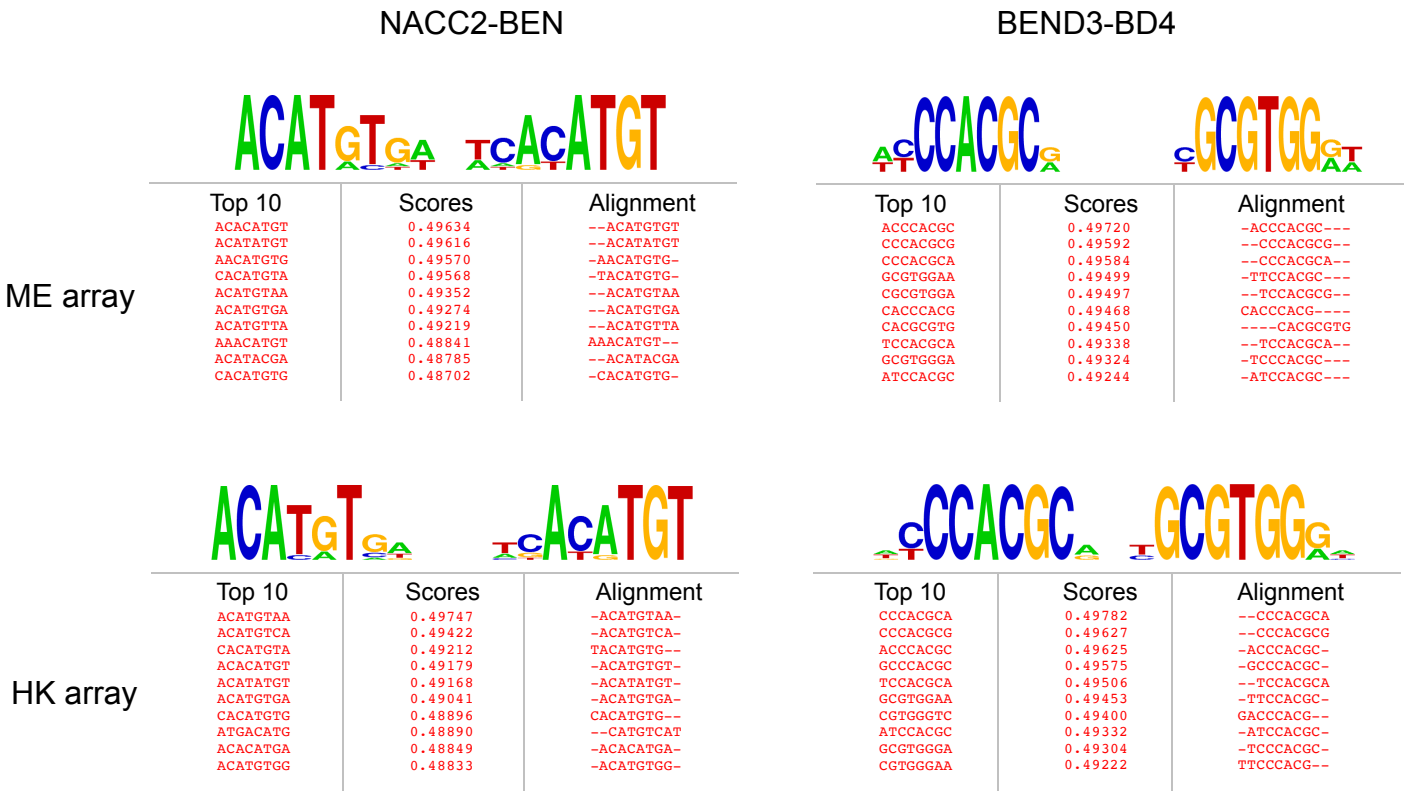
