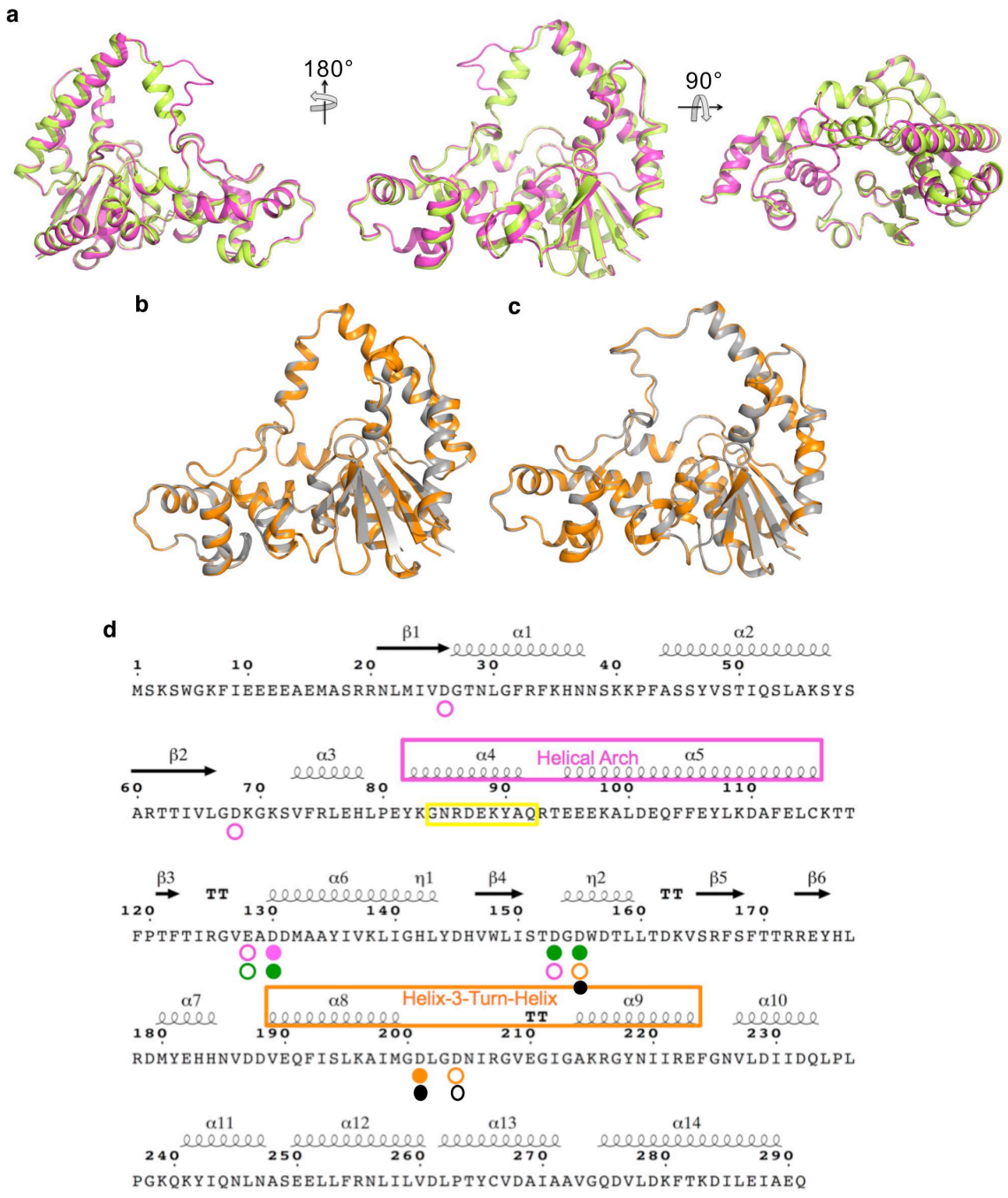
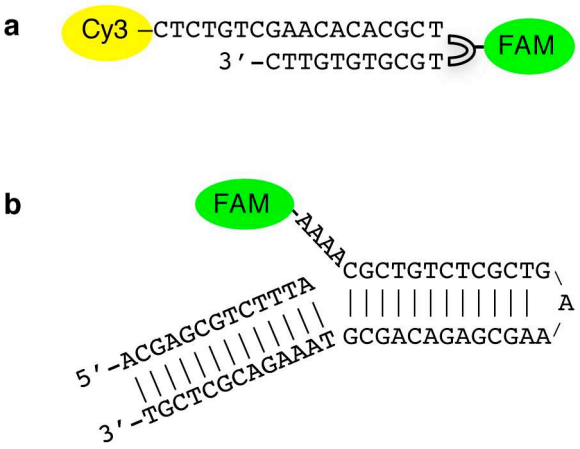


Supplementary Figure 1

Structures of T5Fen and the D153K variant.

