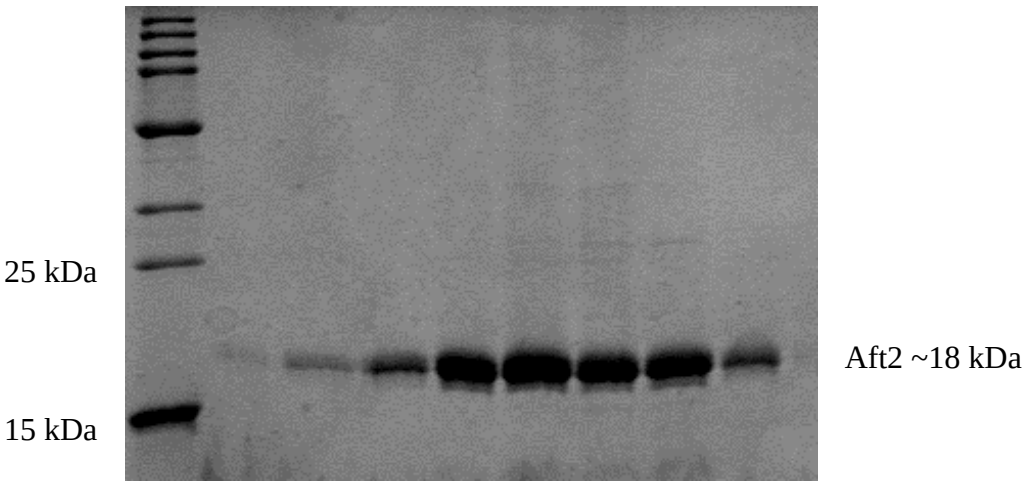


Fig. S1. SDS-PAGE analysis of purified Aft2. The Aft2 protein includes residues 38-193, which is the protein form used for crystallization studies.

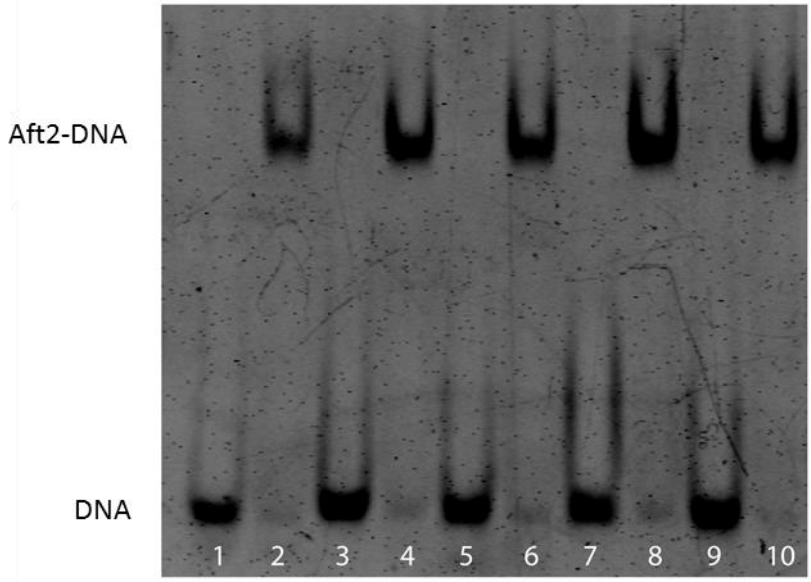


Fig. S2. Aft2 binding to promoter sequences with mutations in the GTGCAC palindrome. Lanes 1, 3, 5, 7, and 9 contain DNA (50nM) only and lanes 2, 4, 6, 8, and 10 contain DNA and Aft2 (200nM). All DNA sequences are double-stranded 16-mers, except where noted below for lanes 1 and 2, which have a double-stranded 15-mer with one nucleotide overhanging on each 5' end. DNA sequences are as follows, with the mutated palindrome indicated in bold: lanes 1, 2 – 5' – AAA**GTGCAC**CCATTTG – 3', 3' – TTCACGTGGGTAAACT – 5'; lanes 3, 4 – 5' – TTT**CTGCAC**CCTGGCC – 3'; lanes 5, 6 – 5' – CAA**GAACAC**CCTGCTA; lanes 7, 8 – 5' – CTT**TGACAC**CCCCGAT – 3'; and lanes 9, 10 – 5' – TAA**CCGCAC**CCTGTTG – 3'. Only a single Aft2-DNA complex is formed in all cases.