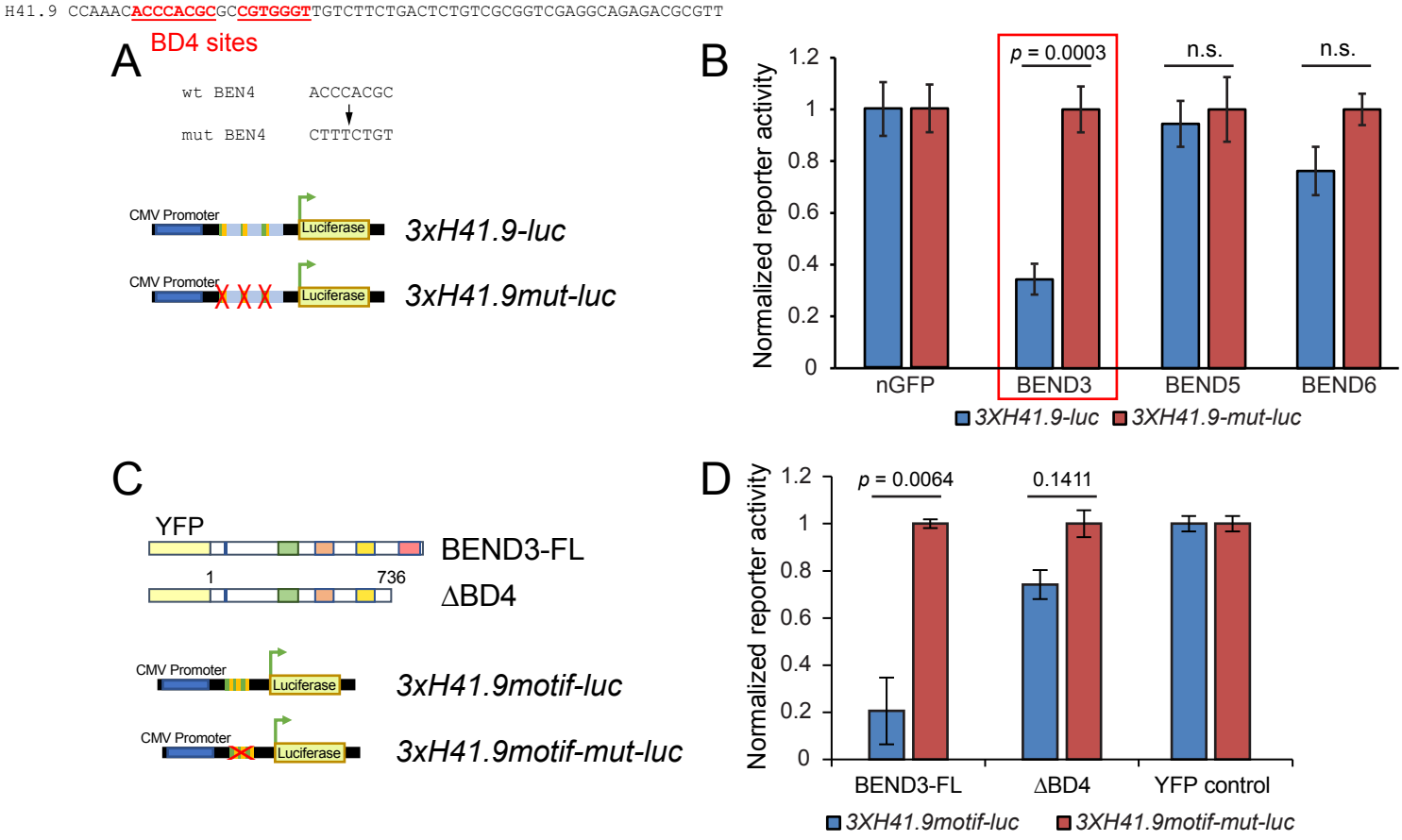


