Holliday Junctions formed from Human Telomeric DNA

**Shozeb Haider^{†, #, *}, Pengfei Li[†], Soraia Khiali[†], Deeksha Munnur[‡], Arvind
Ramanathan[§], Gary N Parkinson^{†, #, *}**

[†] UCL School of Pharmacy, University College London, London WC1N 1AX,
United Kingdom.

[‡] Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United
Kingdom

[§] Computational Science and Engineering Division, Oak Ridge National
Laboratory, Oak Ridge, TN, USA

Senior authors

*Corresponding author: gary.parkinson@ucl.ac.uk

Abstract

Cells have evolved inherent mechanisms, like homologous recombination (HR) to repair damaged DNA. However, repairs at telomeres can lead to genomic instability, often associated with cancer. While most rapidly dividing cells employ Telomerase, the others maintain telomere length through HR-dependent alternative lengthening of telomeres (ALT) pathways. Here we describe the crystal structures of Holliday junction intermediates of HR-dependent ALT mechanism. Using an extended human telomeric repeat, we also report the crystal structure of two Holliday junctions in close proximity, which associate together through strand exchange and forms a hemicatenated double Holliday Junction. Our combined structural results demonstrate that ACC nucleotides in the C-rich lagging strand (5'-CTAACCCTAA-3') at the telomere repeat sequence constitute a conserved structural feature that constrains crossover geometry and is a preferred site for Holliday junction formation in telomeres.

Introduction

Telomeres are nucleoprotein complexes that play an important role in cell replication, by protecting the ends of chromosome from degradations, fusions and checkpoint recognition and provide a genomic stable environment for the cell to divide and replicate ¹. Similar to other chromosomal regions telomeric regions are also replicated by semi-conservative DNA replication. However, the 5' to 3' unidirectional processing ability of DNA polymerase results in ever shortening telomere lengths. In human telomeres, the DNA sequence consists of hexanucleotide repeats of 5'-TAGGGT on the leading strand, ranging up to several kb in length, while the complementary C-rich lagging strand has the repeat sequence 5'-ACCCTA. A significant numbers of telomeric proteins have been identified associated with specific DNA structures at the ends of chromosomes, which reflects the complexity of replication machinery and the difficulty faced in mammalian cells to insure fidelity and progression of replication within telomeric regions ². Examples of telomere maintenance mechanisms (TMM) include the management of G-quadruplex (G4) formation ³, D-loops, (Figure 1a), R-loops ⁴, T-loop processing ⁵, topological stress ⁵, replication origins ⁶, the passage of replication forks ⁶, correct fork restart after replication stress ⁶, and dissolution of post-replicative structures.

In addition to replication, the maintenance of telomere length is critical in dividing cells ⁷. In the absence of telomere maintenance mechanisms, the end of telomeres in normal somatic cells become shorter during each replication cycle until the cells respond by either cellular senescence or undergo cell crisis. Telomere shortening is a major determinant of the cellular replicative potential in normal somatic cells ⁸. However, stem and cancer cells can replicate indefinitely by the up-regulation of the enzyme telomerase a ribonucleoprotein that synthesises new telomeric repeats to the 3' end of the linear chromosome ⁹. About 85% of all human cancers have a detectable level of telomerase that are sufficient to prevent telomere shortening ¹⁰⁻¹¹. While in the remaining cancer cases (~15%), the immortalised cancer cells elongate their telomeres through a mechanism known as the Alternative lengthening of telomeres (ALT) ¹². The resulting telomeres in ALT positive cells are heterogeneous in length, and can contain extra-chromosomal circular telomeric DNA repeats (ECTR) ¹³. The ALT pathway preferentially occurs at telomeric lagging strands ¹⁴, resulting in C-rich

strands being far more prevalent in tumour cells engaged in the ALT pathway of telomeric maintenance¹⁵.

The ALT pathway is a recombination-mediated telomere maintenance mechanism that progresses via the formation of a Holliday junction (HJ) intermediate¹⁶. A Holliday junction is a DNA motif with four interwoven nucleotide strands connecting two quasi-continuous double helices as a mobile 4-way junction¹⁷ (Figure 1). When formed between two homologous DNA duplexes it can undergo branch migration, during which, a sequential exchange of the base pairing occurs such that the branching point is effectively displaced along the DNA sequence¹⁸. The Holliday junction is structurally polymorphic when free in solution¹⁷.

Figure 1. Schematic views of models for HJ formation within human telomeric sequences. Green hue represents G-strand and purple hue the C-strand. (a) Displacement loop (D-loop) model with 3' C-strand overhang insertion into telomeric DNA with transitions to parallel, open and stacked-X anti-parallel HJs. (b) Model for exchange of telomeric sister chromosomal DNA. HJ formation transitions through open to stacked-X anti-parallel topology in the presence of magnesium ions.

In the absence of cations or under low cation salt conditions, the Holliday junction predominantly adopts a square planar open-X structure¹⁷ minimising the repulsion between the negatively charged phosphate groups on the DNA backbone where it can branch migrate¹⁹. Meanwhile, at high cation concentrations, the Holliday junction predominantly adopts an anti-parallel right-handed stacked-X structure that is characterised by the coaxial pairwise stacking of the helical arms, restricting branch migration^{17, 20-21} (Figure 1). The control over the formation of a Holliday junction,

branch migration and its resolution all have important implications in carcinogenesis, particularly in telomere maintenance dependent on ALT mechanisms ²².

The exact structural details that drive homologous recombination (HR) -dependent telomere maintenance in ALT pathways remains poorly understood. This is partly due to the lack of any structural knowledge of the **nucleic acid** substrates, intermediates and products of recombination in this pathway. There are two accepted mechanisms - break-induced replication (BIR) ²³ or DNA double strand-break (DSB) response at ALT telomeres ²⁴. In either case there is loading of ssDNA via RPA/RAD51 ssDNA binding proteins, inter-telomere recombination and associated D-loop formation. The POL δ DNA directed polymerisation commensurate with a migrating D-loop structure ending with the dissolution of the HJ ²⁵. The elongation relies on both, a unidirectional replication fork inside a D-loop and the formation of a HJ near the point of strand invasion. Within the context of telomeres, the opening of dsDNA, D-loop formation and generation of HJs with ALT-mediated telomere synthesis via intra-²⁶ or inter-telomeric recombination ²⁴ all take place in the presence of repeating hexanucleotide telomeric sequences. Additionally, when homologous recombination occurs between two homologous chromosomes or between two homologous sequences, a double Holliday junction (DHJ) can also form, to prevent the loss of heterozygosity or gross chromosomal rearrangements ²⁷⁻²⁸. Under such conditions, the two HJ branch migrate towards one another using the BTR complex (BLM-TopoIII α -RMI1-RMI2) until they form a hemicatenated intermediate that is decatenated to form non-crossover recombinants ²⁷.

