Structural basis for translocation by AddAB helicase–nuclease and its arrest at χ sites

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In bacterial cells, processing of double-stranded DNA breaks for repair by homologous recombination is dependent upon the recombination hotspot sequence χ (Chi)1,2 and is catalysed by either an AddAB- or RecBCD-type helicase–nuclease (reviewed in refs 3, 4). These enzyme complexes unwind and digest the DNA duplex from the broken end until they encounter a χ sequence3, whereupon they produce a 3′ single-stranded DNA tail onto which they initiate loading of the RecA protein4. Consequently, regulation of the AddAB/RecBCD complex by χ is a key control point in DNA repair and other processes involving genetic recombination. Here we report crystal structures of Bacillus subtilis AddAB in complex with different χ-containing DNA substrates either with or without a non-hydrolysable ATP analogue. Comparison of these structures suggests a mechanism for DNA translocation and unwinding, suggests how the enzyme binds specifically to χ sequences, and explains how χ recognition leads to the arrest of AddAB (and RecBCD) translocation that is observed in single-molecule experiments5–9.

We have previously determined the crystal structures of initiation complexes of AddAB and RecBCD bound to a DNA end5–9. We have now crystallized AddAB in a translocation-like state by extending the 5′ and 3′ tails of the substrate to mimic an unwound fork (Fig. 1 and Extended Data Fig. 1). These structures provide additional details of the interaction between the AddA helicase domain and the 3′ tail of the DNA fork (Extended Data Fig. 2). The structure of the complex with the non-hydrolysable ATP analogue ADPNP revealed that AddAB contains two ATP-binding sites (Fig. 1a). One is located between the helicase domains of AddA, and the other is in the equivalent position between the catalytically inactivated helicase domains of AddB. The ATP-binding site in AddA is required for processive DNA translocation and unwinding10, and is essentially the same as that observed in other SF1 helicases11,12. The ATP-binding site in AddB is important for stabilizing the complex between AddAB and χ12. It contains several residues that are similar to those in AddA, and ADPNP is bound in a similar fashion at both sites (Fig. 1b and Extended Data Fig. 3). However, there are important differences in residues associated with hydrolysis rather than binding of ATP. For example, essential catalytic glutamate and arginine residues (E408 and R873 in AddA) are replaced in AddB by a glycine and a serine, respectively. This suggests that AddB has a much reduced ATPase activity compared to AddA, consistent with biochemical studies12. Conservation of an ATP-binding site in AddB further substantiates proposals that the χ-scanning subunits of RecBCD and AddAB (RecC and AddB, respectively) have evolved from bona fide helicase subunits13–15.

The binding of nucleotide to the AddA subunit induces conformational changes in the motor domains (1A and 2A) comparable to those seen for other SF1 and SF2 helicases16 (Fig. 1a). By contrast, the conformation of the AddB subunit remains unaltered by the binding of ADPNP other than local changes in amino-acid side chains. The conformational changes in AddA suggest both a mechanism for single-stranded DNA (ssDNA) translocation, similar to that proposed for other SF1 helicases11,13, and how the complex might unwind DNA (Fig. 2). Immediately ahead of the ssDNA motor, the ‘arm’ domain of AddA and the carboxy-terminal nuclease domain of AddB contact the duplex on opposite sides such that they almost encircle the DNA. Upon ATP binding, the 1A and 2A motor domains of AddA move together, causing ssDNA to slide by one base across the surface of the 1A domain and the 3′ ssDNA tail to be pulled away from the DNA junction. At the same time, the arm domain pulls the duplex in the opposite direction, thereby creating tension that is relieved by unwinding a base pair at the fork junction. When ATP is hydrolysed, the motor domains revert to the open state, domain 1A slides back along the ssDNA towards the junction and the regions of the protein contacting the DNA duplex also slide backwards, to alter the register of DNA contacts by one base pair (see Supplementary Videos 1 and 2). Hence one

Figure 1 | Structure of the binary and ternary complexes. a. Comparison of the binary and ternary (ADPNP) complexes. AddA is shown in beige (binary) and orange (ADPNP) but with the motor domains of AddA in the ADPNP complex in green and red, AddB in light cyan (binary) and cyan (ADPNP), the DNA is shown in pink (binary) and purple (ADPNP), bound ADPNP molecules in blue sticks, and the iron–sulphur cluster as spheres. b. Overlay of ATP-binding sites in AddA (orange) and AddB (cyan). Although a bound magnesium ion is superimposable in both sites, a bound water molecule, positioned for attack of the γ-phosphate, is only evident in the AddA site.