Supplementary Figure 1. Top PBM-derived motifs for NAC2-BEN and BEND3-BD4 domains.

Independent protein binding microarray (PBM) designs (ME and HK) were used to interrogate sequence-specific binding by GST-BEN proteins from NACC2 and BEND3. The top 10 motifs from each PBM experiment are shown as representatives for the sequence logos derived for NACC2-BEN and BEND3-BD4 domains. E-scores >0.45 are considered as specific binding; thus, the scores shown at 0.48-0.49 are highly significant.

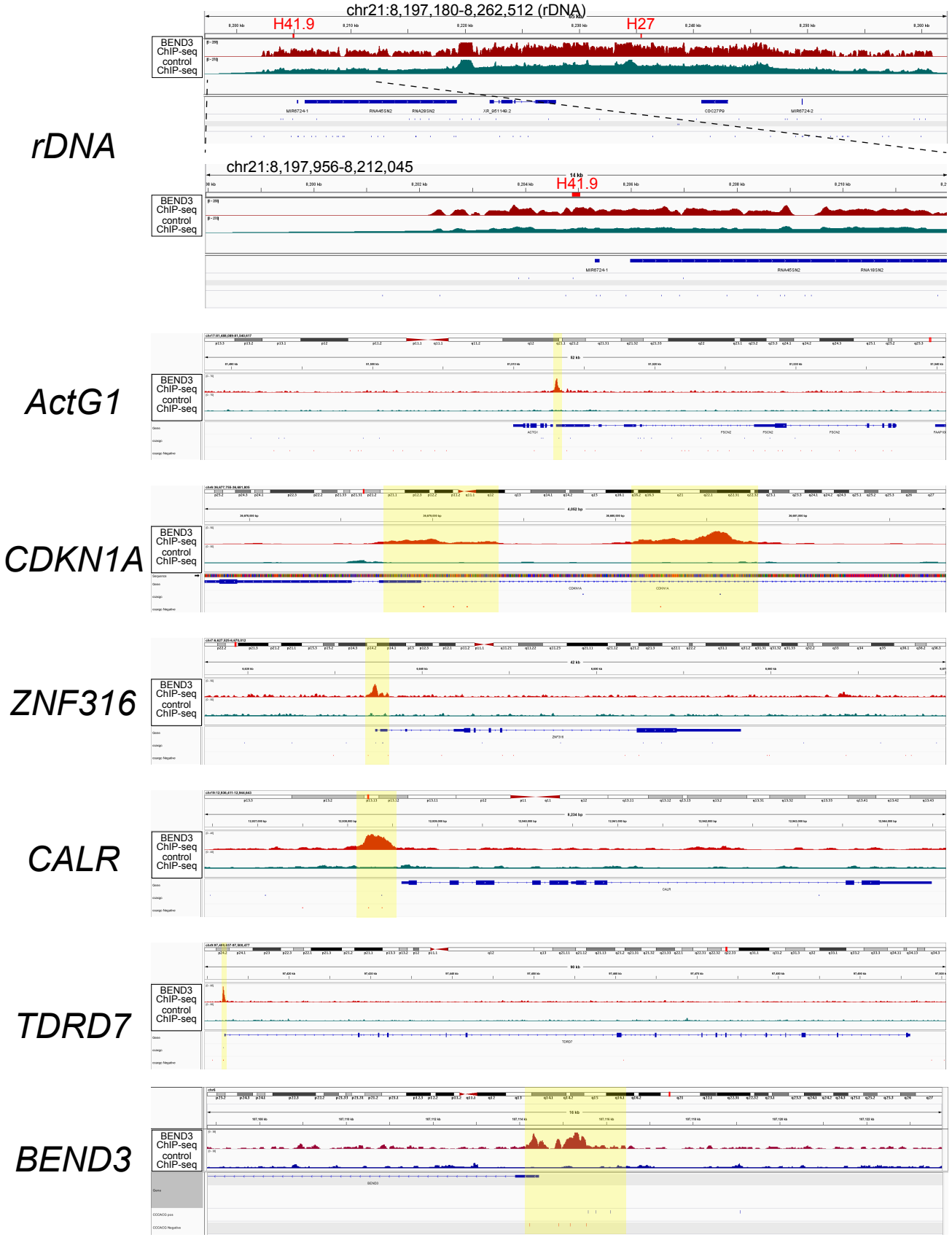
Supplementary Figure 1



Supplementary Figure 2. Specificity of BEND3-mediated repression.

(A) Sequence of wt *H41.9* rDNA region used in a transcriptional reporter, along with mutations introduced in a mutant counterpart. (B) Human BEND3, but not BEND5 or BEND6 proteins, could repress the *H41.9-wt* reporter. (C) Schematics of full-length (FL) and deletion variants of BEND3. (D) Repression of the *H41.9-wt* reporter by BEND3 requires its BD4 domain.

Supplementary Figure 2



Supplementary Figure 3. Genomic binding of BEND3 at BD4-bearing sites.