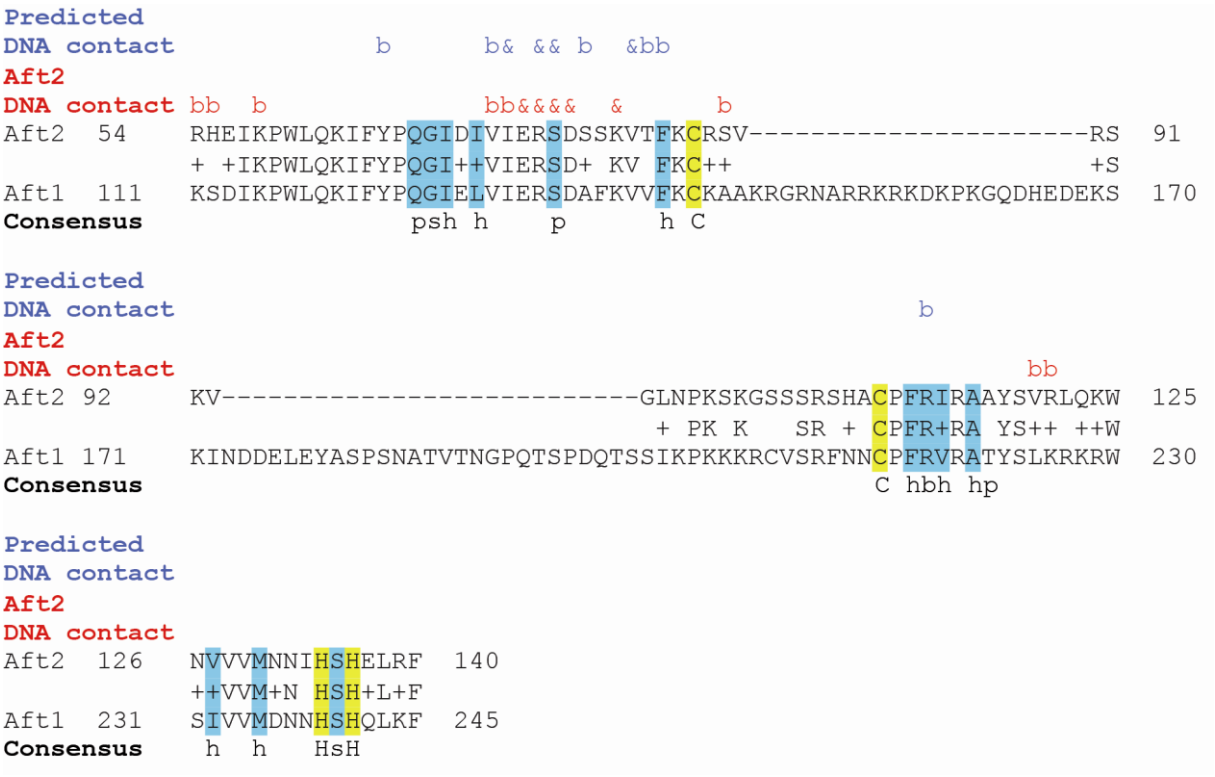


Fig. S3. Sequence alignment of the predicted WRKY domains in Aft2 and Aft1 and DNA contacts. Consensus amino acids are shown in blue and zinc ligands in yellow. Consensus: h: hydrophobic, b: big, p: polar, s: small amino acid. Predicted DNA contacts are shown in blue and DNA contacts observed in the crystal structure of Aft2 are shown in red. DNA contacts: b: backbone, &: base contacts.