(**a**) Three views of the T5Fen structure showing superposition of the two molecules seen in the asymmetric units (chain A, green; chain B, magenta). (**b**) Superposition of the helical arch forms of T5Fen (PDB code 5HMM) (grey) with the iso-structural T5FenD153K (PDB code 5HML) molecule (orange). (**c**) Superposition of looped out forms of wt T5Fen (grey) with the equivalent conformation of D153K molecule (orange). (**d**) T5Fen sequence with secondary structural elements marked (α-helices as coils; β-strands as arrows). Filled and open circles indicate respectively, direct and indirect ligands for Mg1 (magenta), Mg2 (green) and Mg3 (black). Alpha helical regions are numbered and the position of the H3TH motif is shown. The yellow-boxed region shows residues involved in the alternative conformation for the arch or loop region seen in one chain of each asymmetric unit in the DNA free protein structures.



c

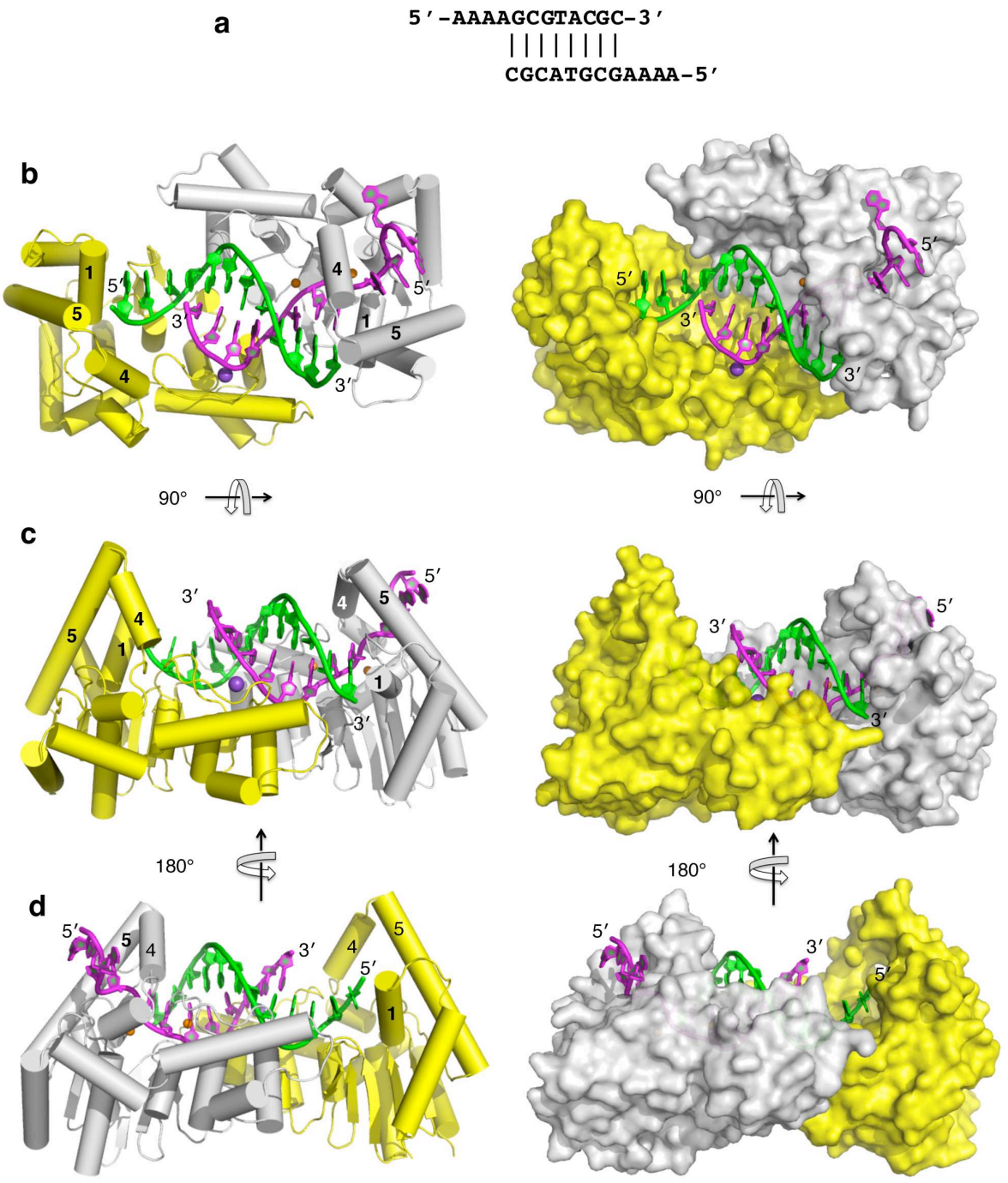
Protein	*Dissociation Constant* (nM)
T5Fen	26.1±4.4
D153K	9.5±1.7
D155K	12.6±2.1

