Supplementary Figures 1 - 8

Structural asymmetry in the *Thermus thermophilus* RuvC dimer suggests a basis for sequential strand cleavages during Holliday junction resolution.

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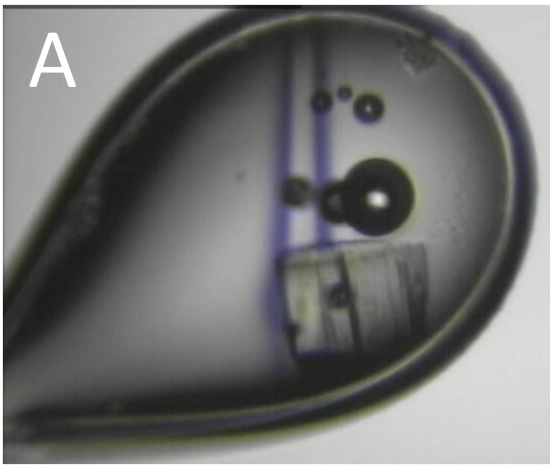
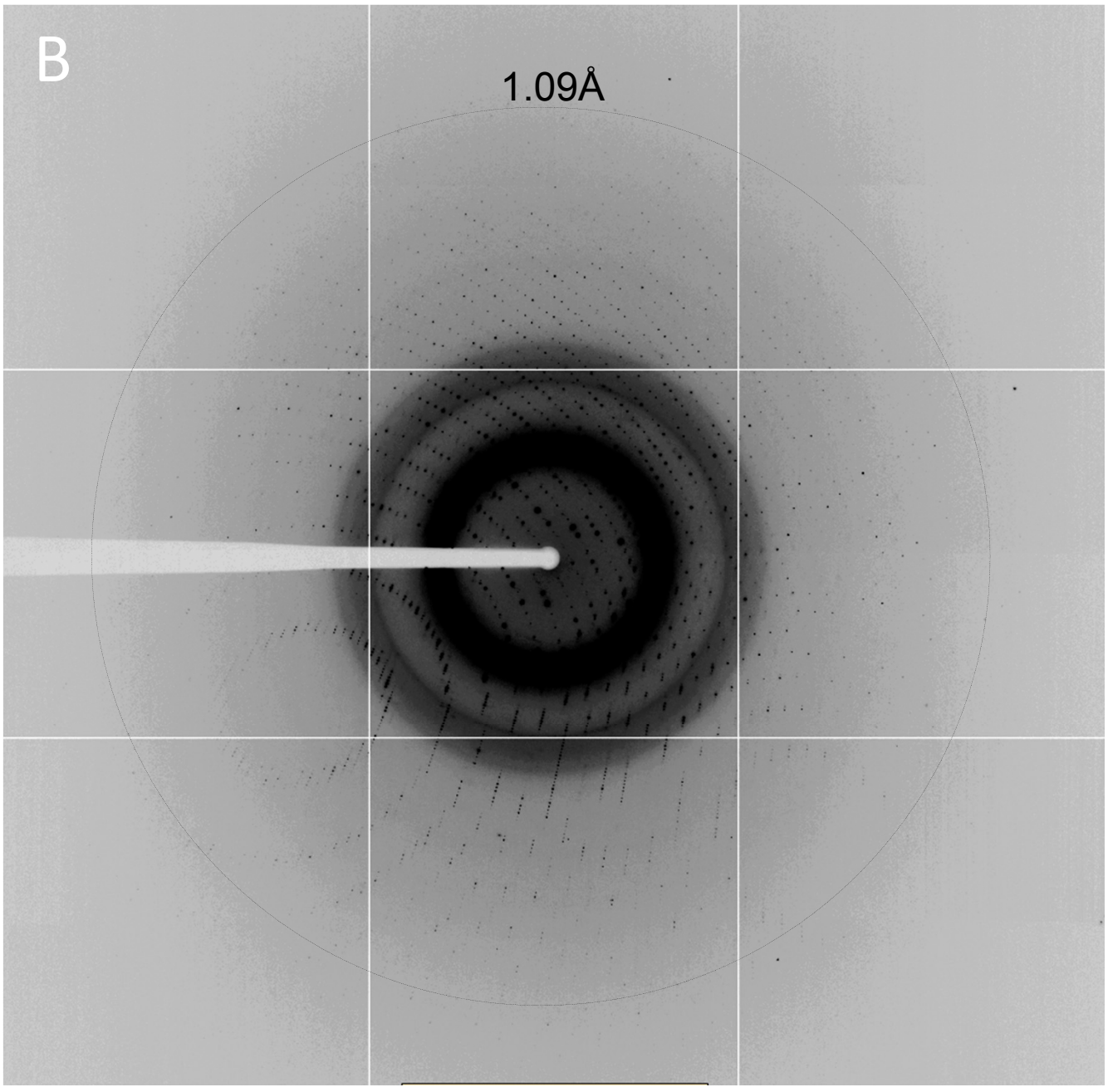
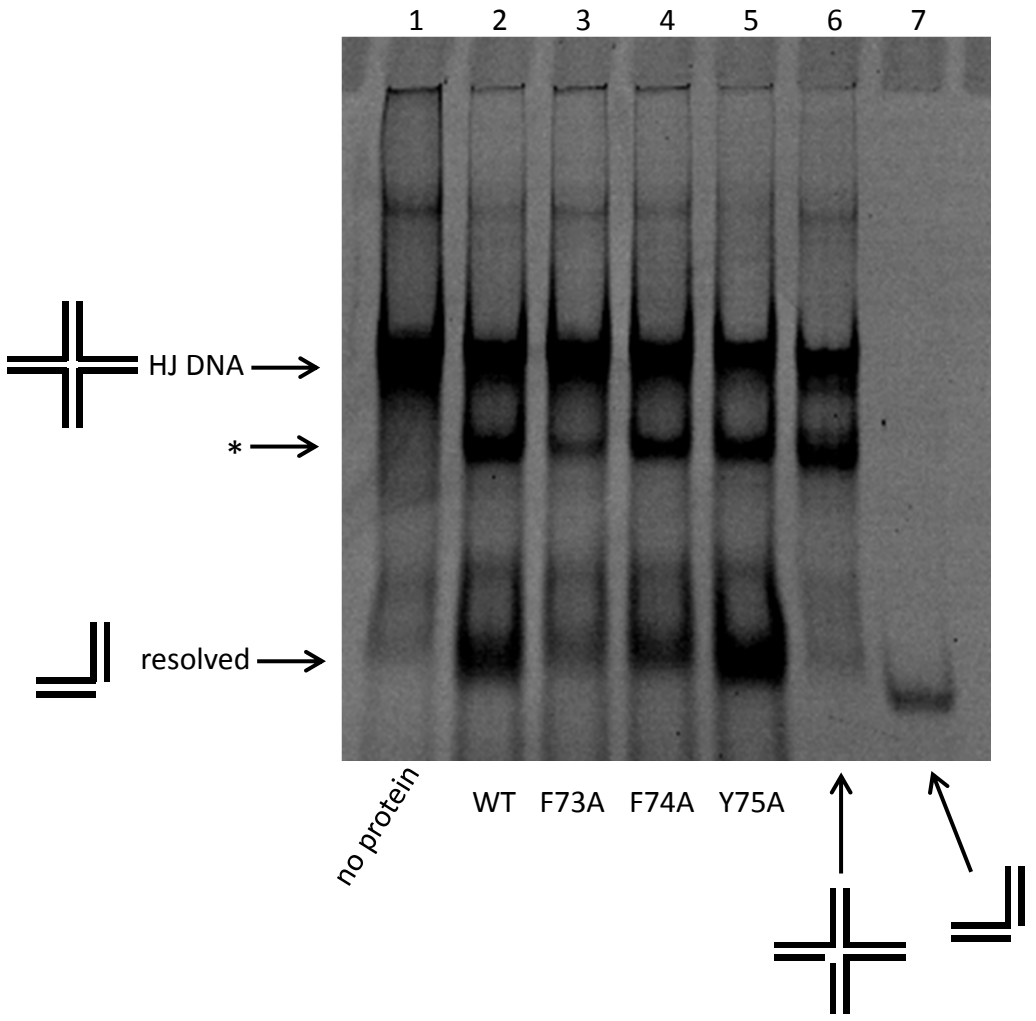
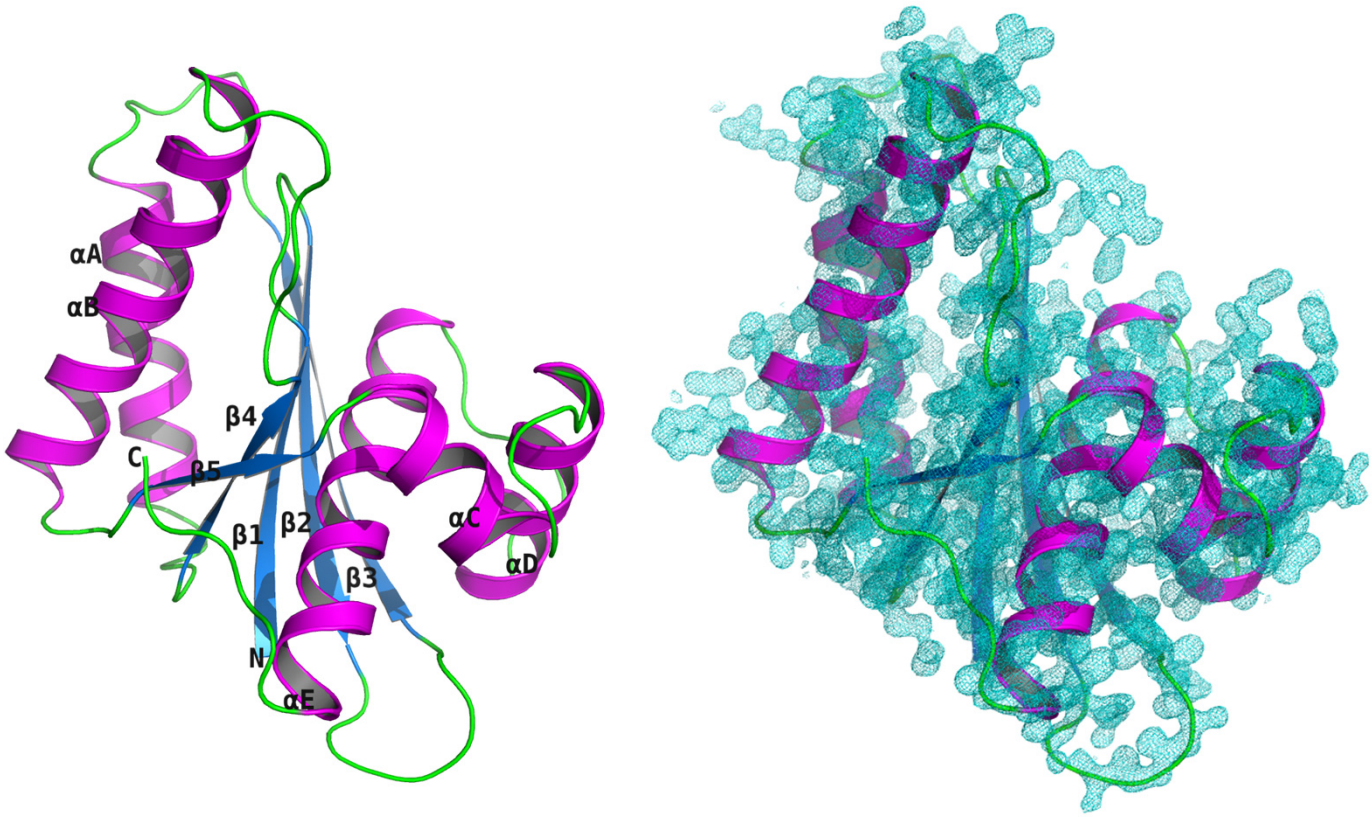