Figure 2. DNA sequences and Holliday junction topologies. (a) Telomeric DNA sequences used in this study and reported crystal structures, the asterisk identifies structures deposited in the PDB. The C-rich telomeric sequences are shown in purple and complementary G-rich sequences in green. The crossover ACC triplet is shown in bold font. (b) Diagrammatic representations showing the sequences hybridised as determined in our crystallisation experiment including single (SHJ) or double (DHJ) Holliday junction (green, G-rich sequence; purple, C-rich sequence). i) Two HJ₁A and two HJ₁B strands associate together to form a SHJ topology and crystal structures SHJ₁₋₂₋₃. (ii) Two HJ₃ strands hybridised to form a hemicatenated DHJ and crystal structure DHJ₃. (c) Non-telomeric HJ forming sequences. (i) A representative 10mer length DNA sequence containing the ACC crossover triplet with the HJ generated from just two independent strands, utilizing 2-fold symmetry to generate the HJ (PDB id 1DCW). (ii) The crossover strand is shown for a sequence containing a TCC triplet (PDB id 3IGT), where the HJ is formed from four independent strands. (iii) The strands hybridize to form a SHJ in space group *P1*. The crossover is shifted by 2 nt when compared to our telomeric HJ forming topologies, an observation consistent with all previous crystallographic determinations. (d) Ribbon cartoon representation illustrating the SHJ topology from the sequence HJ₁AB. (i) Four strands associate to form two duplex DNA helices linked by a crossover at the AC step. (ii) The J_{twist} measures the relative rotation between the two helices.

Human telomeric sequences are polymorphic and can adopt multiple conformations^{3,29}. To understand the structural topology of human telomeric **double stranded** DNA in recombination and ALT processes, we conducted a biophysical and crystallographic analysis, with the aim to explore Holliday junction motifs. The design of our sequences was based on (a) a core sequence of 10 nucleotides (10mer) and (b) an extended human telomeric sequence of 42 nucleotides (42mer) that could self-assemble to form a contiguous Holliday junction. In this study, we report on five crystal structures of Holliday Junction intermediates of ALT mechanism, formed from human telomeric sequence. In two of the five structures, we describe and explore the topology of a hemicatenated double Holliday junction in close formed at telomeric ends. We identify conserved structural features that constrain crossover geometry in both, single and consecutive Holliday junctions. And finally, we provide a structural model on how strand exchange could be centred on ACC trinucleotides within telomeric Holliday junction folded motifs.

Results

We have determined three interrelated single Holliday junction structures formed from human telomeric 10mer strands (A strand = 5'-CTAACCCTAA-3'; B strand = 5'-TTAGGGTTAG-3'; Figure 2, S1a). Two of the Holliday junctions are packed in space group *P1*, consisting of either a large unit cell containing 8 strands folded into two independent Holliday junctions within the Asymmetric Unit (ASU), or a smaller unit cell where the *c*-axis is halved, which results in 4 strands folded into a single Holliday junction (Figure 3, Supplementary movie 1). The third packing arrangement for the SHJ₁-2 is within the monoclinic space group *C2*, which also contains 4 strands folded into one Holliday junction per ASU.

The structure that we observe in the *C2* space group is similar to a reported structure of sequence 5'-CCGGTACCGG-3' (PDB-id 1DCW, and related 1DCV with the sequence 5'-CCGGGACCGG-3')³⁰. Here 1DCW HJ is formed from an inverted repeat sequence, enabling the formation of a four-stranded HJ from one 10mer sequence utilizing dimerization and an internal 2-fold symmetry axis (Space group *C2*, Figure S1b). In all three cases the telomeric sequence folds into SHJ₁ structures with a right-handed (positive) rotation angle as seen previously for crystallised non-

telomeric sequences. The arms of the HJ are B form DNA, in a right-handed stacked-X conformation with an interduplex angle of ~67 degrees and with the crossover step associated with the ACC trinucleotide in the sequence 5'-CTAACCCTAA-3', (Figure 3).

The general consensus sequence in crystallised HJs is a trinucleotide Purine-Pyrimidine-Cytosine (PuPyC) core at the junction crossover³¹. Our analysis of the crystallised HJ structures in the PDB revealed a conserved motif containing the sequence CCnnnN₆N₇N₈GG, where n is any nucleotide and N₆N₇N₈ is typically represented by an ACC triplet and is in accord with Hays et al.³². The structural importance of this sequence is consistent with the observation that cytosine at position N₈ can form a direct hydrogen bond to the 5' crossover phosphate (denoted by Pn-1) at the U-turn of the crossing strands (Figure 3e,f). The structure adopted by the ACC triplet is the focal point of the crossover geometry. The PuPyC triplet core have been shown to promote HJ formation in solution and seen by atomic force microscopy to define the crossover points in HJs in 2D-lattice^{31, 33-34}. In our context, the telomeric C-rich strand contains the ACC triplet 5'-CTAACCCTAA-3'.

Table 1: Geometric parameters of telomeric Holliday junction crystal structures. The calculations of the geometric parameters of IDA, J_{twist}, J_{roll} and J_{slide} were adapted from Watson et al.³⁵

Structure ID	IDA (degrees)	J _{twist} (degrees)	J _{slide} (Ang)
SHJ ₁ -1	65.5	66.8	0.26
SHJ ₁ -2	69.6	68.7	0.30
SHJ ₁ -3	67.9	67.4	0.18
DHJ ₂ (i)	74.9	77.3	0.47
DHJ ₂ (ii)	68.6	79.3	0.28
DHJ ₃ (i)	76.9	77.3	0.47
DHJ ₃ (ii)	68.2	75.9	0.22

The placement of the AC step in our 10mer telomeric sequence (HJ₁AB) results in a relative shift in the spatial position of the AC step and consequently a change in the lengths of the duplex arms when compared to previous reported 10mer HJ structures (Figure 2bi, ciii). Our survey of the PDB for successfully crystallised Holliday junctions consisting of 10mer sequences reveals that these sequences are commonly designed to place the HJ AC step between nucleotides 6-7 nt, measured from the 5' end, using a single inverted repeat sequence. These inverted repeat sequences (Fig 2c) typically fold into three related structural arrangements which all pack in space group *C2*. Alternatively, HJs have also been formed using four independent strands. The Ho group has reported on a HJ that packs into space group *P1*, PDB-ID 3IGT³⁶, similar to our *P1* packed HJs (Figure 2ciii). In this arrangement, the HJ arms take on a normal B-form DNA conformation, where the junction is in a stacked-X conformation with a crossed angle 65.5 degrees, a right-handed X, with the step associated with the ACC trinucleotide sequence (5'-CCGGTACCGG-3'). Figure 4a shows SHJ₁₋₃ in space group *P1* overlaid with 3IGT structure, where again the AC step can be seen shifted 2nt relative to the 5' and 3' positions, but with a similar B-form DNA conformation for the arms.