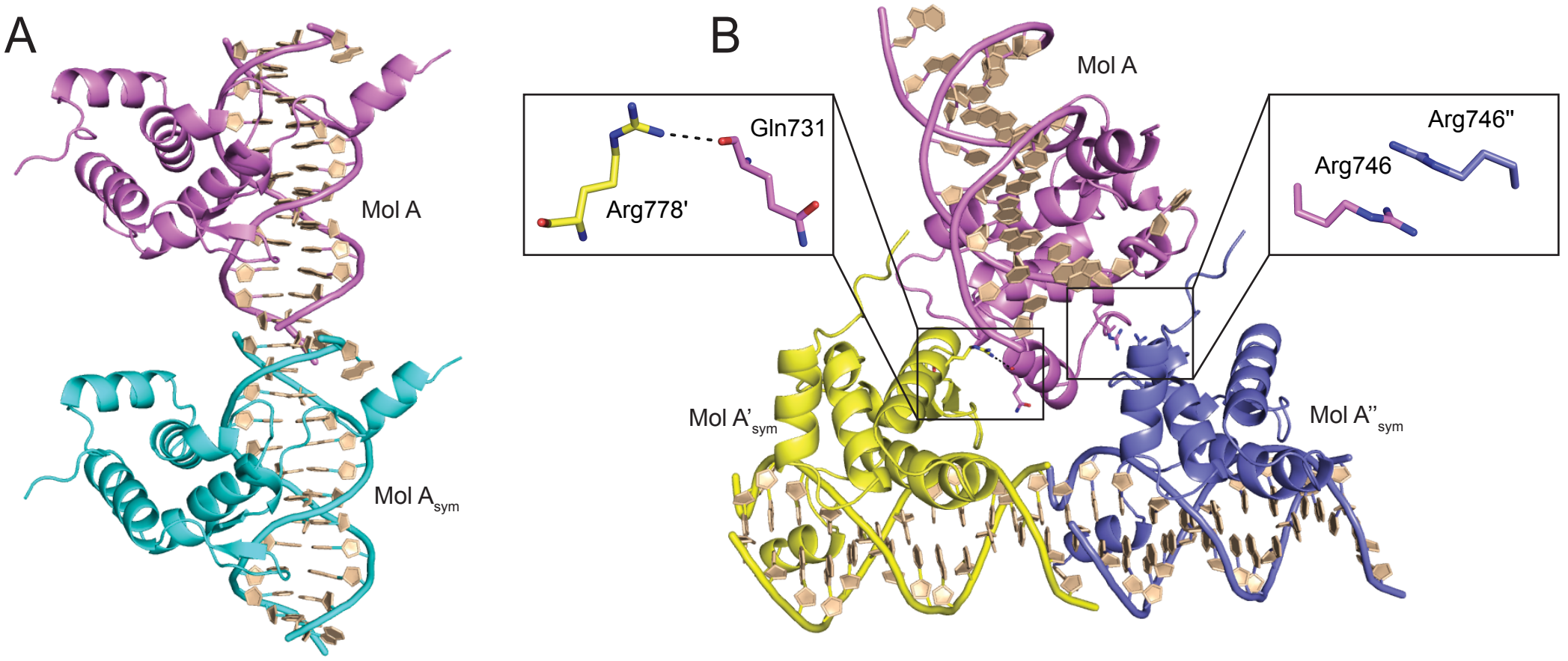
Shown are IGV screenshots of BEND3 and control ChIP-seq data at target genes analyzed in this study. It was difficult to observe specific signals at the highly repetitive rDNA locus, but 6 other targets bear highly focal BEND3 ChIP-seq signals that coincide with one or more matches to the BD4 PBM motif.

Supplementary Figure 3

Supplementary Figure 4. BEND3 is higher expressed in pluripotent cells than most differentiated cell types.

Summary of BEND3 expression across human cell types from bioGPS (http://biogps.org/). Highlighted in yellow (top) are various types of ES cells and iPS cells, which express much higher levels of BEND3 than all other cell types in the database.