A


B
1.09Å

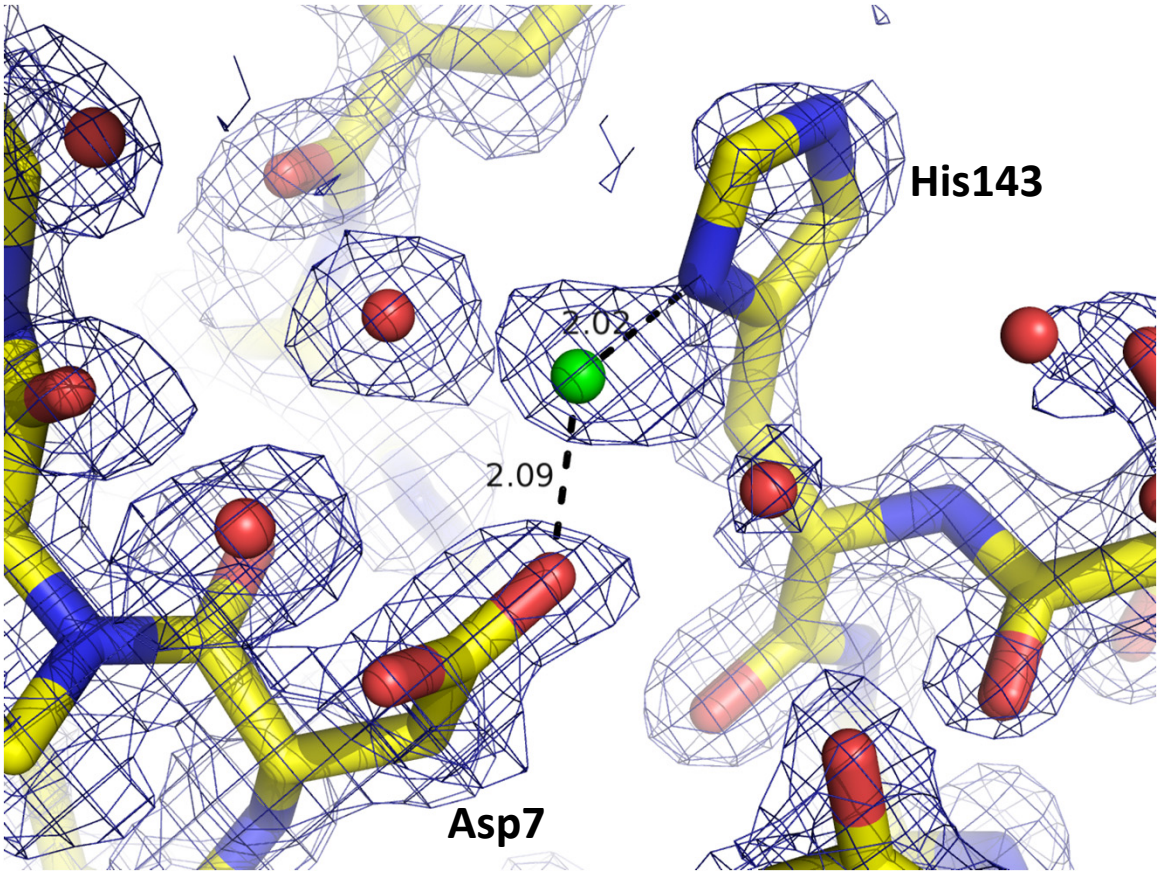
Supplementary Fig. 1 (previous page) | **X-ray diffraction by the *T.th*. RuvC crystal** (**A**) Picture of a frozen *T.th*. RuvC crystal (form I) mounted in a nylon loop, taken after a brief x-ray exposure. The vertical streak line penetrating through the loop marks the path of the x-ray beam. (**B**) An x-ray diffraction image from the crystal shown in (A). The dotted circle corresponds to a Bragg spacing of 1.09Å. The diffraction image was collected at the beamline 24-ID-C of the Advanced Photon Source.