Figure 3. Holliday junctions formed from HJ₁AB and HJ₃ telomeric containing sequences. 2Fo-Fc electron density maps (1 σ) of (a) SHJ₁-1 crystallised in space group *P1*, containing two HJs in the ASU and (b) DHJ₃ formed from two HJ₃ strands within the ASU, crystallised in space group *P2*₁. DHJ₃ telomeric DNA sequences are coloured in purple/pink (C-rich) and green/olive green (G-rich). (c) Superimposition of SHJ₁-3 containing four 10mer strands, (green/G-rich/HJ₁B, purple/C-rich/HJ₁A) onto the 3IGT structure (yellow, strands A-D). The arrows illustrate the shift of 2nt in the 5' direction relative to the ACC triplet. (d) SHJ₁-1 drawn with 2Fo-Fc electron density map (1 σ) around the crossover junction. The location of the A₆-C₇-C₈ motif has been labelled. (e) A close-up view of the crossover geometry around the ACC triplet. The N4 atom of C₇ and C₈ forms strong electrostatic interactions with the phosphate backbone atom of A₆. Pn-1 denotes the 5' crossover phosphate (f) 2Fo-Fc electron density map (1 σ) of one DHJ₃ HJ crossover highlighting the electrostatic interactions between N4 atoms of C₁₇ and C₁₈ and the phosphate backbone of A₁₆.

Multiple crystals were screened and three interrelated unit cells were identified (Table S1). The selection of *P1* space group for SHJ₋₁ and 3 structures was based on data collection statistics for merging R-factors and overall refinement statistics. The large *C2* cell (SHJ₋₂) has four independent strands in the asymmetric unit; unlike previous determinations in space group *C2* where the symmetry is broken by our two independent complementary strands, A and B. HJ structures consistently crystallize in the *C2* space group while the *P1* space group has only been observed once before (PDB id 3IGT). The cell dimensions of 3IGT are similar to that observed in SHJ₋₃. The helical axis is parallel to the B-axis and the internal duplex angles are also comparable with our telomeric structure packing in a similar way. It is interesting to note that the position of the ACC crossover shifts the arms ± 2 nucleotides along the helical axes irrespective of packing in *P1* or *C2* space groups (Figure 3c).

The 42mer HJ₂ and 3 sequences were designed to hybridise and self-assemble into a single stable intramolecular HJ (Figure 2a) with a mixture of G-rich and C-rich strands linked by CTTG hairpins, however a more complex arrangement is observed in the crystal lattice. Unexpectedly, we observe a strand exchange between residues T₁₃ and G₂₂ of strands A and B resulting in the formation of a dimer (Figure 2bii, 3b, Supplementary movie 2). Crystallising in space group *P2₁* the dimer consists of two 42mer strands (HJ₃) packed into the ASU, hybridizing together through G-rich and C-rich strands. The intramolecular (Figure 2bii) folded dimer forms a complex structural arrangement resulting in the formation of two Holliday junctions positioned symmetrically about an extended helical axis, (Figure 3b, 4d). It is important to note that the two Holliday junctions are independent of crystallographic symmetry operators.

Figure 4. Comparisons of Holliday junction geometries. (a) Crystal structure of SHJ₁₋₃ (green/G-strand/HJ₁B, purple/C-strand/HJ₁A) overlaid on to the HJ DNA extracted from the resolvase HJ/protein complex (yellow) PDB id 2WJO. (b) Crystal structure of our single HJ, SHJ₁₋₃ (green/G-strand/HJ₁B, purple/C-strand/HJ₁A) superimposed on to our hemicatenated double HJ structure DHJ₃, coloured (monomer A/gold, monomer B/blue). (c) Superposition of DHJ₃ structure containing two HJ₃ strands (C-rich/purple/pink, G-rich/green/olive green) on to the T4 Endonuclease protein/DNA complex (strand A gold, strand B orange, PDB id 2QNC), aligned by overlapping the first 20nt of the DHJ₃. The arrow indicates the opening of the HJ crossover by T4 Endonuclease in 2QNC complex. (d) DHJ₃ formed from two HJ₃ strands A and B displaying the close proximity of two HJs and associated unpaired thymines. The solid lines indicate the helical axis. (e) The core DHJ₃ structure is extended on the 5' and 3' ends to illustrate the overall geometry of two HJs in close proximity. The overall topology resembles that of a hemicatenated double Holliday junction.

The paired nucleotides adopt a regular B-form duplex DNA conformation allowing us to define four helical axes as two right-handed stacked-X conformations representing a model for a double HJ. Both junctions are in a right-handed stacked-X conformation, with the step positioned in the ACC trinucleotide sequence within the C-rich telomeric sequence. The two longer 11nt DNA strands adopt a regular B-DNA form and are linked by two unpaired thymines (T₁₃) pushed out from the helical axis to associate with the minor grooves of adjacent symmetry related helices, facilitating lattice stabilisation (Figure 4d). The short 7nt duplex strands form semi-

continuous helices (Figure 4), for both HJs adopting a regular B-form DNA, distorted locally at the terminating short hairpin loops (CTTG). The introduction of a short 4nt hairpin loop was crucial to ensure a standard dsDNA B-conformation for the short 7bp sequences in the HJ arms (Figure 3b). Thymine T₂₀₋₂₁ and T₂₉₋₃₀ in the short hairpin loops successfully transition the two 7nt duplex DNA stems with the 5' thymine tucked into the minor groove of the DNA, displaying consistently the previously observed folded topology.

