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## Tfam, a mitochondrial transcription and packaging factor, imposes a U-turn on mitochondrial DNA

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### SUMMARY

Tfam, a DNA binding protein with tandem HMG (high mobility group)-box domains, plays a central role in expression, maintenance, and organization of the mitochondrial genome. It activates transcription from mitochondrial promoters and organizes the mitochondrial genome into nucleoids. Using X-ray crystallography, we show that human Tfam forces promoter DNA to undergo a U-turn, reversing the direction of the DNA helix. Each HMG-box domain wedges into the DNA minor groove to generate two kinks on one face of the DNA. On the opposite face, a positively charged  $\alpha$ -helix serves as a platform to facilitate DNA bending. The structural principles underlying DNA bending converge with those of the unrelated HU family proteins, which play analogous architectural roles in organizing bacterial nucleoids. The functional importance of this extreme DNA bending is promoter-specific and appears related to the orientation of Tfam on the promoters.

### Keywords

Tfam; HMG-box; transcriptional activation; mtDNA; nucleoid

### INTRODUCTION

Among eukaryotic protein complexes, the mitochondrial oxidative phosphorylation (OXPHOS) machinery is unique in having a bigenomic origin. Most of the OXPHOS

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#### Supplementary Information

Supplemental Information includes Supplementary Fig. 1–6 and Supplementary Methods.

**Accession codes.** Protein Data Bank: Atomic coordinates and structure factors for the Tfam–mtDNA complex have been deposited under the accession code 3TMM.

#### Author Contributions

H.B.N. and D.C.C. designed the experiments, analyzed the data, and wrote the paper. H.B.N. carried out the crystallography and performed the experimental work. J.T.K. helped with the crystallographic analysis.

#### Competing financial interests

None

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machinery is encoded by the nuclear genome, but 13 essential subunits of respiratory chain complexes I, III, IV, and V are encoded by the 16 kb mitochondrial genome. Tfam (transcription factor A, mitochondrial; also known as mtTFA), a DNA binding protein in mitochondria, is a central player in expression and maintenance of mitochondrial DNA (mtDNA), and therefore is essential for ATP production via OXPHOS<sup>1, 2</sup>. The mammalian mitochondrial genome contains three promoters--the light strand promoter (LSP), the heavy strand promoter 1 (HSP1), and the heavy strand promoter 2 (HSP2)--that drive expression of mtDNA transcripts. Transcription from LSP and HSP1 has been reconstituted *in vitro*, and normal levels of transcription require Tfam<sup>1-4</sup>. Moreover, because truncated RNA transcripts from LSP are used to prime DNA replication, Tfam is secondarily essential for mtDNA replication. Mice lacking *Tfam* therefore show impaired mtDNA transcription and loss of mtDNA, leading to bioenergetic insufficiency and embryonic lethality<sup>5</sup>.

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Upstream of both the LSP and HSP1 transcriptional start sites, Tfam recognizes a binding site that has been defined by DNase I footprinting experiments<sup>3, 4</sup>. Tfam contains two HMG-box domains followed by a short C-terminal tail<sup>6</sup>. HMG-box domains are DNA binding motifs that bind to the minor groove of DNA and, in some cases, result in DNA bending<sup>7</sup>. Tfam belongs to the HMG-box subgroup that contains tandem HMG-box domains<sup>7</sup>. Several proteins in this subgroup, like Tfam, play important structural roles in DNA organization, but there is currently no information about how two HMG-box domains can be spatially coordinated to affect DNA structure. The C-terminal tail of Tfam is essential for transcriptional activation<sup>8</sup> and also for its physical association with Tfb2m<sup>9</sup>, another transcription factor required for mtDNA transcription. As a result, it has been proposed that Tfam binding allows recruitment of Tfb2m via the C-terminal tail.

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In addition to its transcriptional function, Tfam is thought to play a major role in mtDNA packaging<sup>10, 11</sup>. Although Tfam functions as a sequence-specific transcription factor, it also has high affinity for non-specific DNA. Unlike nuclear DNA, mtDNA is not associated with histones. mtDNA genomes within the mitochondrial matrix are organized into compact DNA-protein complexes termed nucleoids<sup>12</sup>. Tfam is one of the most abundant proteins associated with mtDNA nucleoids<sup>13</sup> and its levels have been estimated to be sufficient to coat the entire mitochondrial genome<sup>14</sup>. The levels of Tfam correlate with the levels of mtDNA<sup>15</sup>. The yeast ortholog of Tfam, Abf2, has no role in transcription and its major function is thought to be in organization of the mitochondrial genome<sup>16</sup>.

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To understand how Tfam mediates these multiple functions on mitochondrial DNA, we have solved the structure of human Tfam in complex with the LSP binding site. The structure shows how Tfam coordinates its two HMG-box domains to impose a dramatic U-turn on the DNA. To bend DNA, Tfam utilizes structural principles similar to that found in the HU family of prokaryotic nucleoid proteins, which, like Tfam, play architectural roles in genome organization. Moreover, we find this DNA bending is more important for transcriptional activation at LSP than HSP1.

## RESULTS

### Structure determination

We solved the 2.5 Å crystal structure of human Tfam bound to a 28 bp DNA fragment derived from LSP (Table 1 and Fig. 1a–d). The recombinant Tfam used (residues 43–246) represents the full-length, mature Tfam after cleavage of the N-terminal mitochondrial leader sequence<sup>6</sup>. The DNA fragment includes a ~22 bp sequence identified as a high-affinity Tfam binding site by DNase footprinting and has 2 half-sites that interact with the HMG-box domains<sup>3</sup>. A selenomethionine-substituted Tfam–mtDNA complex was used for structure determination at 2.5 Å by multi-wavelength anomalous diffraction (MAD) analysis. The crystallographic statistics of data collection and refinement are presented in Table 1. The electron density map was of sufficient quality to build almost all of the protein (residues 43–237) and all 28 base pairs of mtDNA. Model building and refinement produced a final structure with excellent stereochemistry, with an  $R_{\text{free}}$  of 24.7% and an  $R_{\text{work}}$  of 19.8%.

This crystal structure is the first one of a native tandem HMG-box protein in complex with DNA. In a previous study<sup>17</sup>, the NMR structure was solved of a chimeric molecule consisting of the HMG-box domain of SRY (a single HMG-box protein) fused to one of the two HMG-box domains of HMGB1 (a tandem HMG-box protein). This artificial molecule is nonphysiological, and its structure in complex with DNA does not resemble the structure described here.

### Tfam imposes a severe bend on LSP mtDNA

The most striking feature of the structure is that binding of a Tfam monomer dramatically distorts the DNA into a U-shape, causing a reversal in the direction of the DNA helical axis (Fig. 1c, d). Each HMG-box folds into a 3-helix motif whose concave surface intercalates between the bases in the minor groove of an LSP half-site (Fig. 1c). These two intercalations result in two sharp kinks on one face of the DNA helix. The buried contact area of the first HMG-box domain (box A) with DNA is 1566 Å<sup>2</sup>, and the corresponding surface area of the second HMG-box (box B) is nearly as extensive at 1404 Å<sup>2</sup> (Fig. 1e, f). The linker connecting the two HMG-box domains forms an  $\alpha$ -helix around which the DNA wraps (contact area 864 Å<sup>2</sup>) (Fig. 1c, d, and f). As described in detail later, basic side chains in the linker interact with the negatively charged phosphates in the bent DNA backbone. The C-terminal tail also contacts DNA (580 Å<sup>2</sup>) and the first part of this region extends the third helix of the second HMG-box domain. Therefore, all four regions (Fig. 1a) of Tfam--the two HMG-box domains, the linker, and C-terminal tail--make extensive contact with the DNA.

The structure agrees well with previous DNase I footprinting and methylation interference experiments probing the binding of Tfam to LSP DNA<sup>3, 18</sup>. The Tfam monomer accounts for the large recognition site identified by a combination of DNase I footprinting and sequence analysis<sup>3, 4, 8</sup>. Each HMG-box domain binds to one of the two half-sites identified by sequence analysis<sup>8</sup>. In previous methylation interference experiments<sup>18</sup>, the methylation by dimethylsulfate (DMS) of selected adenines was associated with reduced binding of Tfam. DMS methylates adenine at the N3 atom, which is located within the minor groove

and would sterically block subsequent Tfam binding. In our crystal structure, all the adenines identified by Clayton and colleagues<sup>18</sup> reside in a position where Tfam contacts the DNA minor groove and causes widening (Supplementary Fig. 1). In contrast, methylation of adenines located outside the contact area did not affect Tfam binding.