Supplementary Fig. 2 | HJ-DNA resolution by *T.th*. RuvC analyzed on a native gel
The HJ-DNA substrate was incubated with the wild-type or mutant forms of *T.th*. RuvC, and the products were analyzed on a non-denaturing polyacrylamide gel. The right most lane (lane 7) has the expected resolved product assembled from three oligonucleotides that would be generated as a result of symmetrical strand cleavages at the positions indicated by arrows in Figure 1B. The intermediate band (*) is presumed to be generated as a result of single-strand nicking, as a band of the same mobility is present in the pre-nicked HJ-DNA substrate prepared by mixing 5 oligonucleotides (lane 6).



Supplementary Fig. 3 | Electron density map for *T.th.* RuvC
Ribbon drawing for a monomer of *T.th.* RuvC in the higher resolution $P2_12_12_1$ crystal form (form I), with the secondary structure elements labeled (left, same as Fig. 2A) or the σA weighted 2Fo-Fc electron density map contoured at 1.2σ superimposed (right).



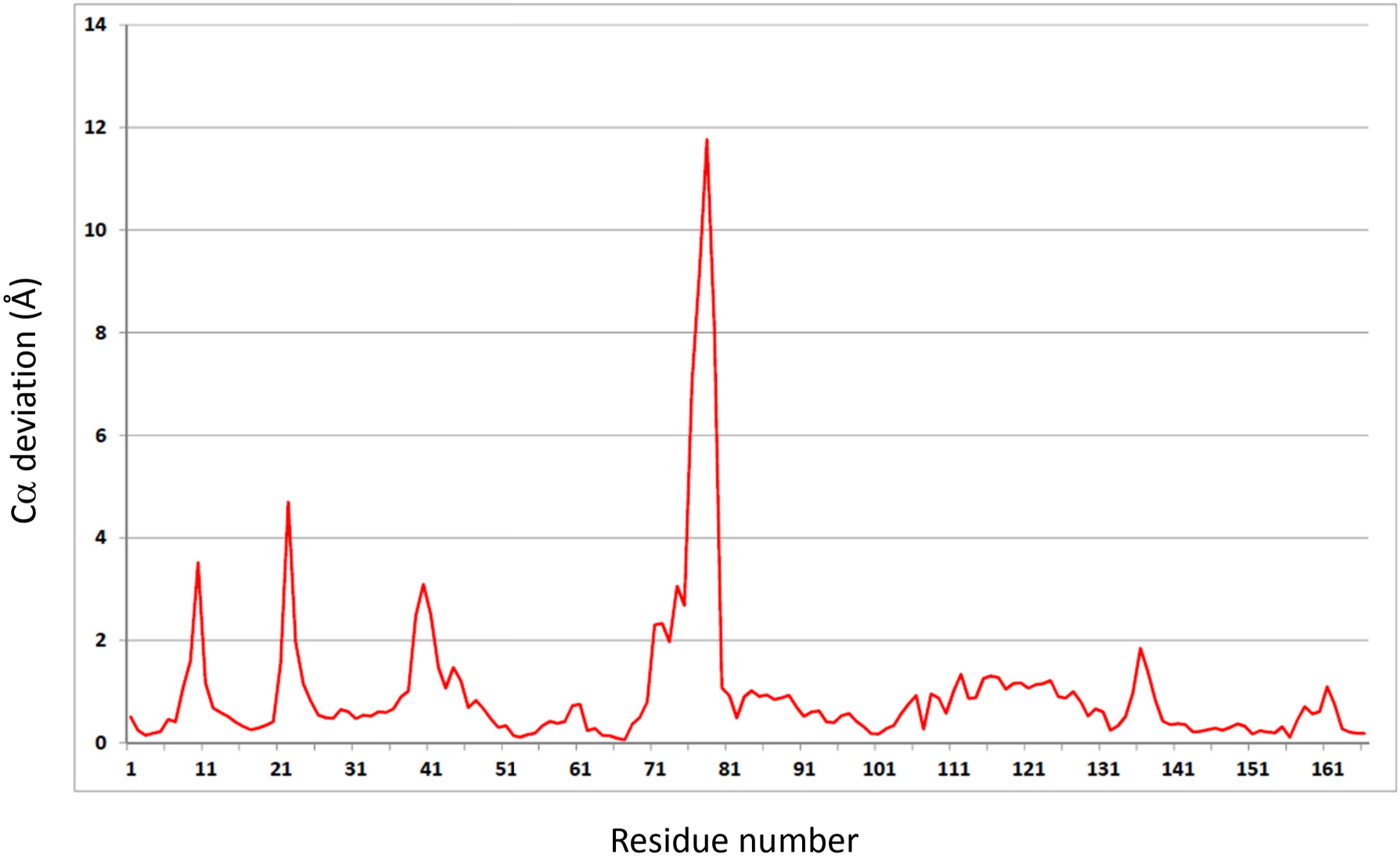
Supplementary Fig. 4 | His143 in the active site of *T.th*. RuvC.
A subset of RuvC orthologs including *T.th*. RuvC have His at this position instead of Asp as in *E. coli* RuvC. The catalytic residues His143 and Asp7 coordinate a metal ion (green sphere), putatively assigned as Mg^{2+} or Na^{+} based on the coordination distances. Simulated annealed composite omit 2Fo-Fc electron density is shown by blue meshes at a 1.0σ contour level. The red spheres represent water molecules.