Consistent with the shorter 10mer sequence (Figure 3d) we observe the same ACC trinucleotide sequence involved in formation of HJs for both the HJ₂ and ₃ constructs (Figure 3f). All three sequences, independent of packing arrangements, display the same folding topology with clear and unbroken electron density (Figure 3a,b,d,f), consistent in all cases with well-ordered arrangement of nucleotides in the lattice and the formation of a stable HJ. To help describe the spatial relationship observed for the two HJs formed from the association of the two HJ₃ 42mer strands we have coloured the G-rich sequences with green hues and C-rich sequences with purple hues (Figure 4d, S1e and schematic 2bii), and omitted drawing unpaired nucleotides. To further help visualise two consecutive HJs, as a generic model for a hemicatenated double Holliday junction (DHJ), we extended all four dsDNA ends of DHJ₃ (Figure 4e, Supplementary movie 3), highlighting the dsDNA-A G-rich strand (green) extending from across the consecutive two HJs linking to the dsDNA-A' strand, equivalent to the second G-rich strand dsDNA-B (olive green), linking across to dsDNA-B'. The C-rich strands shown in pink form the central core of a plausible hemicatenated DHJ motif.

To understand the HJ sequences increased thermal stability and to characterise the possible folded topologies for all five HJ sequences both thermal melt experiments and PAGE studies were conducted. Experimental conditions were chosen to be similar to the buffers used in the crystallisations containing the same monovalent metal ions with the addition of either, Mg²⁺ or Ca²⁺ ions. A summary of the melt experiments are shown in Figure S2 and described in detail in the supplementary information section. In summary the HJ₁AB shows strong divalent metal ion dependence, with a T_m of 29°C when annealed without divalent metal ions, increasing by +9°C to 38°C in the presence of 30 mM Ca²⁺. Similarly HJ₃ annealed without

divalent metal starts with a T_m of 54°C show a strong dependence reaching a maximum of +14°C to 68°C in the presence of 30 mM Ca^{2+} ions and +19°C in 30 mM Mg^{2+} . In order to understand the possible role of the ACC crossover triplet observed in the formation of a consecutive Holliday junction, we designed modified sequences, where the ACC triplet was replaced by TGC at positions 16 and 17 (HJ₃-M1) and a double substitution at positions 16, 17 and 34, 35 (HJ₃-M2) (Figure S3). The TGC sequence is not represented as a junction-forming triplet in any structure currently available in the PDB. The modified sequences HJ₃-M1 and HJ₃-M2 were first annealed without Mg^{2+} and both displayed a significantly reduced thermal stability (T_m of around 48°C). The addition of 30 mM Mg^{2+} increased their thermal stability to around 63°C indicating the strong dependence on divalent metal ions and possible HJ formation. Attempts to crystallize both modified HJ₃-M1 and -M2 sequences with and without divalent salts proved unsuccessful. The HJ₃ series of sequences were also subjected to native PAGE (Figure S7), confirming HJ formation as cruciform, with only the HJ₃ indicating potential DHJ formation in the presence of Mg^{2+} ions, experimental results are discussed in the supplementary section.

Discussion

We have structurally demonstrated that the human telomeric sequence can form Holliday junctions. Our design of experiments was based on the published findings that telomeric sequences are involved in ALT mechanism via homologous recombination forming a HJ intermediate and the presence of the ACC triplet in the C-rich strand repeated every 6 nucleotides. A review of telomeric sequences for all Groups and Organisms with linear DNA shows that either the ACC or the complementary sequence GGT can be seen in all identified telomeric sequences³⁷ except for Roundworm, *Ascaris lumbricoides*. Telomeric sequence 5'-TTAGGC(GCCTAA)-3' in this organism contains an alternative N₆N₇N₈ HJ forming step³², within an amphimorphic sequence. The telomeric ACC triplet may have a significant but an unidentified role in telomere recognition and maintenance, associated with a greater propensity to form Holliday junctions, which would need to be managed to prevent unwanted strand exchange or cleavage. There is indeed significant recruitment of helicases to ALT telomeres experiencing replicative stress, such as Fanconi anaemia group J protein (FANCI), SMARCA1 (SWI/SNF Related,

Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A-Like 1) that stabilizes replication forks during DNA damage and Bloom syndrome helicase (BLM) a member of the helicase family that can resolve G4 DNA, D-loop structures, and Holliday-junction DNA in vitro. From this idea,³⁸ it was proposed that a favoured junction motif might then provide a suitable stable substrate for recognition and binding by proteins involved in recombination and DNA integration processes.

We successfully generated diffracting crystals by altering the position of the ACC triplet to nucleotides 4-5-6, in the 10mer sequences compared to those used in previous investigations, reproducing the stacked-X form. This also resulted in a new packing arrangement for the 10mer oligonucleotides. We were also successful in our crystallisation trials with a 10mer human telomeric sequence with the ACC step positioned at steps 6-7-8 but this resulted in poorly diffracting crystals and no further analysis was performed. HJs intrinsically have two-fold symmetry (Figure 3a and 3b). HJs have been reported in two conformations - the stacked-X form closed conformations as observed in our crystallographic studies, and the open conformation (Figure 1). The relative populations of these two conformers depend on salt concentrations and the central eight base pairs near the crossover junction. HJs are dynamic. Single-molecule measurements have shown that the junction can exist in multiple structural isoforms³⁹, which have to transition via an open conformation (Figure 1). Our schematic representation of the HJs corresponding to the 3D structural topologies for SHJ and DHJ are illustrated in Figure S4. **A comparison of telomeric HJ topologies with that of HJs co-crystallised in the presence of DNA junction-resolving enzymes, T4 Endo (VII) and T7 Endo (I) reveals an overall similarity of topology between the HJs particularly for the T4 Endo (VII) complex, as shown for the DHJ (Figure 4c). While for the T7 Endo (I) complex it would be of interest to determine if the HJ₃ telomeric sequence has the necessary flexibility to adopt the distorted “induced fit” HJ topology.⁴⁰**

Sequence Dependence and HJ Conformations

A comprehensive analysis of the N₆N₇N₈ step sequence³², also identified three amorphous core motifs GCC, ACC and CCC that are stabilized by divalent cations in Holliday junctions, which retain the cytosine at position N₈ to phosphate (Pn-1) hydrogen bond. The HJ formed from 4 strands (PDB-id 3IGT)³⁶ containing

the TCC step shows the loss of this stabilizing interaction with Cyt₈-N4 amino atoms to the 5' crossover phosphate (Pn-1) at around 4.8 Å, while for the CTG step it is about 4.9 Å from the 5' crossover phosphate (Pn-1). In all our SHJ₁ and DHJ₃ crystal structures the equivalent Cyt₈-N4 amino atoms are > 6 Å from the 5' crossover phosphate (Pn-1).