Our crystal structure indicates that Tfam binds mtDNA as a monomer. Human Tfam without DNA is monomeric, but it has been suggested that Tfam assembles into dimers upon DNA binding<sup>19</sup>. The latter conclusion is tenuous, because it was based on a gel mobility assay that used extremely high concentrations of Tfam and DNA and does not give a definitive assessment of stoichiometry. To independently test the 1:1 stoichiometry found in our crystal structure, we analyzed Tfam and the Tfam–mtDNA complex in solution by size exclusion chromatography with in-line multi-angle light scattering analysis (SEC-MALS). The measured molar masses indicated that Tfam in isolation is monomeric and when complexed with DNA, forms a 1:1 complex (Fig. 2).

### Protein-DNA interactions

As in other HMG-box structures, each Tfam HMG-box domain folds into an L-shape composed of 3  $\alpha$ -helices, with the third helix forming the long axis (Fig. 1c). A hydrophobic core composed of Tyr57, Phe60, Trp88, and Tyr99 stabilizes the L-shaped configuration of the first HMG-box (Fig. 3a; Supplementary Fig. 2). Similarly, buried residues Tyr162, Tyr165, Trp189, and Tyr200 stabilize the second HMG-box domain (Fig. 3b; Supplementary Fig. 2). The overall fold of both HMG-box domains superimpose well with other HMG-boxes whose structures in complex with DNA have been solved (Fig. 3c).

In the Tfam–mtDNA complex, most of the side-chain–DNA contacts are not sequence-specific and occur on the sugar-phosphate backbone of the DNA. However, a small number of contacts to bases within the minor groove can be seen. HMG-box domains generally contain 1 or 2 hydrophobic residues that intercalate into the minor groove (highlighted in Supplementary Fig. 3). Consistent with this generalization, the HMG-box A of Tfam contains the first of these intercalating residues at position 58 (Leu58), which interacts with A8 (strand B) (Fig. 3d, red residue). A previous crystal structure of the isolated, HMG-box B of Tfam raised the issue of whether it was a non-canonical HMG-box domain with unusual binding properties<sup>19</sup>, because it appeared to lack both intercalating hydrophobic residues. Our Tfam–mtDNA structure clarifies this issue by showing that HMG-box B does contain DNA binding residues at these same positions, even though the residues are not nonpolar. In the first position, HMG-box B contains Asn163, which reaches into the minor groove and contacts the underlying thymine (T7, strand A). In the second position, Pro178 similarly inserts into the minor groove and contacts a guanine (G9, strand A) (Fig. 3e, red residues). In comparison to the previous structure of HMG-box B without DNA<sup>19</sup>, Pro178 has shifted  $>2$  Å to make this contact with the DNA base.

Besides the interactions indicated above, several other contacts to DNA bases are apparent. In HMG-box A, contacts are observed between Ile81 and T19 (strand A), Tyr57 and G20 (strand A), and Ser61 and G20 (strand A) (Fig. 3d; Supplementary Fig. 2c). In addition, Ser61 and Ser55 indirectly interact with C9 (strand B) and T21 (strand A), respectively, via water molecules. In the HMG-box B (Fig. 3e; Supplementary Fig. 2d), contacts are observed

between Arg157 and T24 (strand B), and Gln179 and C19 (strand B). The linker does not directly interact with DNA bases. However, it makes substantial contacts with DNA via charged or polar interactions (Fig. 3f, g). Lys147 contacts G16 (strand A). His137 and Arg140 both make contacts to the phosphate backbone. Other lysine residues in the linker region (Lys136, Lys139, and Lys146) make longer-range contacts ( $>3.35\text{\AA}$ ) with the sugar-phosphate backbone.

### Similarity to HU and IHF nucleoid proteins

The conformations of the two half-sites bound by Tfam deviate substantially from canonical B-DNA (Fig. 4a–d). At each location, intercalation by the HMG-box results in substantial widening of the minor groove (Fig. 4a). There is local DNA unwinding, as indicated by sharp minima in the base twist (base step parameter) at the sites of intercalation (Fig. 4c). Globally, however, the DNA is not underwound, with an average helical twist of  $\sim 36^\circ$ . The roll angle profile (Fig. 4b) shows two sharp peaks, which reflect distortions of base stacking due to acute DNA bending.

The mode of DNA bending in the Tfam–mtDNA structure shows remarkable parallels with the HU protein family, which consists of DNA minor groove binding proteins that play architecture roles in prokaryotic DNA nucleoids<sup>20, 21</sup>. Integration host factor (IHF), HU, and Hbb are HU family proteins that contort their bound DNA into a U-shape<sup>20, 21</sup>. These proteins form dimers in which each subunit uses a "β-ribbon arm" to intercalate into the DNA minor groove (Fig. 4a, e). The dimerization interface between the two subunits is rich in positive residues and serves to neutralize the negative charges of the bent DNA backbone. The DNA fragments in the Tfam and Hbb complexes show similar profiles in the minor groove width, with two broad peaks corresponding to minor groove intercalations (Fig. 4a). The roll angles also show two peaks that signify the sharp bending of DNA. The peaks are slightly closer together in the Hbb ( $\sim 9$  bps apart) versus the Tfam structure (Fig. 4b). Superimposition of the DNA fragments reveals the similarity in overall geometry (Fig. 4f).

### Both HMG-boxes and the linker are critical for DNA bending

To monitor DNA bending by Tfam, we developed a FRET (fluorescence resonance energy transfer)-based assay. The crystal structure shows that after binding of Tfam, the ends of the 28 bp LSP DNA fragment are brought to within  $55\text{\AA}$  (measuring from the 5' phosphate of one strand to the 5' phosphate of the other strand), compared to the  $95\text{\AA}$  separation found in a rod-like DNA fragment of identical length. To construct the FRET sensor, Cy3 (donor) and Cy5 (acceptor) fluorophores were covalently attached to opposite ends of the LSP fragment. Addition of Tfam to the labeled, double-stranded DNA resulted in a dose-dependent increase in acceptor emission and a decrease in donor emission (Table 2; Supplementary Fig. 4a, b). Control experiments confirmed that the acceptor emission depended on the presence of both the donor fluorophore and Tfam. The maximum FRET efficiency measured with wild-type Tfam corresponds to a calculated fluorophore separation of  $59\text{\AA}$ , in good agreement with the crystal structure. The ability of Tfam to bend DNA is not restricted to the LSP template. Tfam was able to bend a DNA template lacking promoter sequences, and also one corresponding to HSP1 (Supplementary Fig. 4c, d).

Analysis of a panel of Tfam mutants indicated that coordinated binding of both HMG-box domains is important for effective bending of DNA (Table 2). HMG-box A alone bound to LSP DNA with the same affinity as full-length Tfam, consistent with previous studies<sup>19, 22</sup>. However, it showed a large reduction in DNA bending. HMG-box B alone showed much weaker affinity for LSP DNA ( $K_D \sim 400$  nM) and also showed a large reduction in DNA bending. In addition, we tested Tfam mutants with single point mutations in HMG-box residues that contact DNA (Table 2; Supplementary Fig. 4b). T77A, containing a mutation in HMG-box A, and Y162A, containing a mutation in HMG-box B, showed moderate reductions in DNA bending. Each of these residues makes contacts with the DNA backbone (Fig. 3d, e). Finally, we tested the effect of mutations in the positively charged residues in the linker helix. Single point mutations had little effect (data not shown). We therefore made a mutant, L6, in which 6 positively charged residues in the linker region were replaced by alanine. The L6 mutant showed >30% reduction in FRET, indicating that the linker region is important for DNA bending. All these mutants were well-folded, based on secondary structure analysis by circular dichroism (Supplementary Fig. 4e). In addition, the T77A, Y162A, and L6 mutants retained high affinity for DNA, as indicated by an assay monitoring the quenching of intrinsic tryptophan fluorescence upon DNA binding (Table 2; Supplementary Fig. 5).

### Tfam bending mutants show promoter-specific defects

With mutants that reduce DNA bending by Tfam, we used *in vitro* transcription reactions containing mitochondrial RNA polymerase (Polrmt) and Tfb2m to test whether DNA bending is important for its transcriptional activation function (Fig. 5). Neither HMG-box A nor HMG-box B alone was able to activate transcription from LSP or HSP templates. In addition, Tfam containing both HMG-boxes but lacking the C-terminal tail was unable to activate transcription (Fig. 5a, b). These results are expected, because previous studies indicated that the C-terminal tail of Tfam is essential for transcriptional activation<sup>8</sup>. Interestingly, we found that both the T77A and the Y162A mutants were less efficient in promoting transcription from the LSP template. Y162A, which has a more severe bending defect, was more affected. Finally, the mutant L6, which has the strongest bending defect, showed a severe defect in transcriptional activation. The transcriptional defects were similar whether full-length or truncated LSP transcripts were quantified (Fig. 5c, d).

To test whether these Tfam mutants were generally defective in transcriptional activation, we examined their activity with an HSP1 template (Fig. 5b, e). In DNA bending measurements, these mutants showed defects in bending the HSP1 DNA template (Supplementary Fig. 4d), as was found previously with the LSP template. In transcriptional activation assays, however, the Y77A, Y162A, and L6 mutants were all efficient at stimulating transcripts from HSP1 (Fig. 5e). Quantification showed that all three mutants showed a similar transcriptional activation profile compared to wildtype Tfam.