* **

Supplementary Figure 2

Substrates used for DNA binding and nuclease assays.

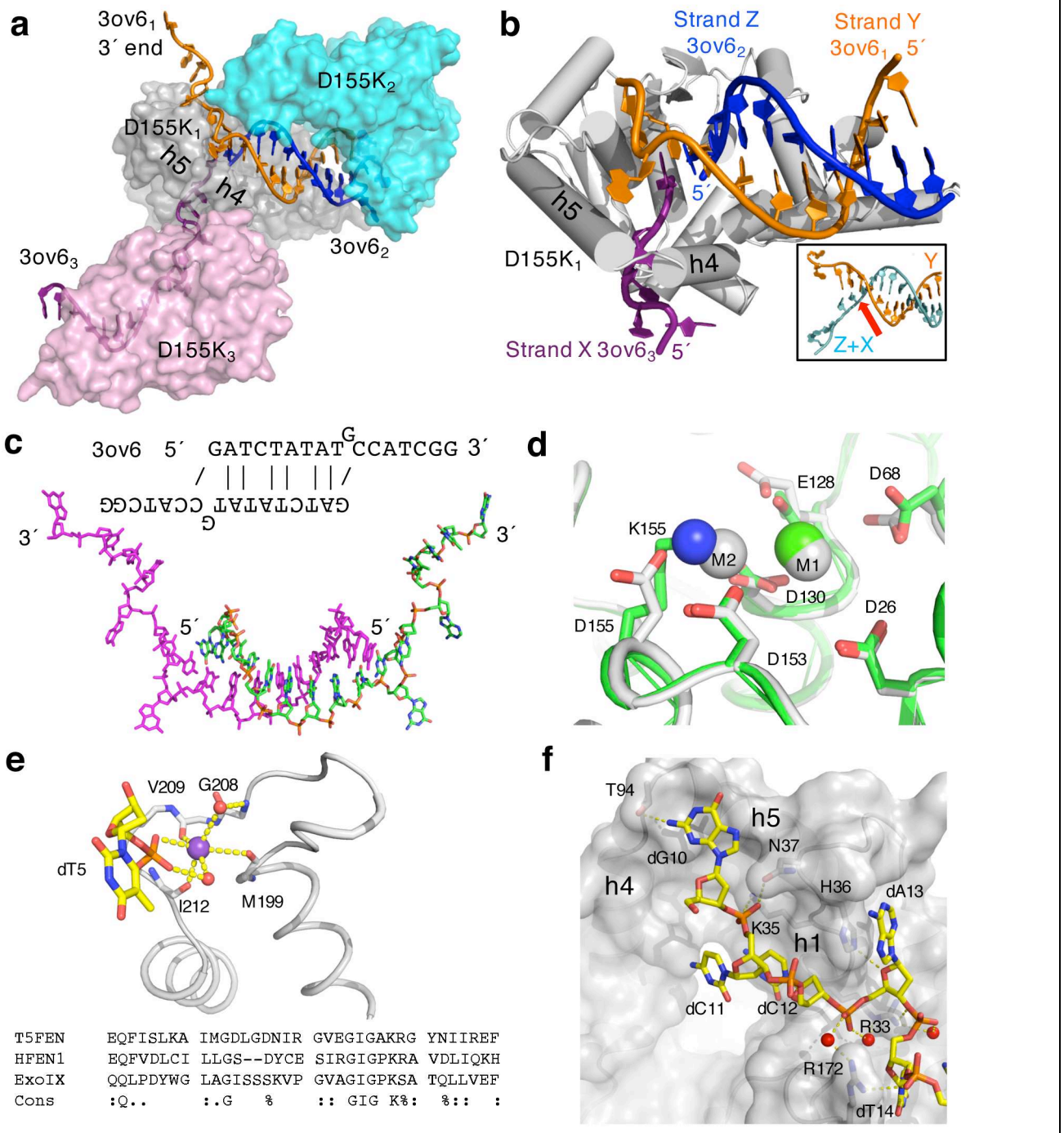
(**a**) Dual-labelled single-turnover substrate OHP2 used for real time endonuclease FRET assay. (**b**) Diagram of fluorescein-labelled flap substrate used for DNA binding studies. (**c**) Flurorescence anisotropy was used to determine dissociation constants for the three proteins indicated using the flap substrate shown in (**b**) in the absence of divalent metal ions (n=3, ±SEM). * P=0.0245, ** P=0.0504 using a 2-tailed t-test.