The conformations observed for the 42mer HJ₂ and HJ₃ sequences correspond to the conformation that leads into adopting a hemicatenated DNA structure. Each strand of duplex DNA is associated with its complementary strand in the other duplex through base pairing and topological linking resulting in two consecutive HJ structures (Figure 4d,e). Our finding is consistent with tandem repeating sequences at telomeric ends, where Holliday junctions have been identified as intermediates of double stranded break-promoted homologous recombination (HR) repair mechanism²⁴. Although no double Holliday junctions have yet been reported to occur in telomeric DNA, the structure that we observe closely resembles the topology of a hemicatenated double Holliday junction and can be representative of these motifs formed elsewhere in the genome.

The exact structural mechanism that drives HR-dependent telomere maintenance in ALT mechanism remains poorly understood. There is limited **structural** knowledge of the **nucleic acid** substrates, intermediates and products of recombination in this pathway. The biological relevance of our Holliday junction structures is supported by initial evidence from Reddel group, who describes the presence of single-stranded C-rich DNA circles as indicators for an active ALT mechanism¹³. It still remains unclear how such circles are generated. Subsequently, Oganessian and Karlseder reported that mammalian C-rich strand were far more prevalent in tumour cells engaged in the ALT pathway of telomeric maintenance, which relies on HR machinery, thus implicating the involvement of C-rich strand in the HR-dependent pathway of telomeric maintenance¹⁵. This is consistent with our structural finding that the crossover junction is centered on ACC trinucleotides within the C-rich strand. Additionally, it has been demonstrated from *in vitro* strand invasion assays that artificially constructed 3' G-rich and 5' C-rich overhangs of vertebrate telomeric sequences can invade double-stranded DNA with equal efficiency⁴¹. Indeed, rare surviving ALT cells derived from TERC (TR) gene knockouts were preferentially

elongating their telomeric lagging strands as 5' C-rich overhangs ¹⁴. This elongation on the C-rich strand is only possible via HR-dependent pathway involving a Holliday junction intermediate structure as revealed in this study.

In conclusion, we have solved multiple crystal structures of the HJs formed from the human telomeric sequence with two HJs in close proximity, which resembles structural features of a hemicatenated double Holliday junction. We identify the C-strand 5'-CTAACCCTAA-3' telomeric repeat sequence, containing the ACC nucleotide motif, as an essential prerequisite for the structure to adopt the crossover geometry, which is confirmed in both single and consecutive HJ structures. The 3' G-strand overhang is particularly susceptible to damage from UV light and other oxidative agents due to its heterochromatic nature ⁴²⁻⁴⁴. It is therefore tempting to speculate that the selective pressure to preserve the TERRA coding C-rich strand is of importance and that ALT positive cells have evolved and adopted this as a mechanism in absence of telomerase activity.

Methods

Sample preparation and crystallization

All sequences used in these studies were purchased from Eurofins, HPLC purified: a) strand A, 10mer 5'-CTAACCCTAA-3' HJ₁A, strand B, 10mer 5'-TTAGGGTTAG-3' HJ₁B, containing the human telomeric sequence, were designed to directly mimic previous successful crystallisations of 10 mer DNA Holliday junction sequences, which required the addition of a complimentary sequences to form the intermolecular Holliday Junction (Figure 1). While the 42mer sequence 5'-GGTTAGGGTTCTTGAACCCTTGGGTTACTTGTTACCCTAACC-3' (HJ₂) and the modified 42mer 5'-GGTTAGGGTTCTTGAACCCTTGGGTTACTTGTAACCCTAACC-3' (HJ₃) were designed to self-assemble intramolecularly to form a single Holliday junction (Figure S1c, d). The G-rich strands are connected by complimentary C-rich strands, using the stable CTTG mini hairpin loop sequence ⁴⁵, which should allow self-assembly into a HJ, similar to the NMR structure PDB-ID 2F1Q. All oligonucleotides were initially dissolved in water to a final concentration of 2.5 mM before annealing. The HJs were assembled by means of annealing in presence of salts and buffer, 50

mM KCl, 20 mM potassium cacodylate pH 6.5 and diluted to final concentrations of 1.0 mM dsDNA for HJ₁AB and 0.5 mM for HJ_{2,3}. The resulting mixtures were slowly cooled from 90°C to 20°C overnight to induce HJ formation.

Typically, crystallizations were carried out using small sample volumes (1.0 μ L, of preformed HJ DNA at concentrations around 0.5 mM), and combined with the reservoir solutions at 1:1 ratios. Screening of many known conditions allowed us to identify conditions for the growth of crystals HJ₁AB. In particular well-formed crystals formed with salts between (50mM for MgCl₂, 20mM for CaCl₂) and a higher (150mM for MgCl₂ and 80mM for CaCl₂), combined with an MPD concentration that ranged from 32% to 38% incubated at 12°C. Over a period of about a week, typically large flat plate like crystals formed. Crystals containing HJ₂ sequence were identified in the Natrix (Hampton Research) screen condition 16, 0.04 M Magnesium acetate tetrahydrate, 0.05 M Sodium cacodylate trihydrate pH 6.0, 30% v/v (+/-)-2-Methyl-2,4-pentanediol. Large flat plate-like crystals were harvested in n-paratone oil and frozen in N₂ gas stream or in liquid N₂ for storage, screening and remote data collections at undertaken at the Diamond synchrotron. Crystals containing the HJ₃ sequence were grown from conditions similar to those identified for the HJ₂ sequence.