RUVC_CALS8 -------MRVLGIDPGIALTGYGIIESKN--GSEFKVIDYGRIETSSSLKKSMRLLHLYT 51
RUVC_THEMA ----MGSLRILGVDPGYGIVGIGIIEVS---GNRISHVFHGTIETPKNLPAEKRLKRIYE 53
RUVC_THEEB -------MRILGLDPGLATLGYGCIEVY---RDTCQVRDFGVITTSADLPTGDRLQSLYN 50
RUVC_HELPY -------MRILGIDPGSRKCGYAIISHASN-KLSLITAGFINITTTR---LQEQILDLIE 49
D3P8S6_DEFDS -------MIIFGIDPGLNKTGVGVLELADN-NNSFRCVDYTVLKNKSSLSTIDKIAEITD 52
RUVC_THET8 -------MVVAGIDPGITHLGLGVVAVEGKGALKARLLHGEVVKTSPQEPAKERVGRIHA 53
RUVC_THEAQ -------MIVLGVDPGITHLGLGVVEAEEKGALKARLLHGEVVRTSHKEKAEARVGRIHA 53
D7BFJ8_MEISD -------MIVLGIDPGITNMGLGVVEQSGK---AHRLLHARLVKTAHSDPAPQRVGEIFQ 50
D3PTI4_MEIRD -------MIVLGIDPGITNLGIGVVEQAGK---QTRMLHAEVVKTTHGETAPERVGKLYR 50
RUVC_DEIRA -------MRVLGIDPGLANLGLGLVEGDVR--RAKHLYHVCLT-TESAWLMPRRLQYLHE 50
RUVC_DEIGD -------MIVLGVDPGLANLGLGLVEGDVR--KARHLYHVCLT-TESAWLMPRRLQYLHE 50
C1CYQ3_DEIDV ---MLSDMIVLGIDPGLANLGLGLVDGDIR--KARHLHHVCLT-TESAWVMPRRLQYIHE 54
D7CUV7_TRURR MNRTRPPLTVVGLDPGLANLGLGAVR-EVG--RAVTLLGAKLVRTSPSAEQSARLETLYR 57
RUVC_MYCTU -------MRVMGVDPGLTRCGLSLIESGRG--RQLTALDVDVVRTPSDAALAQRLLAISD 51
RUVC_ECOLI ------MAIILGIDPGSRVTGYGVIRQVGR---QLSYLGSGCIRTKVD-DLPSRLKLIYA 50
RUVC_HYDTT ------MSRIMTIDPGYSALGYAVGEEN-------KLIEYGTVYFKGK--EETRLTEIYL 45
ELCSIISLYQPDVVAIEELFFSKN-SKTAITIGEARGVIILTCIQNNLSIYEYTPLQVKQ 110
EFLKVLERFSPDECAMEKLFFVKN-VTTAIGVGEARGVLFLALAEKNIPVFEYAPNEVKV 112
DLHTLIPILQPDLVALERLFFYR--MSHTIGVAQARGVILLVLSQRHCPLLELTPPQVKQ 108
ALDCLLDRYEVNEVAIEDIFFGYN-PKSVIKLAQFRGALSLKILERIGNFSEYTPLQVKK 108
SLSKYVKRYSPLYAAVEESFYSVN-VKTAISLGMARGAIISTLLSHSVKVFQFTALQIKK 111
RVLEVLHRFRPEAVAVEEQFFYRQ-NELAYKVGWALGAVLVAAFEAGVPVYAYGPMQVKQ 112
RAKEALLAFRPEALAVEEQFFYRQ-NELAYKVGWALGAVLVAAFEAGVPVYAYGPMQVKQ 112
NLRIVIAEFRPQAIAVEEQFFYRQ-NELSYKVGWAMGAVFIAANEWGLEVYGYGPMKVKQ 109
AVYQVAAAYRPQAIAVEEQFFYRQ-NELAYKVGWAMGAVFLVADQLEIPVYGYGPPKVKQ 109
ELTRLLTEYRPDAVAIEDQILRRQ-ADVAFKVGQAFGVVQLACAQAGVPIHAYGPMQVKK 109
EVARLLAEYRPDAVAIEDQILRRQ-ADVAFKVGQAFGVVQLACAQAGVPIHAYGPMQVKR 109