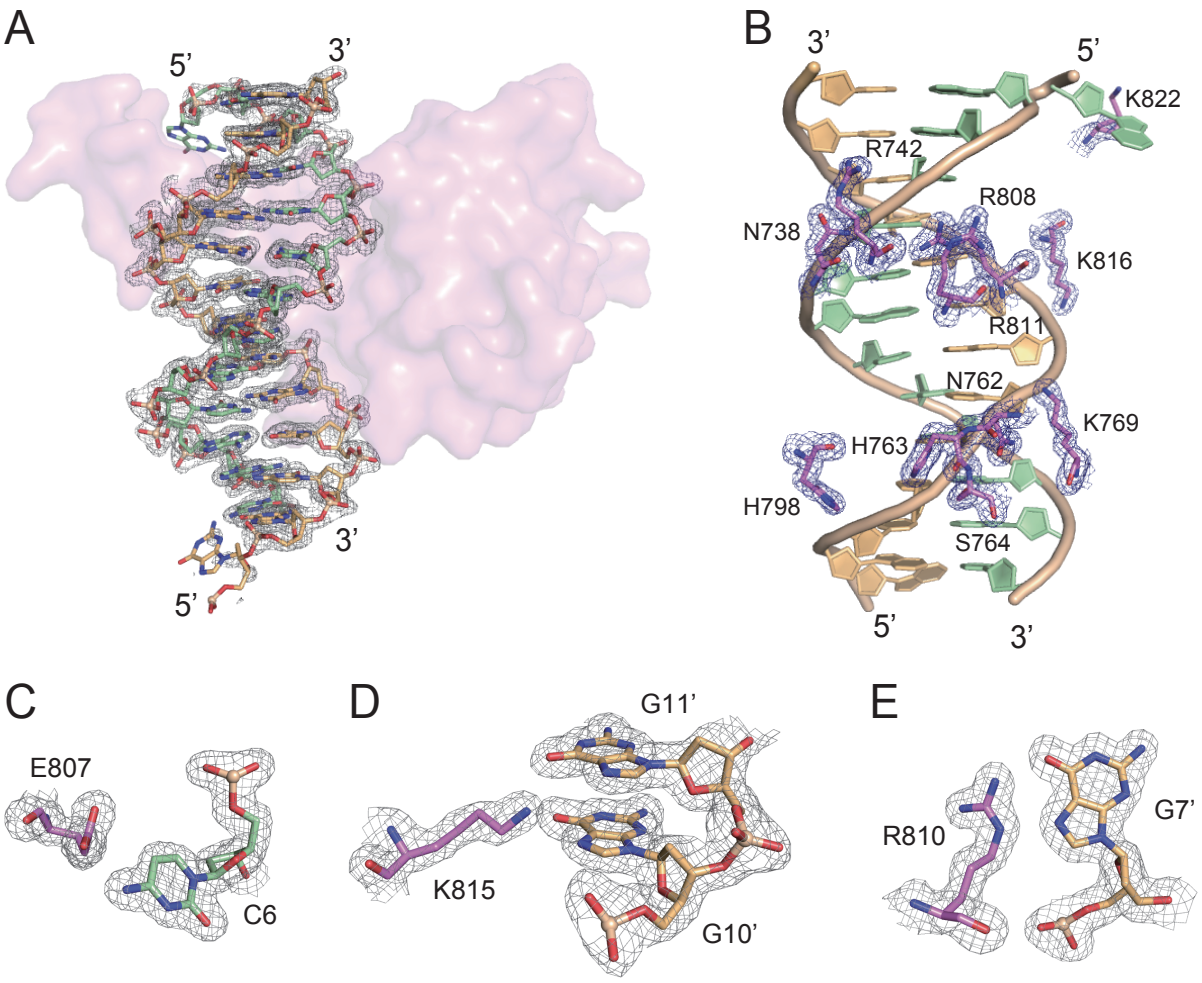
Supplementary Figure 4



Supplementary Figure 5. Intermolecular interaction of BEND3-BD4/DNA complexes.

(A) Overall views of two symmetry-related BEND3-BD4/DNA complexes. Mol A forms end-to-end stacking interaction with Mol Asym along with the DNA duplex in tertiary structure. (B) Protein-Protein interactions in the BD4/DNA complexes. Gln731 from Mol A forms one hydrogen bond to Arg778' of Mol A' sym, while Arg746 from Mol A forms stacking interaction with Arg746'' of Mol A'' sym.