Data collection and structure solution

In all cases crystals were harvested using loops transferred to paratone-N oil and successfully cryo-protected by flash freezing in Nitrogen. X-ray data collections and screenings were conducted at either the Diamond Light Source synchrotron facilities or at our in-house X-ray source using an Oxford Diffraction Xcalibur Nova system, with a hi-flux Cu X-ray micro-focus source, and a Titan CCD area detector (Table S1). Titan CCD image data were processed by CrysalisPro⁴⁶ (Agilent Technologies), and synchrotron data sets were processed and scaled by using XDS, SCALA and XIA2 programs. In all cases molecular replacement methods were used successfully to determine the relative orientation and position of the HJs in the asymmetric unit using the PHASER program⁴⁷. The HJ₁AB annealed sequence crystallised into three interrelated packing arrangements, a larger P₁ cell containing 8 strands in the ASU (SHJ₁-1), a smaller P₁ cell with 4 strands in the ASU (SHJ₁-3), and in the monoclinic space group C₁₂₁ with 4 strands in the ASU (SHJ₁-2). While DHJ₂ and DHJ₃ both

crystallised in a monoclinic space group P_{121} with 2 strands in the ASU. Both SHJ₁₋₁ and DHJ₂ were collected on the home source ($\lambda=1.5418$ Å at 105 K), whilst the rest were collected at the Diamond synchrotron source: SHJ₁₋₂ at beamline IO4-1, ($\lambda=0.91741$ Å), SHJ₁₋₃ at I24 ($\lambda=0.92016$ Å), and DHJ₃ at IO4-1 ($\lambda=0.91587$ Å) all at 100 K. The SHJ₁₋₁ structure was determined using PDB model id 3HS1, using residues 1-10 from chain B and residues 1-6 from chain A, while DHJ₃ and SHJ₁₋₂ structures were solved using a truncated and refined SHJ₁₋₁ model consisting of the core DNA duplex decamer of human telomeric sequence. Model building and refinement were performed using COOT⁴⁸ and REFMAC5⁴⁹ programs. Mg²⁺ ions were located using difference Fourier maps (Fo-Fc). Final R and R_{free} values are in Supplementary tables 1 and 2. Atomic Coordinates and structure factors are available from the PDB as entries (SHJ₁₋₁, PDB-id 6DGH; SHJ₁₋₃, PDB-id 6DGS; DHJ₃, PDB-id 6DGN). The figures were generated using ICM-Pro⁵⁰, PyMol⁵¹⁴⁸ and Chimera⁵².

HJ Stability experiments

The oligonucleotides (HJ₁AB, HJ₃) were prepared in 20 mM K cacodylate buffer (pH 6.5) containing 50mM KCl at a concentration of 0.85 mM and 0.5mM respectively without Mg²⁺ and annealed by heating at 90°C for 5 min, cooling to 4°C at 1°C per minute prior to thermal melt experiments. Thermal melt experiments were carried out on a CARY spectrophotometer using quartz cells of 1 cm path length, at 260 nm. Samples were first diluted in a 50 mM Na cacodylate buffer (pH 7.0) and water at a concentration of 1,3µM and 1,5µM respectively, and titrated from 0 to 30 mM divalent metal ions. The signals were collected after each step of increasing temperature by the rate of 1°C /min from 15 to 65°C for HJ₁AB and from 20°C to 90°C for the HJ₃ family of sequences.

Mobile telomeric Holliday junction design

Mobile telomeric Holliday junctions (tHJ) (Table S4), were constructed using two component partial duplexes (strands A, A', B, B'), and designed for the PAGE and FRET-based assays. The tHJ construct include regions of sequence homology so spontaneous branch migration could occur. The 5' tail of the FAM-labelled partial duplex is complementary to the 3' tail of the TAM-labelled partial duplex; similarly, the 3' tail of FAM-labelled partial duplex is complementary to the 5' tail of the TAM-

labelled partial duplex. As such, once the annealing of the component partial duplexes via the complementary single-strand tails was completed, a mobile telomeric Holliday junction intermediate would spontaneously branch migrate. The branch migration would only traverse the length of the duplex region. Termination of the branch migration results in the irreversible dissociation of the Holliday junction intermediate into two duplex products AB and A'B' (Figure S5). The component partial duplexes were labelled with either the reporter dye carboxyfluorescein (FAM) or the quencher dye carboxy-tetramethylrhodamine (TAM). The HPLC-purified oligonucleotides were purchased from EUROFINs and were re-suspended dd- H₂O to a final concentration of 100 μ M.

Polyacrylamide Gel Electrophoresis (PAGE) and Branch Migration Assay

To help characterise the HJs folded topologies in the presence or absence of Mg²⁺, oligonucleotides (HJ₁AB, HJ₃, HJ₃-M1, HJ₃-M2), were selected and prepared in buffers to mimic crystallisation conditions, 20 mM K cacodylate buffer (pH 7.0) containing 50mM KCl at a concentration of 0.5 mM or 0.01 mM and annealed by heating at 90°C for 5 min, cooling to 4°C at 1°C per minute prior to PAGE. Gel electrophoresis was carried out at 10°C using 17 % polyacrylamide gel (29:1 polyacrilamide/bis-acrylamide) that contained either 0% or 5 mM of a chosen divalent cation salt to stabilise HJ formation during separation. The native TB buffer (90 mM Tris-base, 90 mM boric acid, pH 8.2) was used. To ensure most of the substrates migrated into the gel rapidly, a constant voltage of 120 V was applied. The gel was stained using SYBR-Green-I Stain (brand) for 30 minutes before it is visualised under the Molecular Imager® Gel Doc™ XR System and examined using ImageLab. Four additional PAGE assays were carried out with the same PAGE assay mixtures but also contain 5 mM of either MgCl₂ or CaCl₂: these PAGE assays were used to compare the relative folded topologies and their retarding effect as a function of different cation types and their concentrations.

The PAGE based branch migration assay was carried out using four extended DNA oligonucleotides each containing three human three telomeric repeats within 5' and 3' complimentary regions (A, A', B, B') such that they can be annealed sequentially to form a mobile Holliday junction with a telomeric sequence at its core. A PAGE assay mixture containing 10 μ M partial duplex AA' and BB' was set up by diluting

an equimolar of the partial duplexes with ice-cold dd H₂O. The assay mixture was immediately incubated at 37°C to commence the branch migration. 0.5 µL of the assay mixture was withdrawn in each chosen time interval and was diluted in 50 mM ice-cold divalent cation salt solution to a final volume of 15 µL. All of the samples, collected over a period of 30 minutes, were placed on ice prior to electrophoresis. The PAGE was carried out at 10°C using 8 % polyacrylamide gel (29:1 polyacrilamide/bis-acrylamide) that contained 10 mM of a chosen divalent cation salt to retard branch migration during separation. The native TB buffer (90 mM Tris-base, 90 mM boric acid, pH 8.2) was used. To ensure most of the substrates migrated into the gel rapidly, a constant voltage of 300 V was applied initially for 5 minutes. Subsequently, a constant ampere of 15 mA was applied to minimise gel heating. The gel was stained using SYBR-Green-I Stain (brand) for 30 minutes before it is visualised under the Molecular Imager® Gel Doc™ XR System and examined using ImageLab. Four additional PAGE assays were carried out with the same PAGE assay mixtures but also contain 20 mM or 50 mM of either MgCl₂ or CaCl₂ Figure S6: these PAGE assays were used to compare the branch migration retarding effect as a function of different cation types and their concentrations.