## DISCUSSION

Based on previous structural studies, a single HMG-box domain can bind to the DNA minor groove and sometimes cause bending of the DNA double helix. For example, the

prototypical HMG-box protein Sry, which contains a single HMG-box domain, bends DNA  $\sim 70\text{--}80^\circ$  upon binding to the minor groove<sup>23</sup>. This mode of DNA bending (Fig. 6a) superficially resembles that of the TATA binding protein Tbp, where binding of a  $\beta$ -sheet to the DNA minor groove again induces moderate bending towards the opposite direction<sup>24, 25</sup>.

In comparison to these structures, the Tfam–mtDNA complex illustrates how spatial coordination of tandem HMG-box domains can be harnessed to impose even more extreme distortion onto DNA (Fig. 6b). Tfam belongs to the subset of HMG-box proteins that contain tandem HMG-box domains. These HMG-box proteins generally show broad DNA binding and play important roles in regulating chromatin structure and function<sup>7</sup>. For example, Hmgb1 is an architectural protein on chromatin that has been implicated in transcription, chromatin organization, and genome stability<sup>26</sup>. In Tfam, the  $\alpha$ -helical linker plays a key role by spatially coordinating the two HMG-box domains, so that they bind the DNA minor groove at sites located approximately one helical turn apart. Moreover, the linker further facilitates DNA bending by neutralizing the negative charges on the DNA backbone. Intriguingly, all the other dual HMG-box proteins in the human genome contain a cluster of 5–8 positively charged residues in the short region between the HMG-box domains (Supplementary Fig. 6). It will be interesting to determine whether these residues play a role analogous to that of the linker region in Tfam.

Although Tfam and the HU family of nucleoid proteins do not share sequence or structural homology, our studies indicate that they use remarkably analogous strategies to impose extreme bending onto DNA (Fig. 6b). The similarities between the Tfam–DNA and HU-family–DNA structures are intriguing, given that both proteins are thought to control the architecture of DNA in nucleoids. The DNA in our structure is from LSP and therefore is more directly related to mitochondrial transcriptional activation. However, the structure is likely to also be relevant for the role of Tfam in nucleoid organization, given the ability of Tfam to bend generic DNA (Supplementary Fig. 4c).

Our results indicate that the relative importance of extreme DNA bending by Tfam depends on the mitochondrial promoter. Previous studies indicated that the C-terminal tail of Tfam is essential for transcriptional activation<sup>8</sup> and physical interaction with Tfb2m<sup>9</sup>. In the crystal structure, when Tfam is bound to the LSP promoter, the HMG-box B domain binds at the half-site further upstream from the transcription start site (Fig. 1c, 6c). Without DNA bending, the C-terminal tail would face away from the transcriptional start site (Fig. 6d). However, the DNA U-turn redirects the C-terminal tail towards the transcriptional machinery (Fig. 6c). We speculate that one of the functions of DNA bending by Tfam is to enable the C-terminal tail to interact with the rest of transcriptional machinery. Based on previous results<sup>9</sup>, Tfb2m is a favored candidate for such an interaction. Remarkably, transcription from HSP1 is much less sensitive to DNA bending by Tfam. Based on sequence analysis, the Tfam binding sites in HSP1 versus LSP are in reverse orientations relative to the direction of transcription (Fig. 1b)<sup>4, 8</sup>. When Tfam is bound to the HSP1 promoter, HMG-box B would be expected to bind the half site adjacent to the transcriptional start. The C-terminal tail is therefore in proximity to the transcriptional machinery, regardless of whether the DNA is bent or not. In future studies, it will be important to test this proposal by determining the structure of Tfam in complex with HSP1 promoter DNA.

## Methods

### Tfam purification

The human *TFAM* gene was cloned into the pET28a expression vector (Novagen) between the BamHI and XhoI sites. This construct encodes residues 43–246, corresponding to full-length Tfam after cleavage of the N-terminal mitochondrial leader sequence (residues 1–42). Tfam mutants were constructed by PCR using oligonucleotides encoding mutations. Plasmids were transformed into BL21 (DE3) *E. coli* (Invitrogen). LB broth (20 ml) containing 50  $\mu\text{g ml}^{-1}$  kanamycin was inoculated with a single colony and grown overnight at 37°C. The overnight culture was diluted to 4 liters and grown until an OD<sub>600</sub> of 1.0. After induction with 1 mM isopropyl  $\beta$ -D-1-thiogalactopyranoside, the culture was grown overnight at room temperature. The cells were harvested and stored at –80 °C. Five grams of cells were resuspended in 50 ml lysis buffer (20 mM Tris-HCl, 500 mM NaCl, pH 7.5) and sonicated for 5 min (10 s on and 20 s off) on ice. After centrifugation at  $4.3 \times 10^4 g$  for 30 min at 4 °C, His-tagged Tfam was purified from the supernatant with 3 mls of Talon Cobalt resin (Clontech). The protein was eluted (20 mM Tris-HCl, 500 mM NaCl, 300 mM imidazole, pH 7.5) and further purified by gel filtration chromatography using a Hi-Load Superdex 200 16/60 column (GE Healthcare) pre-equilibrated with running buffer (20 mM Tris-HCl, 300 mM NaCl, 1 mM DTT, pH 7.5) in an AKTA Purifier (Amersham). The peak fraction was collected and concentrated to 17–20 mg ml<sup>-1</sup> using Amicon Ultra-15 concentrators (Millipore) with a molecular weight cutoff of 10 kDa. The protein was flash-frozen in liquid nitrogen and stored at –80 °C. Selenomethionine-substituted Tfam was produced by the metabolic inhibition method<sup>34</sup>, and preparative buffers contained 5 mM  $\beta$ -mercaptoethanol instead DTT. Proteins were analyzed by DNA binding and circular dichroism analysis, as detailed in the Supplementary Methods.

### Crystallization, data collection, and structure determination

The duplex LSP fragment was made by annealing complementary oligonucleotides (5'-TGTTAGTTGGGGGGTACTGTAAAAGT-3' and 5'-ACTTTTAACAGTCACCCCCCACTAACA-3') in buffer (10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA) at a concentration of 0.9 mM. The mixture was incubated at 95°C for 5 min, 75°C for 5 min, and room temperature >5 h.

To form Tfam–mtDNA complexes, Tfam was mixed with duplex DNA in a 1.3:1 molar ratio. The mixture was incubated at room temperature for 30 min and then on ice for 2 h. Crystallization trials by hanging drop-vapor diffusion at room temperature identified a condition [29% PEG 400 (w/v), 0.15 M calcium acetate, 0.1 M sodium acetate (pH 4.2), 400 mM NDSB211 (dimethyl-2-(hydroxyethyl)-(3-sulfopropyl)-ammonium)] that yielded rod-shaped crystals. Diffraction data were collected on frozen crystals on beamline 12-2 at the Stanford Synchrotron Radiation Lightsource. All data were processed with IMOSFLM<sup>35</sup> or XDS<sup>36</sup>, and merged using SCALA<sup>37</sup> as implemented in CCP4<sup>38</sup>. A selenomethionine-substituted-Tfam–mtDNA complex was used for phasing. Using intensity data at 3.0 Å from three wavelengths, all five selenium sites were located by PHENIX<sup>39</sup>. After solvent flattening and density modification in PHENIX, the map revealed clear density for the protein and DNA. Manual model building in COOT<sup>40</sup> using the 3.0 Å experimental map



generated a starting model. Refinement of the best solutions was carried out using PHENIX, with an initial round of rigid body refinement followed by a round of simulated annealing. Refinement against a 2.5 Å data set produced an excellent map with density for most of the side chains. After a few rounds of model adjustment and refinement with TLS obtained from the TLSMD server<sup>41</sup>, the  $R_{\text{work}}$  converged to 19.8% and the  $R_{\text{free}}$  to 24.7%. The final model includes residues 43–237 of Tfam and all the nucleotides. The current model has excellent stereochemistry with no Ramachandran outliers, as assessed by MOLPROBITY<sup>42</sup>.

### FRET experiments

To generate LSP, HSP, and non-promoter templates, the following complementary oligonucleotides were annealed as described above:

LSP: 5'-Cy3-TGTTAGTTGGGGGGTACTGTAAAAGT-3' and 5'-Cy5-  
ACTTTTAACAGTACCCCCCACTAACA-3'

HSP1: 5'-Cy3-GGTTGGTTCGGGGTATGGGGTTAGCAGC-3' and 5'-Cy5-  
GCTGCTAACCCCATACCCGAACCAACC-3'

Non-promoter DNA: 5'-Cy3-GACATTGGAACACTATACCTATTATTCG-3' and 5'-  
Cy5-CGAATAATAGGTATAGTGTCCAATGTC-3'

Additional details of the FRET measurements and analysis of the FRET data are described in the Supplementary Methods.