EVTRLLAEYQPEAVAIEDQILRRQ-ADVAFKVGQAFGVVQLACAQAGVPIHSYGPMQVKK 113
EVSRFFALYEPDVLALEGQYFHRQ-REVAFKVGQACGVCLLAAQEHGLEVVEYGPMQVKQ 116
AVEHWLDTHHPEVVAIERVFSQLN-VTTVMGTAQAGGVIALAAAKRGVDVHFHTPSEVKA 110
GVTEIITQFQPDYFAIEQVFMAKN-ADSALKLGQARGVAIVAAVNQELPVFEYAARQVKQ 109
KIKELIQRHNPDTVLIEDYRVYKDGYKGKHKTAIVVGIICAIAYEHKIKPTFVFHNSWKA 105
SITGYGRADKTQIQKMVKSLLGLSEIPKPDDVADALAVAMCHILSSSS--VLYQEDEV-- 166
SLSGYGRASKKQIQENVKRFLNLSEIPRPDDAADALAIAWCHALQSRARRVTHEKD---- 168
ALTGYGNATKIEVQRAVQRELHLCTLPQPDDAADALAIALT---ASRH--CGHING---- 159
ALTGNGKAAKEQVAFMVKRLLNITSEIKPLDISDAIAVAITHAQRLKLH---------- 157
SVVGYGKADKNQVKKMVEMQLNISLDNVLNDVSDALACGICLAIFLKGKLYDKLY----- 166
ALAGHGHAAKEEVALMVRGILGLKEAPRPSHLADALAIALTHAFYARMGTAKPL------ 166
ALAGHGHASKEEVALMVRSALGLKETPRPSHLADALAIALTHAFYARLGAAKPL------ 166
ALVGYGHAEKEQVAFMVRAILGLKENPKPTHVADALAIALTHLFYLGRGGGNRI------ 163
ALVGTGQADKHQVAYMVRALLGLKSLPKPTHLADALAIALTHCFYQPLAPGQPVS----- 164
SLVGTGRADKEQVIYMVKASLGIRELFN-NHAADALALALTHLAHAPMQERSERLAAAGR 168
SLVGTGRADKEQIIYMVKATLGIRELFN-NHAADALALALTHLAHQPMRAASTRLAQKSA 168
ALVGTGRADKEQVIYMVKASLGVRELFN-NHAADALALALTHLAHQPMQAAA-RLARA-- 169
ALVGTGRASKAQVGYMVRATLGLKETPESHHVADALALALTHLSARQFQLLR-------- 168
AVTGNGSADKAQVTAMVTKILALQAKPTPADAADALALAICHCWRAPTIARMAEATSRAE 170
TVVGIGSAEKSQVQHMVRTLLKLPANPQ-ADAADALAIAITHCHVSQNAMQMSESRLNLA 168
KFQRVYFTIVNRLSEEWKKALGE----GSEHSRDAVMMLLPEVVSLKALLKGG------- 154
RUVC_CALS8 ------------------
RUVC_THEMA ------------------
RUVC_THEEB ------------------
RUVC_HELPY ------------------
D3P8S6_DEFDS ------------------
RUVC_THET8 ------------------
RUVC_THEAQ ------------------
D7BFJ8_MEISD ------------------
D3PTI4_MEIRD ------------------
RUVC_DEIRA AARTGDAPLRR------- 179
RUVC_DEIGD ------------------
C1CYQ3_DEIDV ------------------
D7CUV7_TRURR ------------------
RUVC_MYCTU ARAAQQRHAYLAKLKAAR 188
RUVC_ECOLI RGRLR------------ 173
RUVC_HYDTT ------------------