Supporting Information

Crystallographic collection information, branch migration assays, HJ stability and salt concentration dependence, thermal melt experiments, schematic representations of modified HJ sequences, native PAGE, geometric parameters of HJ, movies of structures

Acknowledgements

A.R. was supported in part by the Laboratory Directed Research and Development Program of Oak Ridge National Laboratory, managed by UT-Battelle, LLC, for the U. S. Department of Energy. This manuscript has been authored by UT-Battelle, LLC under contract No. DE-AC05-00OR22725 with the U.S. Department of Energy. S.H. would like to thank Profs. S.C. West and I.D.Hickson, for their critical comments on the manuscript.

Competing Interests

The authors declare no competing financial interests.

Correspondence to GNP (gary.parkinson@ucl.ac.uk) and SH
(Shozeb.haider@ucl.ac.uk)

References

1. de Lange, T., How telomeres solve the end-protection problem. *Science (New York, N.Y.)* **2009**, 326 (5955), 948-52.
2. Martinez, P.; Blasco, M. A., Replicating through telomeres: a means to an end. *Trends in biochemical sciences* **2015**, 40 (9), 504-15.
3. Parkinson, G. N.; Lee, M. P.; Neidle, S., Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature* **2002**, 417 (6891), 876-80.
4. Rippe, K.; Luke, B., TERRA and the state of the telomere. *Nature structural & molecular biology* **2015**, 22 (11), 853-8.
5. Erdel, F.; Kratz, K.; Willcox, S.; Griffith, J. D.; Greene, E. C.; de Lange, T., Telomere Recognition and Assembly Mechanism of Mammalian Shelterin. *Cell Rep* **2017**, 18 (1), 41-53.
6. Higa, M.; Fujita, M.; Yoshida, K., DNA Replication Origins and Fork Progression at Mammalian Telomeres. *Genes (Basel)* **2017**, 8 (4).
7. Pfeiffer, V.; Lingner, J., Replication of telomeres and the regulation of telomerase. *Cold Spring Harb Perspect Biol* **2013**, 5 (5), a010405.
8. Hastie, N. D.; Dempster, M.; Dunlop, M. G.; Thompson, A. M.; Green, D. K.; Allshire, R. C., Telomere reduction in human colorectal carcinoma and with ageing. *Nature* **1990**, 346 (6287), 866-8.
9. Counter, C. M.; Ailion, A. A.; LeFeuvre, C. E.; Stewart, N. G.; Greider, C. W.; Harley, C. B.; Bacchetti, S., Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *Embo j* **1992**, 11 (5), 1921-9.
10. Greider, C. W.; Blackburn, E. H., A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature* **1989**, 337 (6205), 331-7.
11. Shay, J. W.; Bacchetti, S., A survey of telomerase activity in human cancer. *Eur J Cancer* **1997**, 33 (5), 787-91.
12. Bryan, T. M.; Englezou, A.; Gupta, J.; Bacchetti, S.; Reddel, R. R., Telomere elongation in immortal human cells without detectable telomerase activity. *Embo j* **1995**, 14 (17), 4240-8.
13. Henson, J. D.; Cao, Y.; Huschtscha, L. I.; Chang, A. C.; Au, A. Y.; Pickett, H. A.; Reddel, R. R., DNA C-circles are specific and quantifiable markers of alternative-lengthening-of-telomeres activity. *Nat Biotechnol* **2009**, 27 (12), 1181-5.
14. Min, J.; Wright, W. E.; Shay, J. W., Alternative lengthening of telomeres can be maintained by preferential elongation of lagging strands. *Nucleic Acids Res* **2017**, 45 (5), 2615-2628.
15. Oganessian, L.; Karlseder, J., Mammalian 5' C-rich telomeric overhangs are a mark of recombination-dependent telomere maintenance. *Mol Cell* **2011**, 42 (2), 224-36.
16. Nabetani, A.; Ishikawa, F., Alternative lengthening of telomeres pathway: recombination-mediated telomere maintenance mechanism in human cells. *Journal of biochemistry* **2011**, 149 (1), 5-14.
17. Duckett, D. R.; Murchie, A. I.; Diekmann, S.; von Kitzing, E.; Kemper, B.; Lilley, D. M., The structure of the Holliday junction, and its resolution. *Cell* **1988**, 55 (1), 79-89.