A

Sample	Fe (eq.)	Co (eq.)	Zn (eq.)
Aft2	BDL	BDL	1.03
Co^{2+}-Aft2	BDL	0.88	0.09
Aft2 C187F	-	-	1.15
Co^{2+}-Aft2 C187F	-	0.89	-

B

Sample	Co (eq.)
Aft2	0.88
Aft2 H44F	0.85
Aft2 C187A	0.87
Aft2 C189A	0.82

C

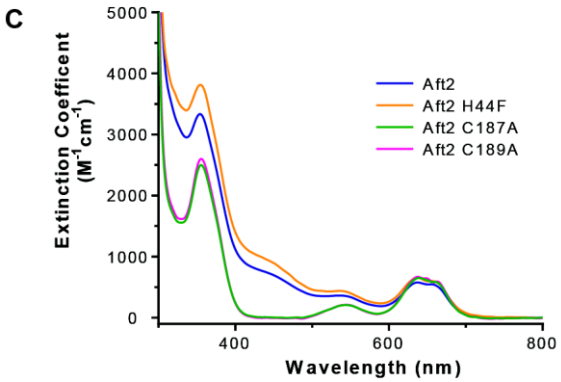


Fig. S4. (*A*) ICP-MS analysis of purified Aft2 protein expressed in LB or minimal medium supplemented with Co^{2+}. BDL: Below detection limit. (*B*) Cobalt(II) loading of different cysteine and histidine Aft2 mutants analyzed by the PAR assay. Results are reported as equivalent of metal bound per protein. (*C*) UV-visible absorption spectrum of Co^{2+}-Aft2 and different cysteine and histidine point mutants.

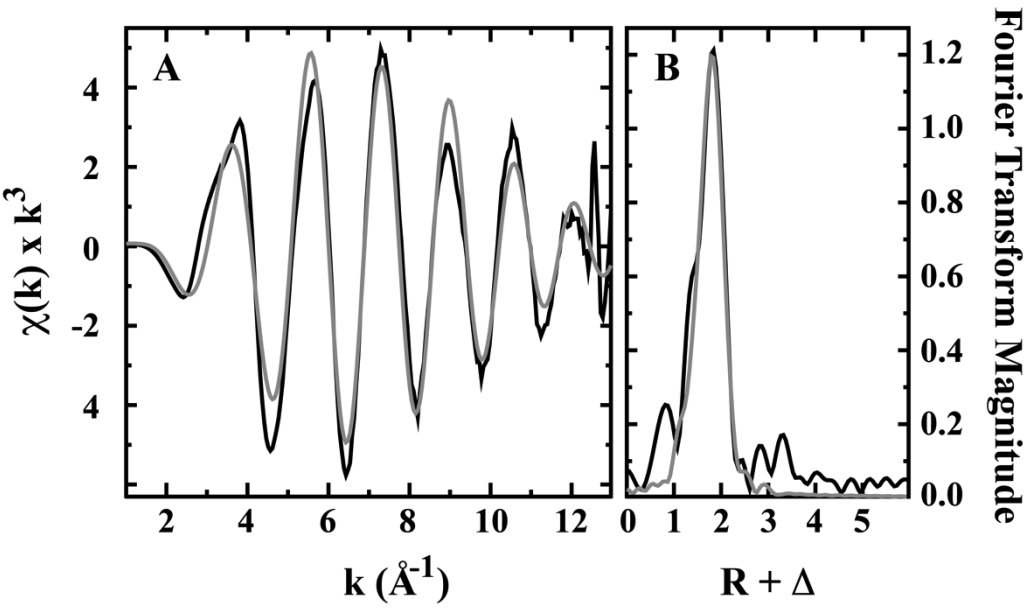


Fig. S5. Zn EXAFS data for Aft2. The best single scattering fit to the data (Fit D below) includes two nitrogens at 2.09 Å and two sulfurs at 2.33 Å. Gray=fit, Black = raw data. Right panel is the Fourier transform and the peaks between 2.5 Å and 3.5 Å can be modeled using multiple scattering imidazole models, also with a 2S/2N coordination environment. Data were analyzed from 1-13 $Å^{-1}$.