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Figure 2 | Proposed mechanism of unwinding and translocation.
a. Superposition of the structures on the arm domain illustrates the stretching of the DNA junction that occurs when ATP-binding induces movement of the 1A and 2A domains. For clarity, only the DNA and domains 1A, 1B and 2A are shown. The binary complex is coloured in beige (protein) and pink (DNA), whereas the ADPNP structure is coloured as labelled in the figure, with the DNA in purple. b. The register of contacts between (1) the arm domain and DNA duplex; and (2) the 2A domain and ssDNA remains the same but their separation increases upon ATP binding. The strain in the duplex is relieved by unwinding of the duplex causing disruption of a single base pair at the junction. Arrows highlight some of the important movements of the DNA. c. Contacts with the ssDNA tail change register by a single base upon ATP binding as the DNA slides across the surface of domain 1A. d. Cartoon summarizing the minimal steps in the unwinding mechanism. Upon ATP binding, the 1A and 2A domains of AddA close and the conformational change of the ssDNA tail across the surface of the 1A domain by one base. At the same time, the spacing between the arm domain and the 2A domain increases, introducing stress into the duplex and causing it to unwind due to a base pair at the junction. After hydrolysis of ATP, the 1A and 2A domains relax to the open conformation, causing the 2A domain to slide across the ssDNA. At the same time, the arm domain and the nuclease domain of AddB slide by one base pair along the duplex. This simple two-step process identifies a minimal set of conformational changes that are required to unwind the DNA but does not preclude other intermediates.

The unexpected and direct role for the arm domain in this mechanism may provide a molecular basis for the enigmatic secondary translocate activity detected in the structurally related RecBC complex. The AddB subunit is responsible for \( \chi \) recognition but the molecular details of this interaction are unknown. To mimic delivery of \( \chi \) to the recognition locus, we next prepared a series of DNA substrates that placed a \( B. subtilis \) \( \chi \) sequence (5’-AGCGG (ref. 2)) on the 3’ strand at increasing distances from the junction by gradually increasing the length of a ‘spacer’ region (Extended Data Fig. 1). These substrates all crystallized with AddAB and ADPNP in the same conditions, space group and unit cell as those lacking \( \chi \). The overall conformation of the protein was the same in all structures, with no electron density attributable to a bound \( \chi \) site, with one exception. In the case of the substrate with a seven-base spacer, there was strong electron density within the AddB subunit corresponding to a bound \( \chi \) sequence (Fig. 3) as well as substantial alterations in the conformation of the AddA motor domains (discussed below).

Both RecC and AddB have the same fold as an SF1 helicase and in AddB even the ATP-binding site is conserved, albeit with changes that probably reduce or abolish ATPase activity (see above). Furthermore, some residues known to affect binding and/or response to \( \chi \) in AddAB and RecBCD are located in positions equivalent to the characteristic helicase motifs. Consequently, we expected the DNA to bind in a manner similar to that observed for SF1 DNA helicases. The \( \chi \)-binding site is indeed located in the same groove as the ssDNA-binding site in SF1 helicases. Moreover, residues either side of the \( \chi \) sequence interact with the protein principally via the phosphodiester backbone, which is largely similar to ssDNA binding in other DNA helicases that need to bind DNA in a non-sequence-specific manner. However, the manner in which the five bases of the \( \chi \) sequence interact with the protein is entirely different. Starting with the first residue (adenine), the DNA is flipped by 180° such that the base points towards, rather than away from, the protein surface. As a result the entire interface with \( \chi \) is via the bases, but bounded by the phosphate groups on either side that also make important contacts (see below). This mode of binding is thereby optimized for specificity over affinity: any interaction with the backbone would be sequence-independent, increasing affinity for all DNA sequences rather than specificity for \( \chi \). All five bases of the \( \chi \) sequence interact with the protein (Fig. 3c). Several residues in AddB that either contact the \( \chi \) site directly (Q42, T44, R70, F210) or order residues that contact the bases (F68, W73) had been implicated previously in the recognition or response to \( \chi \) (ref. 10). However, this structure reveals a role for several additional residues, including some, surprisingly, that are in the AddA subunit (Fig. 3c). Many of these have equivalents in RecB and RecC, probably reflecting a commonality in the mechanism of \( \chi \) regulation in AddAB and RecBCD.

The \( \chi \)-binding site extends across from the AddB subunit into the AddA nuclease domain, which has the same fold as \( \lambda \)-exonuclease. The structure of \( \lambda \)-exonuclease complexed with DNA identified the importance of the 5’ phosphate of the substrate for both binding and catalysis. The function of an arginine residue (R28) that has a key role in this context is replaced by a tyrosine (Y1204) in AddA (Fig. 3d). However, in \( \lambda \)-exonuclease, additional contacts with this phosphate moiety involve residues T33–S35, a region that is conserved in the nuclease domain of AddA (S1015–S1017) within the linker connecting the helicase and nuclease domains. These residues coordinate the equivalent
position in the phosphodiester backbone on the 3' side of the final G of the \(\chi\) sequence. A similar motif is conserved in the AddB nuclease domain and probably contributes in an equivalent manner to binding of the 5' tail.