18. Grainger, R. J.; Murchie, A. I.; Lilley, D. M., Exchange between stacking conformers in a four-Way DNA junction. *Biochemistry* **1998**, 37 (1), 23-32.
19. Clegg, R. M.; Murchie, A. I.; Lilley, D. M., The solution structure of the four-way DNA junction at low-salt conditions: a fluorescence resonance energy transfer analysis. *Biophys J* **1994**, 66 (1), 99-109.
20. Duckett, D. R.; Murchie, A. I.; Lilley, D. M., The role of metal ions in the conformation of the four-way DNA junction. *EMBO J* **1990**, 9 (2), 583-90.
21. Ortiz-Lombardia, M.; Gonzalez, A.; Eritja, R.; Aymami, J.; Azorin, F.; Coll, M., Crystal structure of a DNA Holliday junction. *Nature structural biology* **1999**, 6 (10), 913-7.
22. Tarsounas, M.; West, S. C., Recombination at mammalian telomeres: an alternative mechanism for telomere protection and elongation. *Cell Cycle* **2005**, 4 (5), 672-4.
23. Roumelioti, F. M.; Sotiriou, S. K.; Katsini, V.; Chiourea, M.; Halazonetis, T. D.; Gagos, S., Alternative lengthening of human telomeres is a conservative DNA replication process with features of break-induced replication. *EMBO Rep* **2016**, 17 (12), 1731-1737.
24. Cho, N. W.; Dilley, R. L.; Lampson, M. A.; Greenberg, R. A., Interchromosomal homology searches drive directional ALT telomere movement and synapsis. *Cell* **2014**, 159 (1), 108-121.
25. Wilson, M. A.; Kwon, Y.; Xu, Y.; Chung, W. H.; Chi, P.; Niu, H.; Mayle, R.; Chen, X.; Malkova, A.; Sung, P.; Ira, G., Pif1 helicase and Poldelta promote recombination-coupled DNA synthesis via bubble migration. *Nature* **2013**, 502 (7471), 393-6.
26. Doksani, Y.; Wu, J. Y.; de Lange, T.; Zhuang, X., Super-resolution fluorescence imaging of telomeres reveals TRF2-dependent T-loop formation. *Cell* **2013**, 155 (2), 345-356.
27. Bizard, A. H.; Hickson, I. D., The dissolution of double Holliday junctions. *Cold Spring Harb Perspect Biol* **2014**, 6 (7), a016477.
28. Matos, J.; West, S. C., Holliday junction resolution: regulation in space and time. *DNA Repair (Amst)* **2014**, 19, 176-81.
29. Zeraati, M.; Langley, D. B.; Schofield, P.; Moye, A. L.; Rouet, R.; Hughes, W. E.; Bryan, T. M.; Dinger, M. E.; Christ, D., I-motif DNA structures are formed in the nuclei of human cells. *Nat Chem* **2018**, 10 (6), 631-637.
30. Eichman, B. F.; Vargason, J. M.; Mooers, B. H.; Ho, P. S., The Holliday junction in an inverted repeat DNA sequence: sequence effects on the structure of four-way junctions. *Proc Natl Acad Sci U S A* **2000**, 97 (8), 3971-6.
31. Ho, P. S., Structure of the Holliday junction: applications beyond recombination. *Biochemical Society transactions* **2017**, 45 (5), 1149-1158.
32. Hays, F. A.; Teegarden, A.; Jones, Z. J.; Harms, M.; Raup, D.; Watson, J.; Cavaliere, E.; Ho, P. S., How sequence defines structure: a crystallographic map of DNA structure and conformation. *Proc Natl Acad Sci U S A* **2005**, 102 (20), 7157-62.
33. Hays, F. A.; Schirf, V.; Ho, P. S.; Demeler, B., Solution formation of Holliday junctions in inverted-repeat DNA sequences. *Biochemistry* **2006**, 45 (8), 2467-71.

34. Mao, C.; Sun, W.; Seeman, N. C., Designed Two-Dimensional DNA Holliday Junction Arrays Visualized by Atomic Force Microscopy. *Journal of the American Chemical Society* **1999**, *121* (23), 5437-5443.
35. Watson, J.; Hays, F. A.; Ho, P. S., Definitions and analysis of DNA Holliday junction geometry. *Nucleic Acids Res* **2004**, *32* (10), 3017-27.
36. Khuu, P.; Ho, P. S., A rare nucleotide base tautomer in the structure of an asymmetric DNA junction. *Biochemistry* **2009**, *48* (33), 7824-32.
37. Podlevsky, J. D.; Bley, C. J.; Omana, R. V.; Qi, X.; Chen, J. J., The telomerase database. *Nucleic Acids Res* **2008**, *36* (Database issue), D339-43.
38. Khuu, P. A.; Voth, A. R.; Hays, F. A.; Ho, P. S., The stacked-X DNA Holliday junction and protein recognition. *J Mol Recognit* **2006**, *19* (3), 234-242.
39. McKinney, S. A.; Declais, A. C.; Lilley, D. M.; Ha, T., Structural dynamics of individual Holliday junctions. *Nature structural biology* **2003**, *10* (2), 93-7.
40. Hadden, J. M.; Declais, A. C.; Carr, S. B.; Lilley, D. M.; Phillips, S. E., The structural basis of Holliday junction resolution by T7 endonuclease I. *Nature* **2007**, *449* (7162), 621-4.
41. Verdun, R. E.; Karlseder, J., The DNA damage machinery and homologous recombination pathway act consecutively to protect human telomeres. *Cell* **2006**, *127* (4), 709-20.
42. Kruk, P. A.; Rampino, N. J.; Bohr, V. A., DNA damage and repair in telomeres: relation to aging. *Proc Natl Acad Sci U S A* **1995**, *92* (1), 258-62.
43. Oikawa, S.; Tada-Oikawa, S.; Kawanishi, S., Site-specific DNA damage at the GGG sequence by UVA involves acceleration of telomere shortening. *Biochemistry* **2001**, *40* (15), 4763-8.
44. Petersen, S.; Saretzki, G.; von Zglinicki, T., Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Exp Cell Res* **1998**, *239* (1), 152-60.
45. Ippel, J. H.; Lanzotti, V.; Galeone, A.; Mayol, L.; van den Boogaart, J. E.; Pikkemaat, J. A.; Altona, C., Conformation of the circular dumbbell d<pCGC-TT-GCG-TT>: structure determination and molecular dynamics. *J Biomol NMR* **1995**, *6* (4), 403-22.
46. Agilent *CrysAlis PRO*, Agilent Technologies Ltd, Yarnton, Oxfordshire, England: Agilent 2014.
47. McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J., Phaser crystallographic software. *J Appl Crystallogr* **2007**, *40* (Pt 4), 658-674.
48. Emsley, P.; Lohkamp, B.; Scott, W. G.; Cowtan, K., Features and development of Coot. *Acta Crystallogr D Biol Crystallogr* **2010**, *66* (Pt 4), 486-501.
49. Murshudov, G. N.; Skubak, P.; Lebedev, A. A.; Pannu, N. S.; Steiner, R. A.; Nicholls, R. A.; Winn, M. D.; Long, F.; Vagin, A. A., REFMAC5 for the refinement of macromolecular crystal structures. *Acta Crystallogr D Biol Crystallogr* **2011**, *67* (Pt 4), 355-67.
50. Abagyan, R.; Totrov, M.; Kuznetsov, D. A., ICM: A New Method For Protein Modeling and Design: Applications To Docking and Structure Prediction From The Distorted Native Conformation. *J Comp Chem* **1994**, *15*, 488-506.

51. *The PyMol molecular graphics program*, 2.0; Schrödinger, LLC.
52. Pettersen, E. F.; Goddard, T. D.; Huang, C. C.; Couch, G. S.; Greenblatt, D. M.; Meng, E. C.; Ferrin, T. E., UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem* **2004**, 25 (13), 1605-12.

For Table of Contents only