Supplementary Table: EXAFS fitting analysis

	#shells	Scatterer	CN	R	σ^2	F	ΔE_0
A	*1*	N	4	2.13	0.0031	1.22	-12
B	*1*	S	4	2.32	0.0075	0.900	-12
C	*2*	S	3	2.32	0.0055	0.398	-12
		N	1	2.07	0.0014		
D	***2***	**S**	**2**	**2.33**	**0.0031**	**0.351**	**-12**
		N	**2**	**2.09**	**0.0026**		
E	*2*	S	2	2.38	0.0132	0.812	-12
		S	2	2.30	0.0038		
F	*2*	N imidazole	2	2.07	0.0010	0.249	-8
		S	2	2.32	0.0012		-11

Data were analyzed from 1-13 $Å^{-1}$. Best Fit D is shown in the Supplemental Figure 5. Fit F invokes multiple scattering of imidazole for the nitrogen shell.

Sample	Fe (eq.)	S (eq.)	Aft2p:Fe:S
Aft2	0.04	-0.02	1:0:0
Fe^{2+}-Aft2	0.54	0.12	1:0.5:0
[2Fe-2S]-Aft2	1.15	0.90	1:1:1

Fig. S6. Iron and sulfur content of Fe^{2+}-Aft2 and [2Fe-2S]-Aft2.

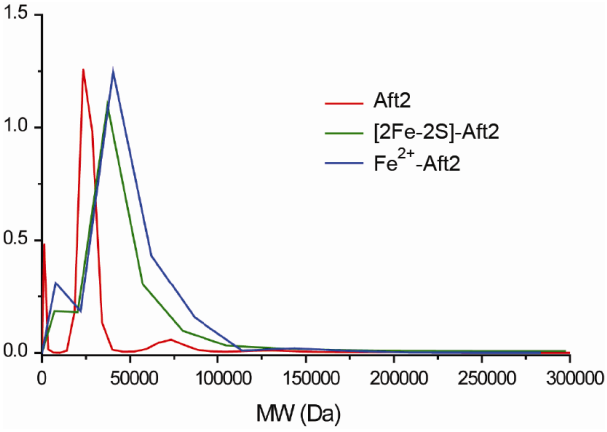


Fig. S7. Fitting curves to the Lamm equation of the ultracentrifugation analyses.