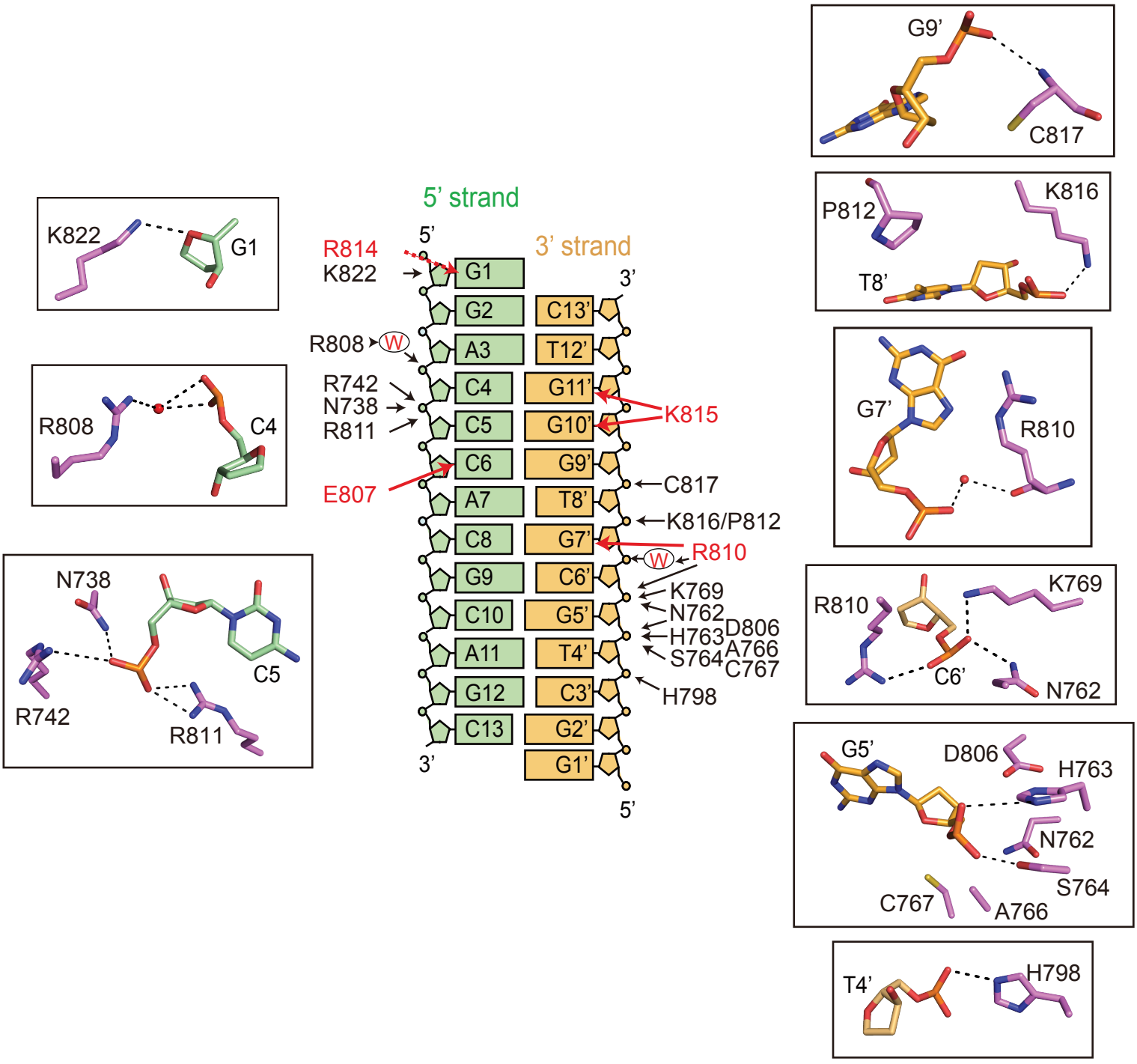
Supplementary Figure 5



Supplementary Figure 6. Composite omit electron density map for BEND3-BD4 in complex with DNA.

(A) Composite omit electron density map of the DNA molecules (contoured at 1.0 σ) bound with BEND3-BD4 protein (shown in surface representation). (B) Composite omit electron density map of the residues (contoured at 1.0 σ) in BEND3-BD4 protein that interacted with the main chain of DNA molecules. (C-E) Composite omit electron density map (contoured at 1.0 σ) of the specific interaction between BEND3-BD4 protein and the base of DNA molecules.