### Size-exclusion chromatography with multi-angle light scattering (SEC-MALS)

SEC-MALS experiments were performed at room temperature by loading samples on a Shodex KW 803 column with a Dawn Heleos MALS detector (Wyatt Technology). The column was eluted with buffer containing 20 mM Tris-HCl (pH7.5), 300 mM NaCl, and 1 mM DTT. A  $dn/dc$  (refractive index increment) value of 0.185 mL  $mg^{-1}$  was used. Bovine serum albumin was used as an isotropic scatterer for detector normalization. The light scattered by a protein is directly proportional to its weight-average molecular mass and concentration.

### *in vitro* transcription reactions

DNA fragments corresponding to LSP (positions 1–477) and HSP1 (positions 499–741) of human mtDNA were cloned into the pSP65 vector at the BamHI and SalI sites. After digestion with BamHI for LSP and SalI for HSP1, the linearized plasmids were used as templates in a transcriptional run-off assay. Transcription reactions were carried out as described<sup>43</sup> with modifications. Template DNA (5 nM) was added to the reaction mix [10 mM HEPES (pH 7.5), 10 mM  $MgCl_2$ , 1 mM DTT, 100  $\mu g\ ml^{-1}$  BSA, and 40 units of RNaseOut (Invitrogen)] for 5 min, and then Tfam, Tfb2m (30 nM, Enzymax), and Polrmt (30 nM, Enzymax) were sequentially added, with a 1 min incubation between each addition. After addition of rNTPs [400  $\mu M$  rATP, 150  $\mu M$  rCTP, 150  $\mu M$  rGTP, 15  $\mu M$  rUTP (Promega), 0.2  $\mu M$  ( $\alpha$ -<sup>32</sup>P) rUTP (3,000 Ci  $mmol^{-1}$ , Perkin Elmer)], the reaction was incubated for 3 h at 33°C, and stopped by addition of 25  $\mu L$  of stop buffer (80% formamide (v/v), 10 mM EDTA, pH 8.0, 0.025% xylene cyanol (w/v), 0.025% bromophenol blue (w/v)). Samples were heated to 90 °C for 5 min and separated on 5% polyacrylamide gels (w/v).

containing 8 M urea in 1×TBE. The gels were fixed in 7% acetic acid (v/v), dried, and exposed to a phosphorimager screen. The data were collected on a Storm 880 phosphorimager (Molecular Dynamics), and quantified using ImageQuant 5.2 Software.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Main References

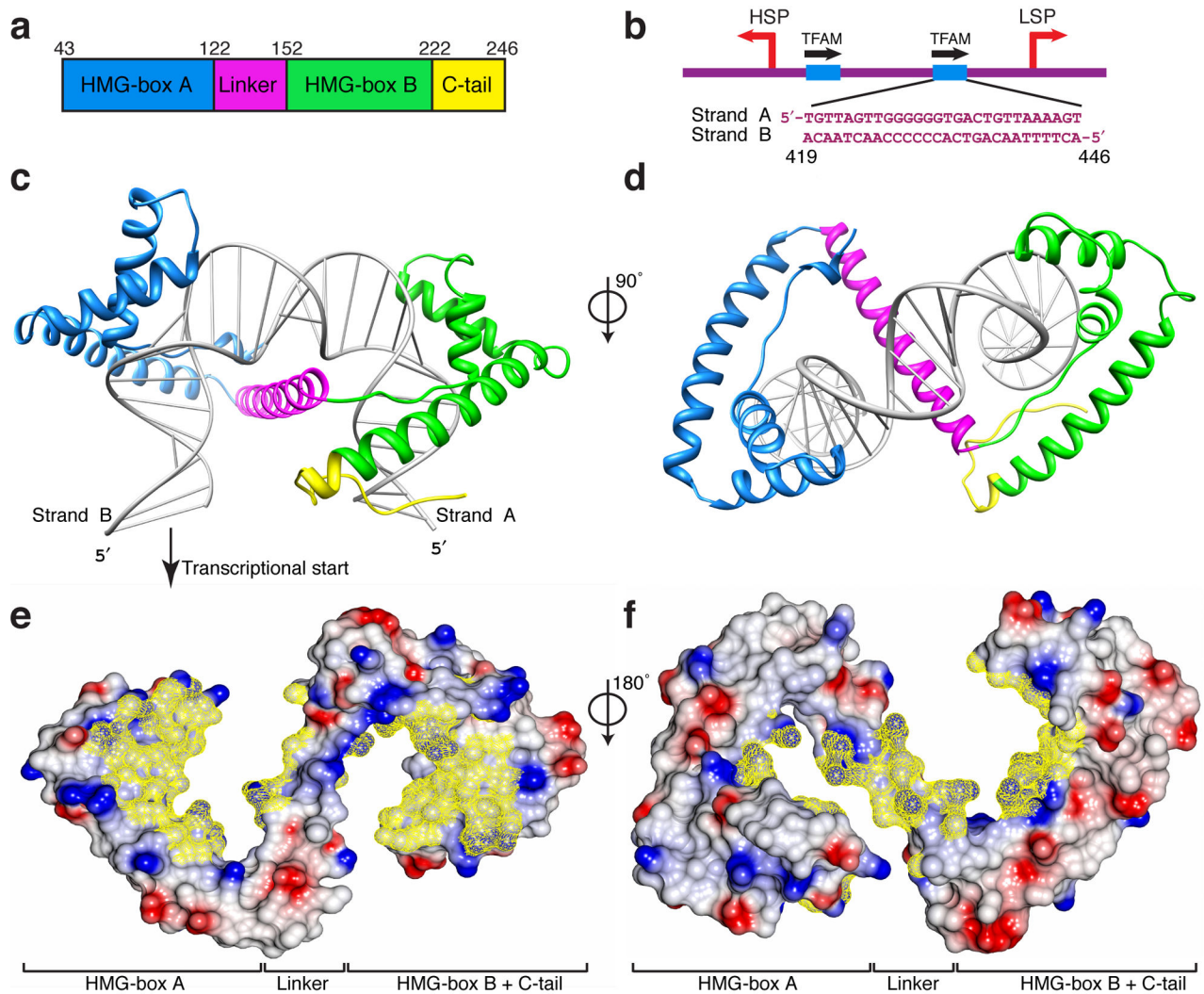
1. Falkenberg M, Larsson NG, Gustafsson CM. DNA replication and transcription in mammalian mitochondria. *Annu Rev Biochem.* 2007; 76:679–699. [PubMed: 17408359]
2. Bonawitz ND, Clayton DA, Shadel GS. Initiation and beyond: multiple functions of the human mitochondrial transcription machinery. *Mol Cell.* 2006; 24:813–825. [PubMed: 17189185]
3. Fisher RP, Clayton DA. Purification and characterization of human mitochondrial transcription factor 1. *Mol Cell Biol.* 1988; 8:3496–3509. [PubMed: 3211148]
4. Fisher RP, Topper JN, Clayton DA. Promoter selection in human mitochondria involves binding of a transcription factor to orientation-independent upstream regulatory elements. *Cell.* 1987; 50:247–258. [PubMed: 3594571]
5. Larsson NG, et al. Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat Genet.* 1998; 18:231–236. [PubMed: 9500544]
6. Parisi MA, Clayton DA. Similarity of human mitochondrial transcription factor 1 to high mobility group proteins. *Science.* 1991; 252:965–969. [PubMed: 2035027]
7. Stros M, Launholt D, Grasser KD. The HMG-box: a versatile protein domain occurring in a wide variety of DNA-binding proteins. *Cell Mol Life Sci.* 2007; 64:2590–2606. [PubMed: 17599239]
8. Dairaghi DJ, Shadel GS, Clayton DA. Addition of a 29 residue carboxyl-terminal tail converts a simple HMG box-containing protein into a transcriptional activator. *J Mol Biol.* 1995; 249:11–28. [PubMed: 7776365]
9. McCulloch V, Shadel GS. Human mitochondrial transcription factor B1 interacts with the C-terminal activation region of h-mtTFA and stimulates transcription independently of its RNA methyltransferase activity. *Mol Cell Biol.* 2003; 23:5816–5824. [PubMed: 12897151]
10. Kang D, Kim SH, Hamasaki N. Mitochondrial transcription factor A (TFAM): roles in maintenance of mtDNA and cellular functions. *Mitochondrion.* 2007; 7:39–44. [PubMed: 17280879]
11. Kaufman BA, et al. The mitochondrial transcription factor TFAM coordinates the assembly of multiple DNA molecules into nucleoid-like structures. *Mol Biol Cell.* 2007; 18:3225–3236. [PubMed: 17581862]
12. Spelbrink JN. Functional organization of mammalian mitochondrial DNA in nucleoids: history, recent developments, and future challenges. *IUBMB Life.* 2010; 62:19–32. [PubMed: 20014006]
13. Bogenhagen DF, Rousseau D, Burke S. The layered structure of human mitochondrial DNA nucleoids. *J Biol Chem.* 2008; 283:3665–3675. [PubMed: 18063578]
14. Alam TI, et al. Human mitochondrial DNA is packaged with TFAM. *Nucleic Acids Res.* 2003; 31:1640–1645. [PubMed: 12626705]