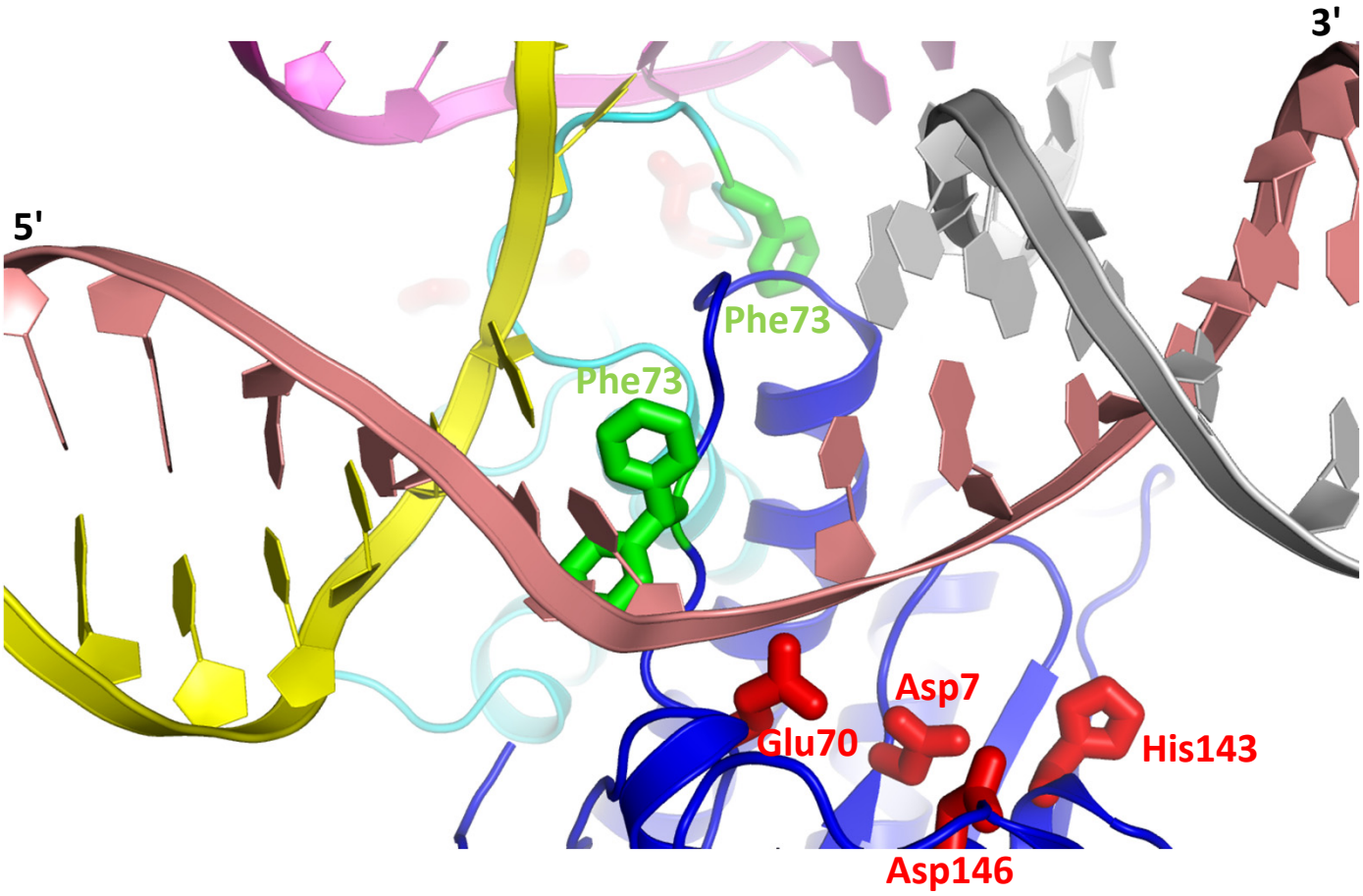
Supplementary Fig. 5 (previous page) | **Sequence alignment of RuvC orthologs.** The highly conserved catalytic residues are highlighted by yellow boxes. The aromatic residues in the asymmetric loop region of *T.th.* RuvC (Phe73, Phe74, and Tyr75), and Phe69 of *E. coli* RuvC that had been shown to be essential for DNA strand cleavage, are highlighted by grey boxes.