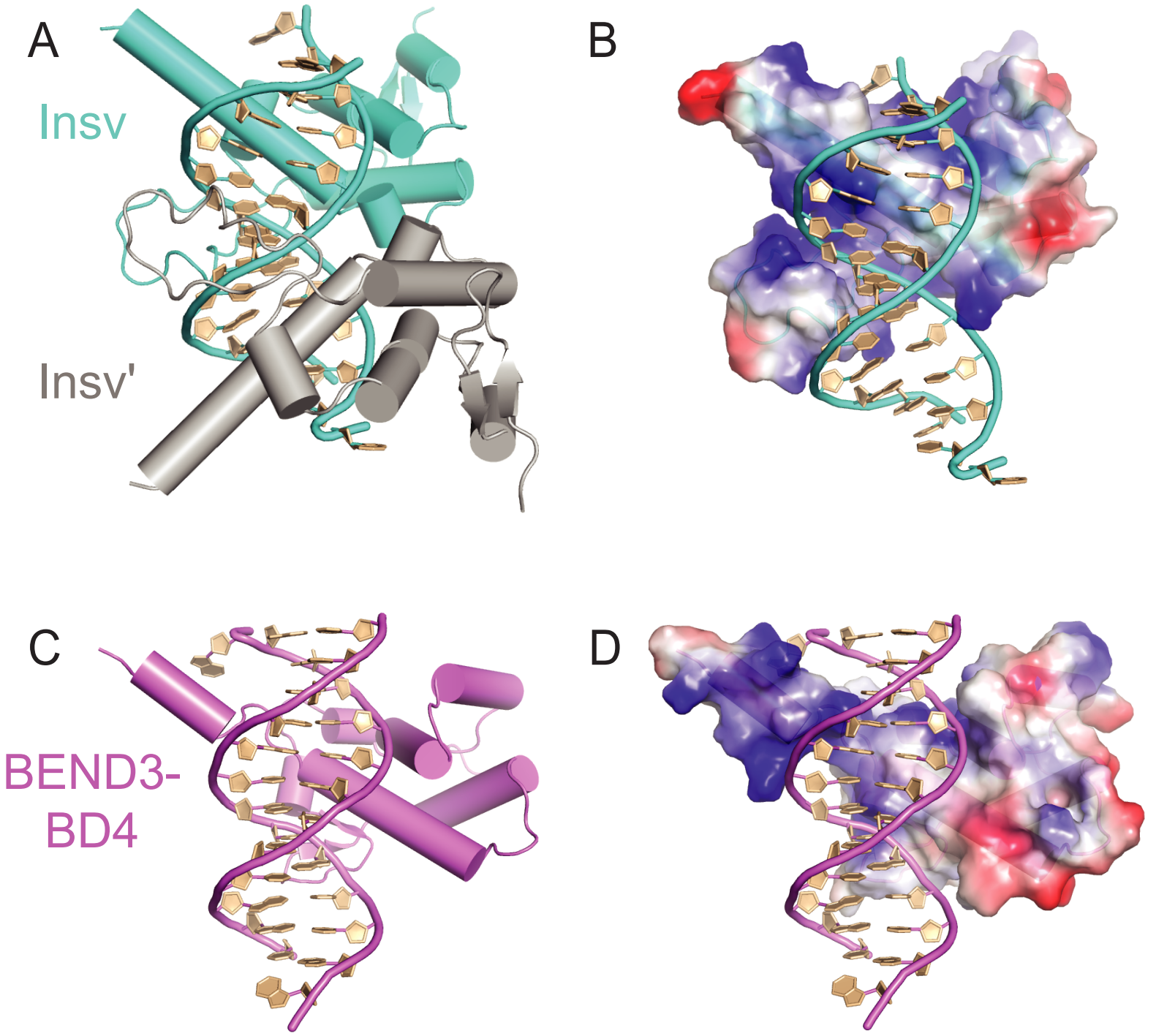
Supplementary Figure 6



Supplementary Figure 7. Structure details of sugar-phosphate interactions in the BD4/DNA complex.

Details of sugar-phosphate intermolecular hydrogen-bonding contacts in the BEN4/DNA complex are shown in the black rectangle. K822 makes one hydrogen bond to the ribose of G1, and the distance is 3.0 Å. R808 forms indirect interaction with C4 through a water molecule labeled red W. Phosphate of C5 is stabilized by N738, R742 and R811. C817 forms hydrogen-bonding interaction and van der Waals contacts with G9'. K816 is hydrogen-bonded to the phosphate of T8', while P812 forms partial stacking interaction with the base of T8'. R810 forms water-mediated interaction with the phosphate of G7'. Phosphate of C6' is anchored by N762, K769 and R810. Phosphate of G5' forms hydrogen-bonding and van der Waals contacts contacts with nearby residues including H763, S764, D806, N762, A766 and C767. H798 interacts with phosphate of T4'.

Supplementary Figure 7



E

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BEND3-BEN domains 1-4 (BD1-4)
BD1(241-360)  241 QP.....P.PEYQLTAAELKQIVDQSLSGGDLACRLLVQLFPELFSDVDFSRGCSACGF
BD2(381-491)  381 DR..SSTI.ASDHVVDTQDLTEFLDEASSPGEFAVFLLHRLFPELFDHRKLGEQYSCYGD
BD3(541-660)  541 IPQPDFEVPGADCLLSKEQLRSIYESSLSIGNFASRLLVHLFPELFTHENLRKQYNCSGS
BD4(710-829)  710 SP..DFPV.PSPYLLSDKEVREIVQQSLSVGNFAARLLVRLFPELFTAENLRLQYNHSGA

BD1(241-360)  294 AAKRKLESLHLQLIRNYVEVYYPSVKDTAVWQAECLPQLNDFFSRFWAQREMEDSQPSGQ
BD2(381-491)  438 GGKQELDPQRLQIIRNYTEIYFPDMQEEEAWLQQCAQRINDELEGLGLDAGSE.......
BD3(541-660)  601 LGKKQLDPSRIKLIRHYVQLLYPRAKNDRVWTLEFVGKLDERCRRRDTE.QRRSYQQQRK
BD4(710-829)  767 CNKKQLDPTRLRLIRHYVEAVYPVEKMEEVWHYECIPSIDERCRRPNRK.KCDILKKAKK