Supplementary Figure 3

Arrangement of molecules in the asymmetric unit of T5FenD153K–DNA crystals

(**a**) Sequence of oligonucleotide 5ov4, which forms an 8 base-pair palindromic duplex with 4 deoxyadenosines at each 5′ end and was crystalized with T5FenD153K (PDB code 5HNK). (**b–d**) Three views showing the DNA plus either cartoon (left panels) or molecular surface representations (right panels) of the two protein molecules (chain A in yellow, chain B in grey). Helices 1, 4 and 5 are indicated as well as the 5′ and 3′ ends of DNA strands X (green) and Y (magenta). Two magnesium and potassium ions (orange and purple spheres, respectively) were identifiable in the complex.

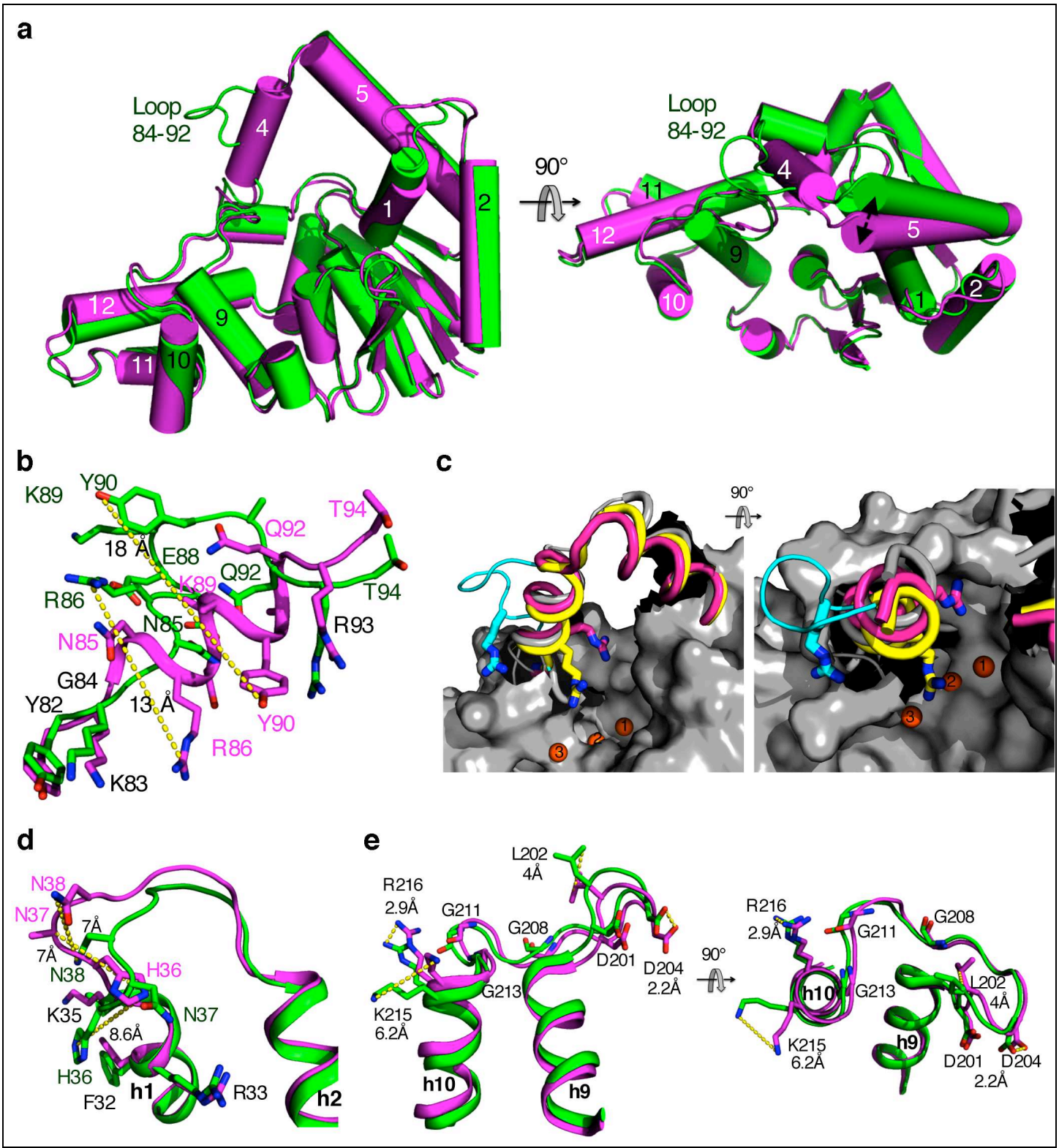


Supplementary Figure 4

Arrangement of protein and DNA molecules in 3´-overhang:T5FenD155K complex.

(**a**) Arrangement of three adjacent asymmetric units. Each consists of one protein and one identical DNA (3ov6) molecule (PDB code 5HP4). Two molecules of oligonucleotide 3ov6$_1$ (orange) and 3ov6$_2$ (blue) form a partially base-paired duplex contacting the central T5Fen molecule (grey surface) while the 3´ end of a third (3ov6$_3$, magenta) threads through its helical arch (helices h4 and h5). (**b**) The central T5Fen protein from (**a**) showing nucleotides from two adjacent AUs that contact it. The strands have been labeled X, Y and Z. Strand Y passed through the helical arch. The resulting assembly resembles a pseudo-product complex such as could have been

derived from hydrolysis of the branched substrate at the indicated phosphodiester (inset, red arrow) – or conceptually, by joining strands X and Z to form the cyan strand. (**c**) The sequence of partially complementary oligonucleotide 3ov6 shown as a duplex. (**d**) Comparison of position of divalent metal ions in T5Fen (grey cartoon) and the D155K variant (green cartoon). Grey stick residues indicate ligands for Mg^{2+} ions in M1 and M2 (grey spheres 1 and 2) in the wt protein. In T5FenD155K DNA–Ca^{2+} complex calcium is positioned at M1 and the ε-amino group of Lys155 (blue sphere) is situated close to the M2 site