RuvC orthologs from the following species were aligned.

RUVC_CALS8: *Caldicellulosiruptor saccharolyticus*
RUVC_THEMA: *Thermotoga maritima*
RUVC_THEEB: *Thermosynechococcus elongatus*
RUVC_HELPY: *Helicobacter pylori*
D3P8S6_DEFDS: *Deferribacter desulfuricans*
RUVC_THET8: *Thermus thermophilus*
RUVC_THEAQ: *Thermus aquaticus*
D7BFJ8_MEISD: *Meiothermus silvanus*
D3PTI4_MEIRD: *Meiothermus ruber*
RUVC_DEIRA: *Deinococcus radiodurans*
RUVC_DEIGD: *Deinococcus geothermalis*
C1CYQ3_DEIDV: *Deinococcus deserti*
D7CUV7_TRURR: *Truepera radiovictrix*
RUVC_MYCTU: *Mycobacterium tuberculosis*
RUVC_ECOLI : *Escherichia coli*
RUVC_HYDTT: *Hydrogenobacter thermophilus*


Cα deviation (Å)
14
12
10
8
6
4
2
0
1
11
21
31
41
51
61
71
81
91
101
111
121
131
141
151
161
Residue number

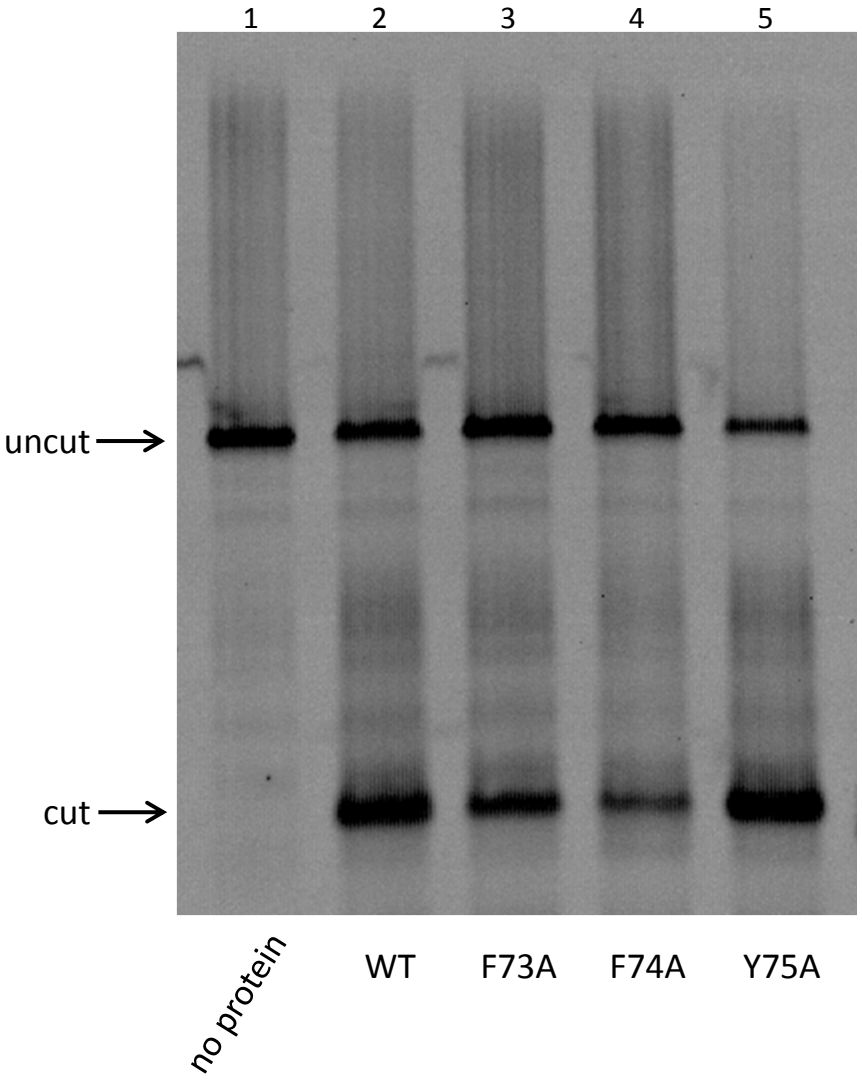
Supplementary Fig. 6 (previous page) | **Structural differences between the two RuvC molecules within the *T.th*. RuvC dimer.**
The two *T.th*. RuvC molecules in the higher resolution $P2_12_12_1$ crystal form (form I) were superimposed, and the deviation of the Cα atom positions was plotted as a function of residue number. The overall rmsd is 0.97Å.



Supplementary Fig. 7 | A close-up view around the junction point of the hypothetical *T.th*. RuvC-HJ model

The view and the color scheme are same as those in Figure 4B. Both the major and minor conformers of the Phe73 sidechain are shown for one of the protein molecules (green sticks). The asymmetric loops penetrate the central opening of HJ.

Note that the model is purely hypothetical, and therefore unlikely to be accurate in all aspects. For instance, the loop in one conformation (cyan) has a steric clash with a DNA nucleobase, implying that the DNA (and/or protein) conformation needs to be different in the real *T.th*. RuvC-HJ complex.



Supplementary Fig. 8 | Cleavage on the pre-nicked HJ substrate by *T.th*. RuvC and its mutants

The pre-nicked HJ substrate with the 5'-phosphate group at the nick (same as that used in Fig. 1C, lane 3) was incubated with the wild-type or mutant forms of *T.th.* RuvC and the products were analyzed on a denaturing polyacrylamide gel as in Fig. 6A.

In contrast to the cleavage on the intact (unnicked) HJ-DNA, the major cutting site by the F74A mutant is same as that by the wild-type enzyme.