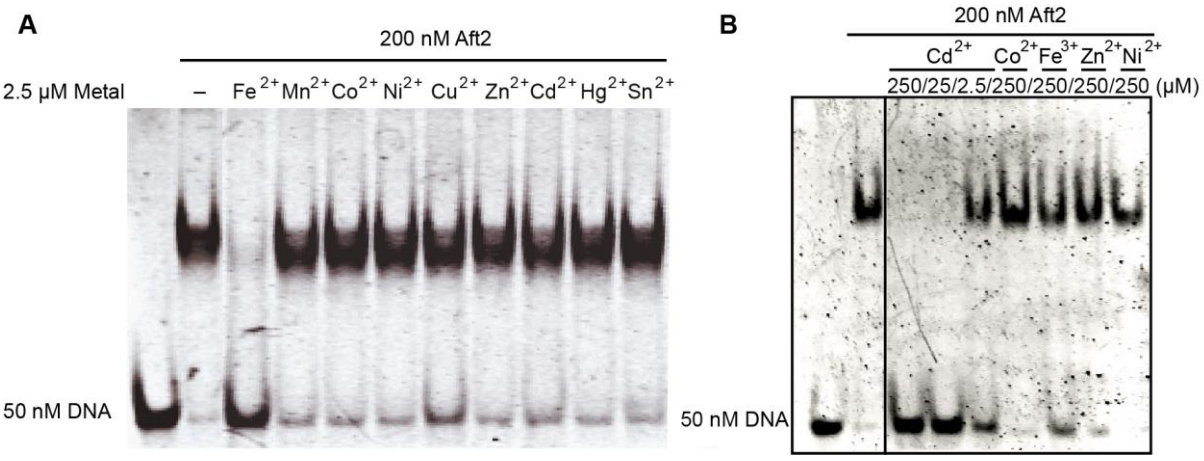


Fig. S8. DNA-binding activity of Aft2 in the presence of metal ions. The electrophoretic mobility shift assay was performed with 50 nM *FET3* promoter sequence, 200 nM Aft2 and indicated concentrations of metals. Addition of Fe^{2+} (*A*) or higher concentrations of Cd^{2+} (*B*) disrupts Aft2 binding to DNA.

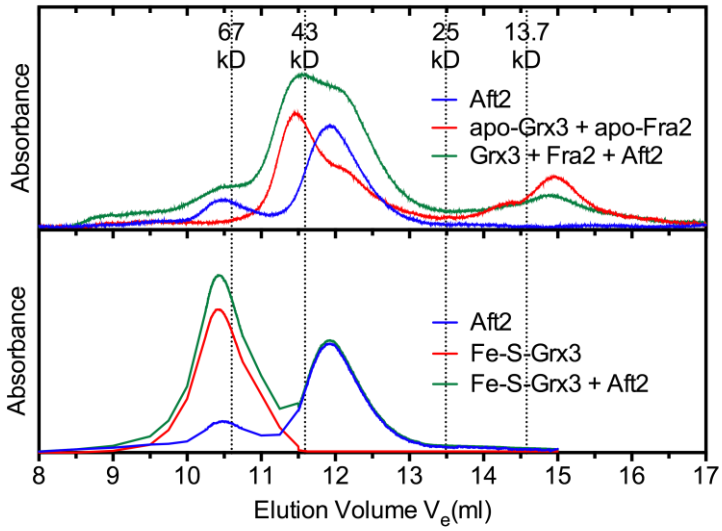


Fig. S9. *Top*, Gel filtration chromatograms of Aft2 (blue), apo-Grx3 + apo-Fra2$^{\Delta 1\text{-}35}$ (red), and Aft2 + apo-Grx3 + apo-Fra2$^{\Delta 1\text{-}35}$ (green). *Bottom*, Gel filtration chromatograms of Aft2 (blue), [2Fe-2S]-Grx3 (red), and Aft2 + [2Fe-2S]-Grx3 (green). The elution positions of molecular mass standards used for column calibration are shown as dotted lines. Addition of Aft2 does not alter the elution profiles of the apo proteins or [2Fe-2S]-Grx3.