making electrostatic interactions with Asp153 and Asp130. (**e**) The H3TH motif (a.a. 191–225, grey cartoon) of T5Fen binds a potassium ion (magenta sphere) which in turn binds the phosphate group of dT5 in the duplex DNA. Sequence alignment of the H3TH motifs: residues 191-224 of T5FEN; 163-197 ExoIX and 219-252 from hFEN1 shown below. Consensus sequence shown with similar (:), hydrophilic (%) and not dissimilar (.) residues indicated. (**f**) Interactions between the 3´ end of one DNA strand with helix 1 (h1). Hydrogen bonds indicated by yellow dashes with water molecules as red spheres. Grey sticks indicate amino acids interacting with DNA.



Supplementary Figure 5

Structural changes in T5Fen upon DNA binding.

(**a**) Schematic showing differences between DNA-bound (complex TC2, magenta) and substrate-free T5Fen structures (green). Residues connecting helices 1 to 2 and 9 to 10 and in helix 4 undergo the largest changes. Helices numbered in white. The junction of helices 4 and 5 is shifted ~ 5-7 Å toward the H3TH motif (h9 and h10) upon binding DNA as shown by the double headed arrow (right panel). (**b**) Rearrangement of residues 84–92 upon DNA binding. Yellow dashes (with distances) show the largest movements. Atoms of residues labeled in black undergo minimal (<2 Å) translations. Colored labels indicate movement of >2 Å. (**c**) Two views showing the range of movement observed for helix 4 residues and Arg86 (sticks) in DNA-free, looped-out conformer (cyan cartoon), and with intact substrate bound in complex C2 (magenta cartoon), and in pseudo-product complex C3 (yellow cartoon). The numbered orange spheres show the position of three metal ions (1 and 2 in Cat1 and ion 3 in Cat2. (**d**) Residues on the helix 1 (h1) and the loop to helix 2 (h2) undergo the next largest rearrangements with His36 playing a role in DNA binding. In one DNA-free form this loop is disordered (not shown). (**e**) Two views of the H3TH motif (helices h9 and h10) showing residues moving >2 Å upon DNA binding. L202 which changes conformation upon engaging substrate and conserved residues shown in stick representation.