15. Ekstrand MI, et al. Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum Mol Genet.* 2004; 13:935–944. [PubMed: 15016765]
16. Kucej M, Kucejova B, Subramanian R, Chen XJ, Butow RA. Mitochondrial nucleoids undergo remodeling in response to metabolic cues. *J Cell Sci.* 2008; 121:1861–1868. [PubMed: 18477605]
17. Stott K, Tang GS, Lee KB, Thomas JO. Structure of a complex of tandem HMG boxes and DNA. *J Mol Biol.* 2006; 360:90–104. [PubMed: 16813837]
18. Fisher RP, Parisi MA, Clayton DA. Flexible recognition of rapidly evolving promoter sequences by mitochondrial transcription factor 1. *Genes Dev.* 1989; 3:2202–2217. [PubMed: 2628167]
19. Gangelhoff TA, Mungalachetty PS, Nix JC, Churchill ME. Structural analysis and DNA binding of the HMG domains of the human mitochondrial transcription factor A. *Nucleic Acids Res.* 2009; 37:3153–3164. [PubMed: 19304746]
20. Mouw KW, Rice PA. Shaping the *Borrelia burgdorferi* genome: crystal structure and binding properties of the DNA-bending protein Hbb. *Mol Microbiol.* 2007; 63:1319–1330. [PubMed: 17244195]
21. Rice PA, Yang S, Mizuuchi K, Nash HA. Crystal structure of an IHF-DNA complex: a protein-induced DNA U-turn. *Cell.* 1996; 87:1295–1306. [PubMed: 8980235]
22. Wong TS, et al. Biophysical characterizations of human mitochondrial transcription factor A and its binding to tumor suppressor p53. *Nucleic Acids Res.* 2009; 37:6765–6783. [PubMed: 19755502]
23. Werner MH, Huth JR, Gronenborn AM, Clore GM. Molecular basis of human 46X,Y sex reversal revealed from the three-dimensional solution structure of the human SRY-DNA complex. *Cell.* 1995; 81:705–714. [PubMed: 7774012]
24. Kim JL, Nikolov DB, Burley SK. Co-crystal structure of TBP recognizing the minor groove of a TATA element. *Nature.* 1993; 365:520–527. [PubMed: 8413605]
25. Kim Y, Geiger JH, Hahn S, Sigler PB. Crystal structure of a yeast TBP/TATA-box complex. *Nature.* 1993; 365:512–520. [PubMed: 8413604]
26. Stros M. HMGB proteins: interactions with DNA and chromatin. *Biochim Biophys Acta.* 2010; 1799:101–113. [PubMed: 20123072]
27. Ohndorf UM, Rould MA, He Q, Pabo CO, Lippard SJ. Basis for recognition of cisplatin-modified DNA by high-mobility-group proteins. *Nature.* 1999; 399:708–712. [PubMed: 10385126]
28. Love JJ, et al. Structural basis for DNA bending by the architectural transcription factor LEF-1. *Nature.* 1995; 376:791–795. [PubMed: 7651541]
29. Remenyi A, et al. Crystal structure of a POU/HMG/DNA ternary complex suggests differential assembly of Oct4 and Sox2 on two enhancers. *Genes Dev.* 2003; 17:2048–2059. [PubMed: 12923055]
30. Murphy, FVt; Sweet, RM.; Churchill, ME. The structure of a chromosomal high mobility group protein-DNA complex reveals sequence-neutral mechanisms important for non-sequence-specific DNA recognition. *EMBO J.* 1999; 18:6610–6618. [PubMed: 10581235]
31. Luscombe NM, Laskowski RA, Thornton JM. NUCPLOT: a program to generate schematic diagrams of protein-nucleic acid interactions. *Nucleic Acids Res.* 1997; 25:4940–4945. [PubMed: 9396800]
32. Lu XJ, Olson WK. 3DNA: a software package for the analysis, rebuilding and visualization of three-dimensional nucleic acid structures. *Nucleic Acids Res.* 2003; 31:5108–5121. [PubMed: 12930962]
33. Pham XH, et al. Conserved sequence box II directs transcription termination and primer formation in mitochondria. *J Biol Chem.* 2006; 281:24647–24652. [PubMed: 16790426]

## Methods-only references

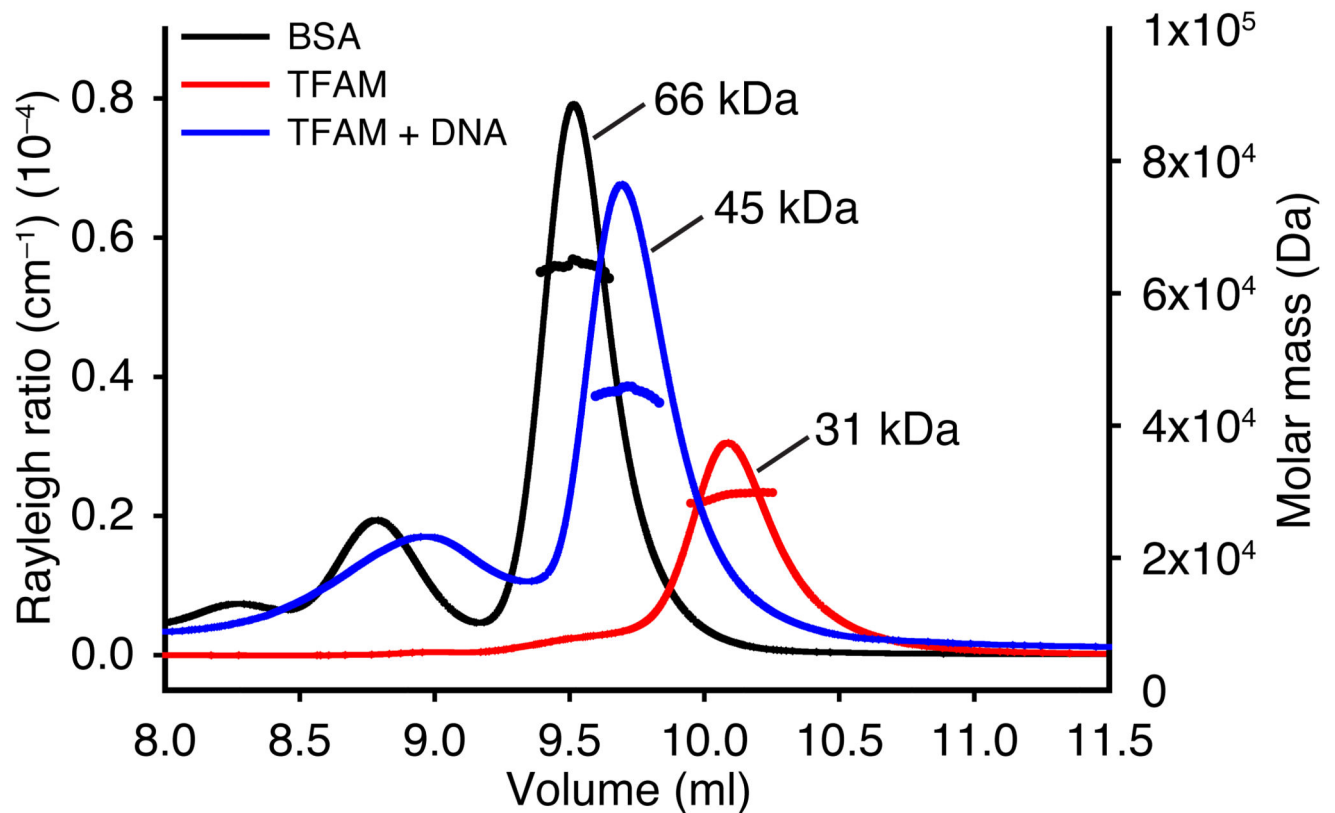
34. Van Duyne GD, Standaert RF, Karplus PA, Schreiber SL, Clardy J. Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *J Mol Biol.* 1993; 229:105–124. [PubMed: 7678431]
35. Leslie, AGW. Joint CCP4 and ESF-EACBM Newsletter on Protein Crystallography. Warrington WA4 4AD, UK: 1992.