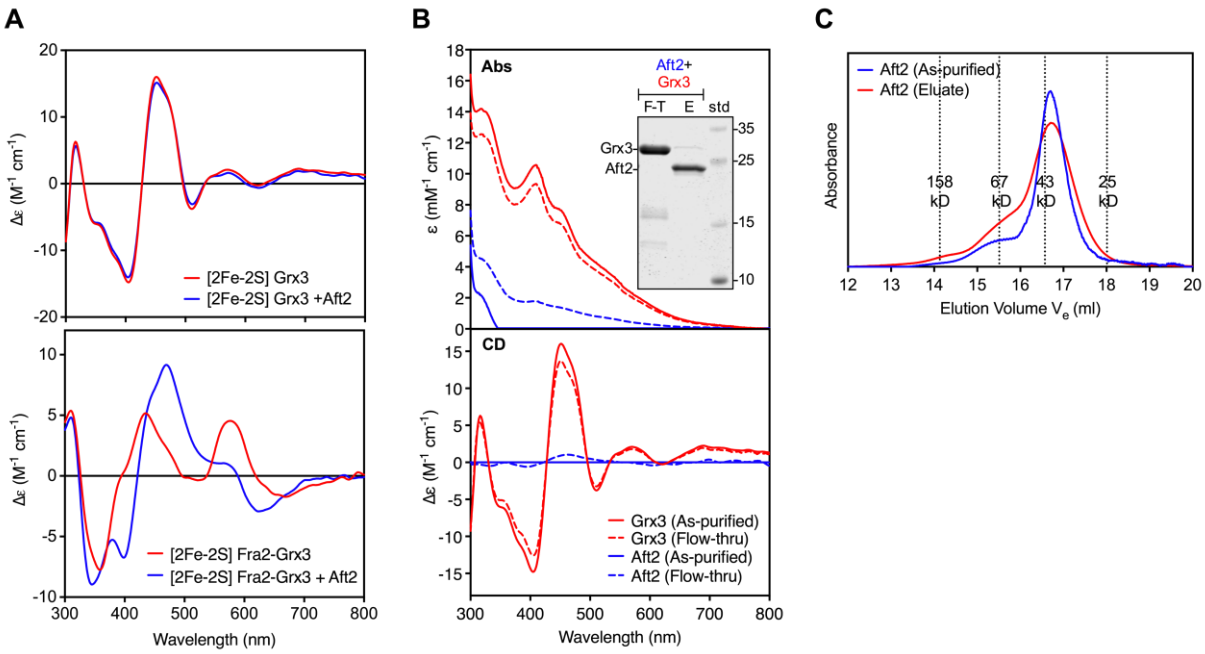


Fig. S10. Interaction between Aft2 and [2Fe-2S]-Grx3 homodimer. (*A*) UV-visible CD spectra of [2Fe-2S] Grx3 versus [2Fe-2S] Grx3 + Aft2 (*top*) and [2Fe-2S] Fra2-Grx3 versus [2Fe-2S] Fra2-Grx3 + Aft2 (*bottom*). [2Fe-2S] cluster concentration was 40 µM while [Aft2]:[2Fe-2S] ratio was 2:1. Δε value is normalized to [2Fe-2S] cluster concentration. (*B*) UV-visible absorption (*top*) and CD (*bottom*) spectra of heparin column flow-through (red dotted line) and eluate (blue dotted line), as compared to as-purified [2Fe-2S] Grx3 (red line) and Aft2 (blue line). ε and Δε values are normalized to Grx3 homodimer or Aft2 homodimer concentrations. *Inset*: SDS-PAGE analysis of the heparin fractions collected. (*C*) Gel filtration chromatograms of as-purified Aft2 and eluate of [2Fe-2S] Grx3 + Aft2 after heparin separation. Dotted lines correspond to the elution positions for selected standard proteins: chymotrypsinogen A (25 kD), ovalbumin (43 kD), albumin (67 kD), and aldolase (158 kD).