BD1(241-360)  354 VASFFEA
BD2(381-491)  491 ......G
BD3(541-660)  660 ......V
BD4(710-829)  826 VE....K
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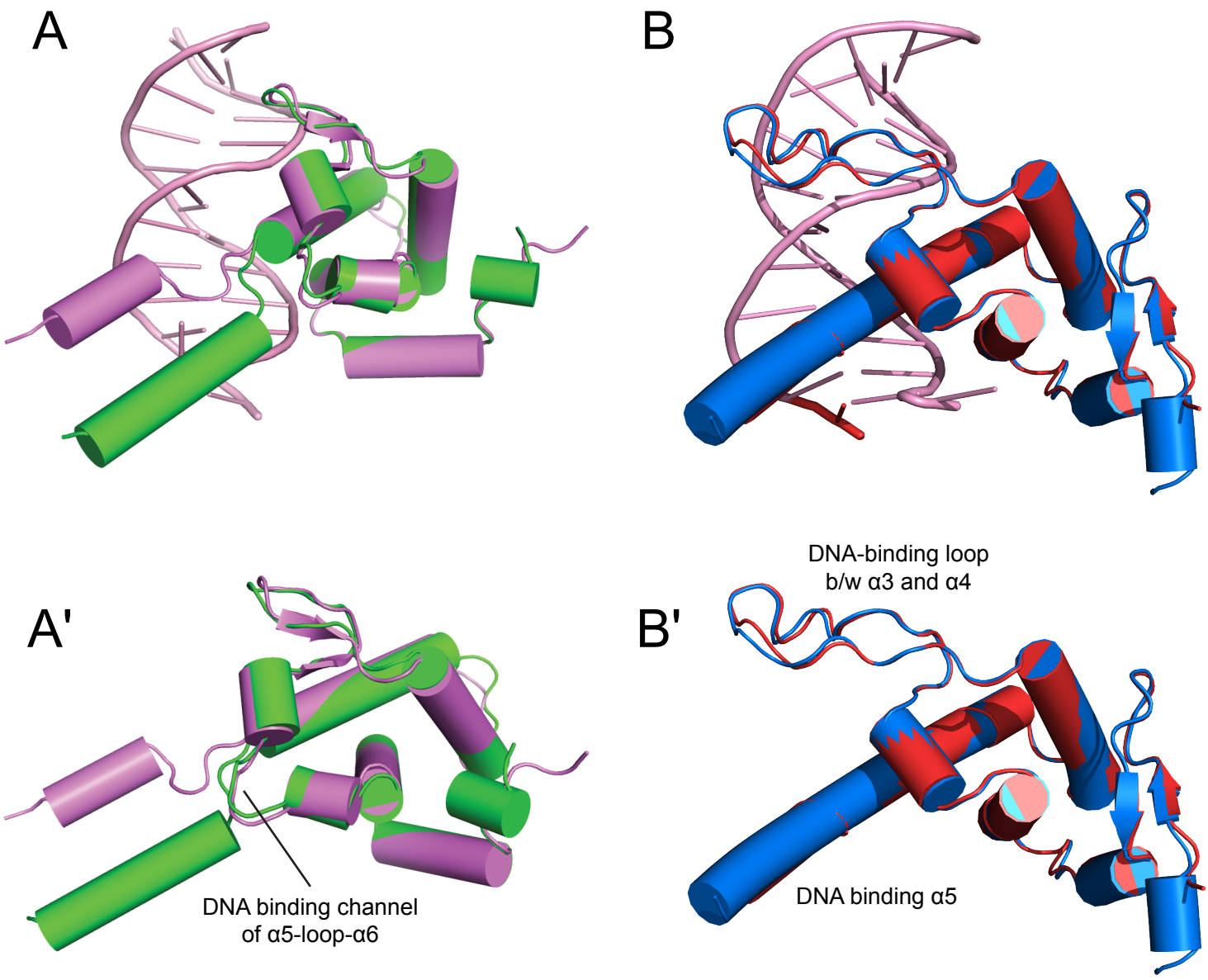
Supplementary Figure 8. Alignment of BEND3-BEN domains and comparison of BD4/DNA and Insv-BEN/DNA complexes.

(A) The overall view of Insv-BEN/DNA complex in a cartoon representation, with one DNA duplex bound to two Insv-BEN protein molecules. One Insv-BEN protein molecule and as well as the DNA backbones are colored light blue, while another Insv-BEN protein molecule is colored gray. The bases of the DNA duplex are colored bright orange. (B) Electrostatic surface representation of the Insv-BEN domain in complex with DNA with only one Insv-BEN protein molecule in the complex. (C) The overall view of BEND3-BD4/DNA complex in a cartoon representation, with one DNA duplex bound to one BD4 protein. The BD4 molecule and the DNA backbones are colored violet, while the bases of the DNA duplex are colored bright orange. (D) Electrostatic surface representation of the BD4 domain in complex with DNA in the same view as B. (E) Sequence alignment of BD1, BD2, BD3 and BD4 domains of BEND3. Conserved residues are colored white in the red boxes. The dark blue stars label positions of residues involved in base-specific interaction in BEND3-BD4 domain.

Supplementary Figure 8



Supplementary Figure 9. Correspondence of predicted BEN domain structures with experimental determinations.

We compared BEN domain structures predicted by AlphaFold2 with our BEN structures from X-ray crystallography. (A-A') Insv-BEN, PDB:4IX7 and (B-B') BEND3-BD4, this study. Structures were determined in complex with their respective high-affinity binding sites; and overlays with predicted structures are shown with and without DNA. These comparisons show overall congruence of experimental and predicted structures, and also emphasize that BD4 (class II BEN domain) lacks the extensive DNA-binding loop between α3 and α4, and instead relies upon the channel formed by the C-terminal α5-loop-α6 region.

Supplementary Figure 9