36. Kabsch W. XDS. *Acta Cryst.* 2010; D66:125–132.
37. Evans, PR. Proceedings of the CCP4 Study Weekend. Data Collection and Processing. Sawyer, L.; Isaacs, N.; Bailey, S., editors. Warrington: Daresbury Laboratory; 1993. p. 114-122.
38. Collaborative Computational Project, N. The CCP4 Suite: Programs for Protein Crystallography. *Acta Cryst.* 1994; D50:760–763.
39. Adams PD, et al. PHENIX: building new software for automated crystallographic structure determination. *Acta Crystallogr D Biol Crystallogr.* 2002; 58:1948–1954. [PubMed: 12393927]
40. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr.* 2004; 60:2126–2132. [PubMed: 15572765]
41. Painter J, Merritt EA. TLSMD web server for the generation of multi-group TLS models. *J. Appl. Cryst.* 2006; 39:109–111.
42. Davis IW, et al. MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. *Nucleic Acids Res.* 2007; 35:W375–W383. [PubMed: 17452350]
43. Lodeiro MF, et al. Identification of multiple rate-limiting steps during the human mitochondrial transcription cycle in vitro. *J Biol Chem.* 2010; 285:16387–16402. [PubMed: 20351113]

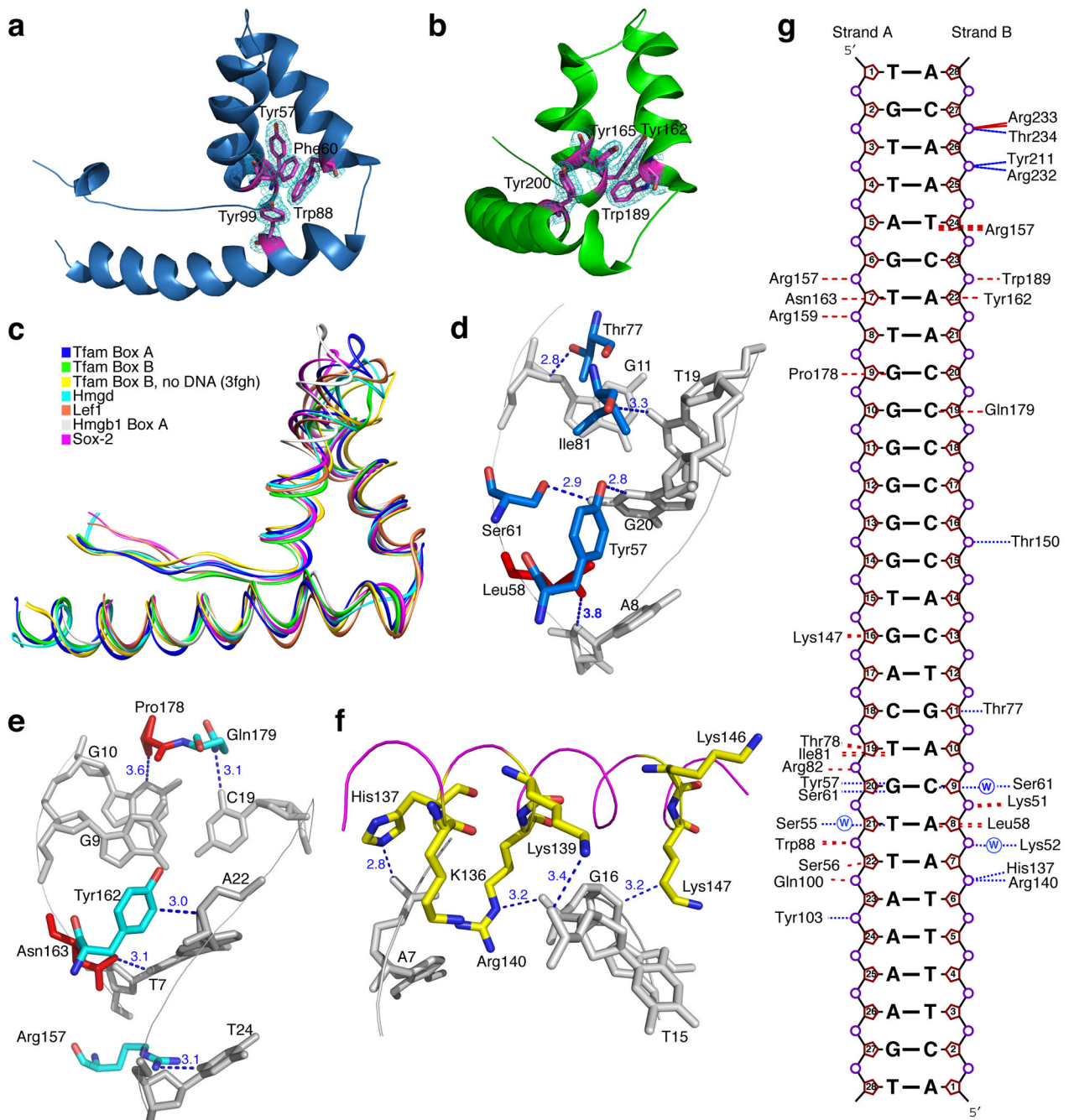


### Figure 1. Overview of the Tfam–mtDNA complex

(a) The domain structure of mature Tfam. Residues 1–42 constitute the mitochondrial targeting sequence that is cleaved upon import of Tfam into the mitochondrial matrix. (b) Organization of the LSP and HSP1 promoters. The two Tfam binding sites are oriented in opposite directions relative to the direction of transcription, based on comparative sequence analysis<sup>4, 8</sup>. The sequence of the LSP DNA fragment used for crystallization is indicated. (c) Side view of the Tfam–mtDNA complex. The Tfam domains are color coded as in panel a, and DNA is colored in gray. The LSP transcriptional start site would be located away from the DNA end on the left, as indicated by the arrow. Note that HMG-box B binds to the half-site further away from the transcriptional start site. (d) A view of the Tfam–mtDNA complex from the top. The protein and DNA are color coded as in panel c. (e) Electrostatic surface potential plot of Tfam. Surface areas of Tfam that are buried upon DNA binding are highlighted in yellow mesh. The HMG-box A, linker, HMG-box B, and C-tail regions are labeled. (f) Electrostatic surface potential plot of Tfam, viewed in the same orientation as in panel d and flipped 180° from panel e. This view emphasizes that the surface of the linker contacts the DNA.



**Figure 2. Molecular mass of the Tfam–mtDNA complex determined by SEC-MALS**  
 Elution profiles of Tfam, the Tfam–mtDNA complex, and BSA (control) examined by SEC-MALS. The horizontal red, blue, and black lines correspond to SEC-MALS calculated masses for Tfam, Tfam–mtDNA, and BSA, respectively. The corresponding theoretical masses are 28,075 Da (Tfam), 45,410 Da (Tfam–mtDNA; 1:1 complex), and 66,776 Da (BSA).