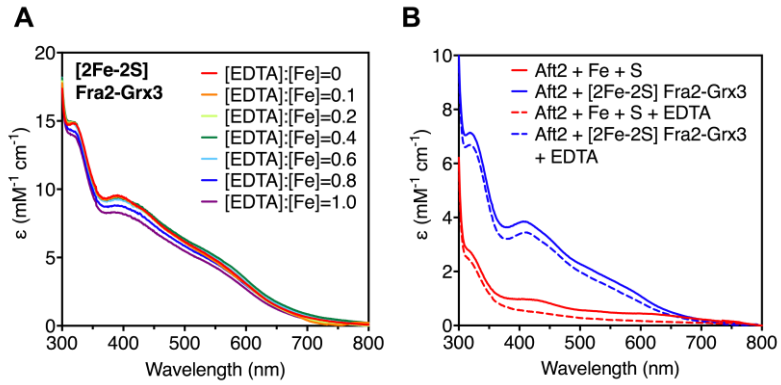
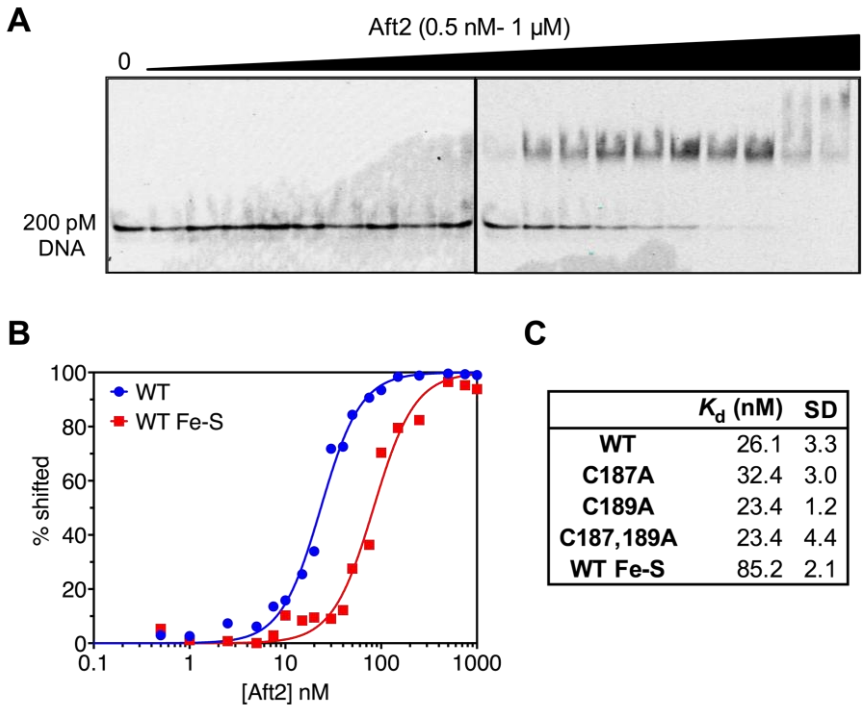


Fig. S11. [2Fe-2S] Fra2-Grx3 transfers an Fe-S cluster to Aft2 without cluster disassembly and reassembly. (*A*) Stability test on [2Fe-2S] Fra2-Grx3 at different [EDTA]:[Fe] ratios (0 to 1) monitored by UV-visible absorption spectroscopy. [2Fe-2S] cluster concentration was kept at 40 µM for all samples. ε value is normalized to Fra2-Grx3 heterodimer concentration. (*B*) UV-visible absorption spectra of heparin column eluates of Aft2 + [2Fe-2S] Fra2-Grx3 in the absence (blue line) or presence (blue dotted line) of EDTA as compared to Aft2 + equivalents amounts of Fe^{2+} + S^{2-} in the absence (red line) or presence (red dotted line) of EDTA. [Fe^{2+}], [S^{2-}] and [EDTA] were 80 µM, with 2:1 [Aft2]:[Fe^{2+}] ratio. ε value is normalized to Aft2 homodimer concentration.



C

	K_d (nM)	SD
WT	26.1	3.3
C187A	32.4	3.0
C189A	23.4	1.2
C187,189A	23.4	4.4
WT Fe-S	85.2	2.1

Fig. S12. Measurements of DNA binding affinity for as-purified Aft2 and Fe-S loaded Aft2. Electrophoretic mobility shift assays were performed with 200 pM *FET3* promoter DNA using 0.5-1 μM as-purified WT, C187A, C189A, and C187,189A (double mutant) Aft2, as well as WT Aft2 loaded with a [2Fe-2S] cluster transferred from Fra2-Grx3. (*A*) Representative EMSA experiment for as-purified WT Aft2. (*B*) Representative DNA binding curves for as-purified and Fe-S cluster loaded WT Aft2. We note that it is difficult to efficiently separate [2Fe-2S]-loaded Aft2 from monomeric Aft2 prior to the EMSA assay. Consequently, the [2Fe-2S]-Aft2 sample used for the assay is only 50-60% loaded with cluster and thus has residual monomeric protein that may account for some of the DNA binding activity. (*C*) Dissociation constants (K_d) and standard deviation (SD) measured for the indicated forms of Aft2. Values shown are the average from 2-5 independent experiments.