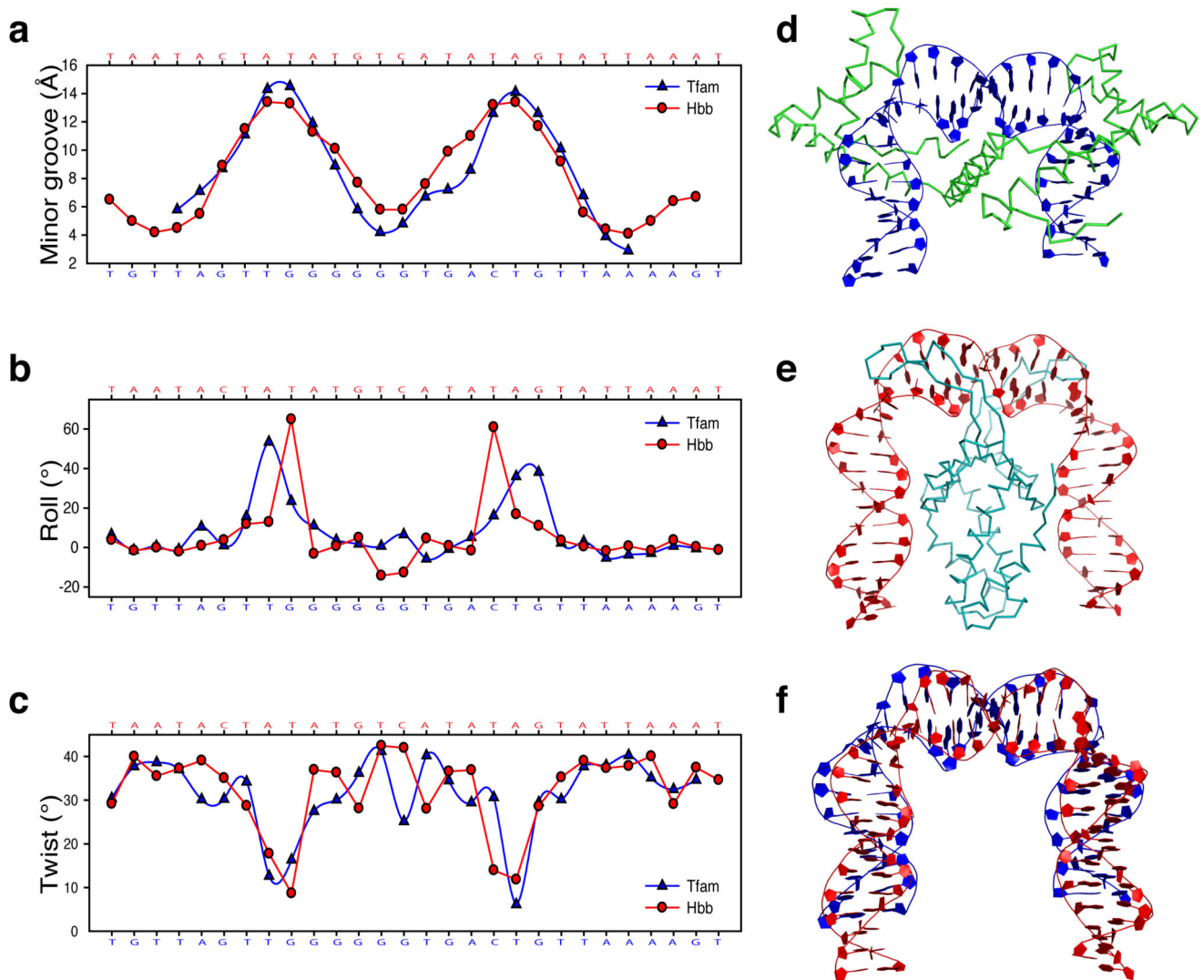


**Figure 3. Interactions of Tfam with DNA**

(a) A ribbon diagram of HMG-box A. Hydrophobic residues that stabilize the core are highlighted, with the 2Fo-Fc electron density map contoured at 1.5  $\sigma$ . (b) HMG-box B, highlighted as in panel a. (c) Superimposition of HMG-boxes A and B of Tfam with other HMG-boxes. Structures correspond to the following accession numbers, and RMSD values, relative to HMG-box A of Tfam, are provided in parentheses: HMG-box B of Tfam without DNA, 3fgh<sup>19</sup> (0.974); Hmgb1 box A, 1ckt<sup>27</sup> (1.101); Lef1, 2lef<sup>28</sup> (1.162); Sox2, 1gt0<sup>29</sup> (1.152); Hmgd, 1qrv<sup>30</sup> (1.127). (d) Interactions of HMG-box A with DNA (gray). Tyr57,

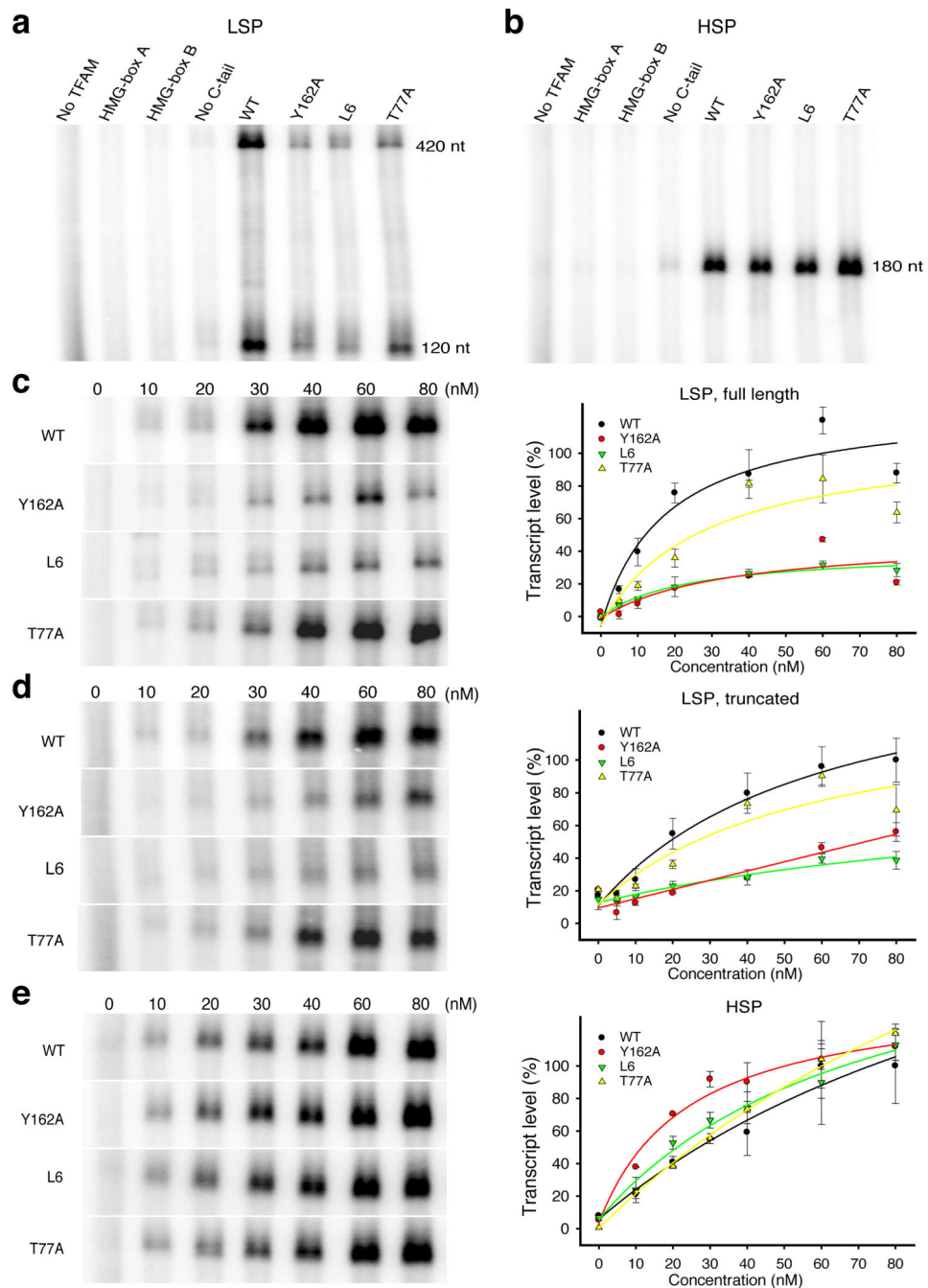
Leu58, Ser61, and Ile81 make contacts with the DNA bases and sugar phosphate backbone, as indicated by dotted lines with distances (in angstroms). Thr77 contacts a deoxyribose in the DNA backbone, and a mutant containing alanine at this position shows reduced DNA bending (Table 2). (e) Interactions of HMG-box B with DNA (gray). Arg157, Asn163, Gln179, and Pro178 make contacts with the bases, as indicated by the dotted lines. Tyr162 contacts a deoxyribose in the DNA backbone, and the Y162A mutant shows reduced DNA bending (Table 2). (f) Interactions of the  $\alpha$ -helical linker with DNA (gray). The backbone of the linker helix is traced in magenta. (g) Interactions between Tfam and DNA, analyzed by NUCPLOT<sup>31</sup>. Blue and red dashed lines represent hydrogen bonded and nonbonded contacts ( $< 3.35\text{\AA}$ ) to DNA, respectively. Circles labeled W indicate water-mediated interaction with DNA. Stereo views of panels **a**, **b**, **d**, and **e** are provided in Supplementary Figure 2.





**Figure 4. Comparison of Tfam and Hbb structures**

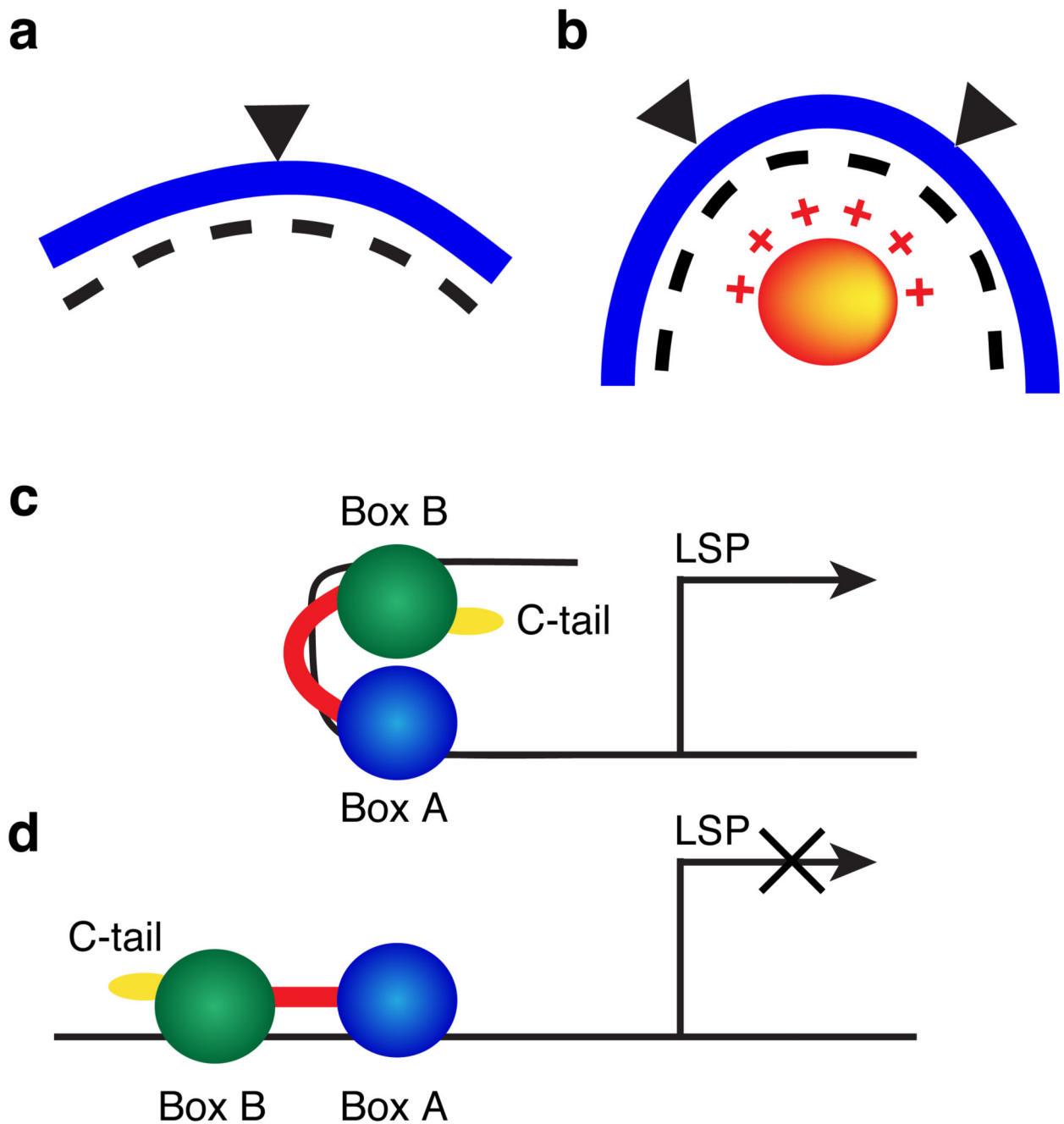
(a) Profiles of minor groove width in the Tfam–mtDNA (blue) and Hbb–DNA (red) structures. (b) Roll angle profiles in the Tfam–mtDNA (blue) and Hbb–DNA (red) structures. (c) Twist angle profiles in the Tfam–mtDNA (blue) and Hbb–DNA (red) structures. (d) Side view of the Tfam–mtDNA complex. The protein is shown in green, and DNA is shown in blue. (e) Side view of the Hbb–DNA complex. Hbb is shown in light blue, and DNA is shown in red. (f) Manual overlay of DNA in the Tfam–mtDNA and Hbb–DNA structures. DNAs from the two structures are color coded as in panels d and e. Analyses of the helical parameters of the DNA molecules were carried out using 3DNA<sup>32</sup>.



**Figure 5. Tfam mutants with a selective defect at LSP**

(a) *in vitro* transcription reactions using an LSP template. Reactions contained 100 nM Tfam or the indicated mutant. HMG-box A: residues 43–122; HMG-box B: residues 153–222; no tail: residues 43–222; L6: K136A H137A K139A R140A K146A K147A. The LSP template generates a 420 nt full-length (run-off) transcript and a truncated 120 nt transcript. (b) Same as panel a, except using an HSP1 template. (c) Generation of full-length LSP transcripts by Tfam and mutants. The left panel shows representative reactions, using the indicated concentrations of protein. Quantification is presented in the right panel, with error bars

representing standard deviations from three independent experiments. **(d)** same as in panel **c**, except that truncated LSP transcripts are shown and quantified. A fraction of LSP transcripts are known to terminate prematurely at the conserved sequence block II (CSBII) site located downstream of the start site<sup>33</sup>. **(e)** same as in panel **c**, except that an HSP1 template was used.



**Figure 6. Models for DNA bending and transcriptional activation**

(a) DNA bending by a single HMG-box. The DNA (blue) is moderately bent by wedging of the HMG-box (triangle) on one face of the DNA. Dashes indicate negative charges on the opposite face of the DNA backbone. (b) Extreme DNA bending by Tfam and HU family proteins. Two wedges (triangles) applied to one face of DNA result in two acute kinks. A positively charged platform (circle) on the opposite face helps to neutralize the negative charges of the DNA backbone. (c) Transcriptional activation at LSP. Based on our crystal structure, HMG-box B binds the half-site further away from the transcriptional start site. The

C-terminal tail nevertheless faces the transcriptional start site because of the extreme DNA bend. **(d)** With Tfam mutants, we suggest that the defect in DNA bending prevents proper orientation of the C-terminal tail.

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**Table 1**  
**Data collection, phasing and refinement statistics**

	Crystal 1 <sup>a</sup>			Crystal 2 <sup>a</sup>
<b>Data collection</b>				
Space group	C222 <sub>1</sub>			C222 <sub>1</sub>
Cell dimensions				
<i>a</i> , <i>b</i> , <i>c</i> (Å)	68.36, 81.35, 160.63			68.44, 81.91, 161.25
α, β, γ (°)	90, 90, 90			90, 90, 90
	<i>Peak</i>	<i>Inflection</i>	<i>Remote</i>	
Wavelength	0.97864	0.97898	0.91837	0.97864
Resolution (Å)	28.8 - 3.0 (3.17-3.0) <sup>b</sup>	28.8 - 3.0 (3.17-3.0) <sup>b</sup>	28.8 - 3.0 (3.17-3.0) <sup>b</sup>	24.2 - 2.5 (2.64-2.50) <sup>b</sup>
<i>R</i> <sub>merge</sub>	0.048 (0.120)	0.046 (0.114)	0.049 (0.215)	0.060 (0.454)
<i>I</i> / σ <i>I</i>	21.9 (9.7)	23.3 (10.0)	21.3 (5.8)	12.2 (2.8)
Completeness (%)	97.7 (91.4)	97.4 (88.8)	98.5 (97.4)	98.3 (99.5)
Redundancy	5.8 (4.2)	5.8 (4.0)	5.8 (4.6)	3.5 (3.6)
<b>Refinement</b>				
Resolution (Å)				24.2 - 2.5
No. reflections				15,795
<i>R</i> <sub>work</sub> / <i>R</i> <sub>free</sub> (%)				19.8/24.7
No. atoms				2,888
Protein				1,641
DNA				1,148
Water				99
<i>B</i> -factors (Å <sup>2</sup> )				
Protein				59.1
DNA				68.7
Water				55.3
R.m.s deviations				
Bond lengths (Å)				0.007
Bond angles (°)				1.03

<sup>a</sup>Two crystals were used for the structure.

<sup>b</sup>Values in parentheses are for highest-resolution shell.

**Table 2**  
**DNA bending and binding of Tfam variants**

The DNA bending and binding properties of Tfam and the indicated mutants were measured. DNA bending was measured with a FRET assay using Cy3-Cy5-labeled LSP DNA. The measured FRET efficiency was used to calculate the distance between the DNA ends. The affinity of Tfam mutants to DNA was monitored via the change in intrinsic tryptophan fluorescence upon DNA binding. Standard deviations from three independent experiments are indicated. L6: K136A H137A K139A R140A K146A K147A, HMG-box A: residues 43–122, HMG-box B: residues 153–222.

Tfam Mutant	FRET efficiency <sup>a</sup> %	Distance <sup>b</sup> (Å)	K <sub>D</sub> <sup>c</sup> (nM)
no protein	6.5 ± 0.7	84	
WT	36.5 ± 0.8	59	6.0 ± 0.9
T77A	31.4 ± 0.2	61	7.6 ± 1.1
Y162A	29.4 ± 0.6	63	12.3 ± 1.8
L6	26.1 ± 0.6	64	10.1 ± 1.2
HMG-box A	12.2 ± 0.4	75	6.5 ± 1.2
HMG-box B	6.2 ± 0.4	84	411.3 ± 46

<sup>a</sup>The FRET efficiency (E) was calculated with the following equation:  $E = (F_{\text{corr}})/(F_{\text{corr}} + D_{\text{corr}})$ , where  $F_{\text{corr}}$  and  $D_{\text{corr}}$  are the corrected FRET and donor signals at 662 and 562 nm, respectively.

<sup>b</sup>The distance was calculated from the FRET efficiency using the following equation:  $E = R_0^6 / (R_0^6 + R^6)$  with  $R_0 = 54 \text{ \AA}$ .

<sup>c</sup>K<sub>D</sub> was calculated from an LSP DNA binding assay, as detailed in the Supplementary Methods.