As the 3' ssDNA tail continues beyond the \(\chi\) sequence and enters the AddA nuclease domain, the conformation flips back by 180° so that the principal contacts are once again with the phosphodiester backbone rather than the bases.

Single-molecule experiments with RecBCD\(^7,8\) and AddAB\(^9\) revealed that both complexes show interesting behaviour when encountering a \(\chi\) sequence. In each case, the complexes are unwinding DNA at several hundred base pairs per second before suddenly pausing at \(\chi\) for several seconds. After this pause, the complexes resume translocation but usually at a lower speed and with modified properties such that the complex is stalled in an altered, but unknown, conformational state until it reactivates to a form that can recommence translocation. However, no information about this \(\chi\)-dependent stalled conformation could be obtained from the single-molecule experiments.

Our structure of the \(\chi\)-bound state of AddAB now reveals the nature of the conformational change that takes place upon encountering \(\chi\). Unexpectedly, the 1A and 2A motor domains of the AddA subunit adopt the conformation for the complex without bound nucleotide, despite being grown under the same conditions as the ADPNP complex and crystallizing in the same space group and unit cell. Importantly, the ATP-binding sites of AddA and AddB are both occupied with a bound ADPNP (Extended Data Fig. 3). Consequently, when \(\chi\) is bound to the AddB subunit, the AddA motor domains are unable to adopt the usual nucleotide-bound closed conformation, thereby blocking

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**Figure 3** The complex of AddAB bound to \(\chi\). 

- **a.** Cutaway space-filling representation of the ADPNP–\(\chi\) complex showing the long channel through the complex, along which the DNA runs. DNA is purple but with the \(\chi\) residues in yellow (inset shows close up of the \(\chi\) site but in ribbon format).
- **b.** Difference electron density \((F_o - F_c, contoured at 2.5\sigma)\) corresponding to the five residues of the \(\chi\) site, with the final structure overlaid for reference.
- **c.** Details of interactions with each base of the \(\chi\) sequence. 
- **d.** Binding site for the phosphate group at the 3' end of the \(\chi\) sequence (carbon atoms yellow).
- **e.** Link between the phosphate binding site at the 5' end of the \(\chi\) residues (yellow) and the latch residues (red).
DNA translocation and explaining the pause seen in single-molecule experiments.\(^2\)\(^-\)\(^4\) We have now determined several structures of \(\chi\)-bound complexes from different crystals and these show that although domain 1A is consistently in an orientation similar to that in the binary complex, the 2A domain adopts a range of non-canonical conformations (Extended Data Fig. 4), suggesting that domain 1A is locked but domain 2A becomes uncoupled and is able to adopt multiple states. Hence, the paused \(\chi\)-bound state may well remain able to hydrolyse ATP without translocating, rather like a motor idling without the gearbox engaged, until a subsequent conformational change allows the complex to reengage and recommence translocation.

Although our structure reveals how the pause is initiated, the reason for the pause remains a point for speculation. One possibility is that the protein requires a slow conformational change to take place before the complex is proficient for the next phase of the reaction, namely the loading of RecA protein. Data for both RecBCD and AddAB suggest this may involve the opening of a protein gate to allow extrusion of an ssDNA loop.\(^1\)\(^6\)\(^,\)\(^17\)\(^,\)\(^25\) Interestingly, the structure reveals a direct link from the \(\chi\)-binding site (R132) to one of the residues (E129) that forms part of a proposed latch (Fig. 3e) responsible for the opening of this exit channel, although the latch remains in the closed conformation in this structure. It might also be that the final DNA cleavage event on the \(\chi\)-containing strand has to take place before translocation resumes and RecA begins to load. This would act as a failsafe mechanism to ensure the production of an appropriate substrate for extension by the action of DNA polymerase following RecA-dependent strand invasion. Consistent with this view, a nuclease-defective RecBCD complex is not able to load RecA protein at \(\chi\). The work we present here captures several intermediates on the AddAB repair pathway (Extended Data Fig. 5), but further work is required to understand how the complex is reactivated following the stall at \(\chi\), thereby resuming DNA unwinding and the loading of RecA protein to initiate homologous recombination.

**METHODS SUMMARY**

Protein and DNA substrates were prepared as described previously.\(^1\)\(^6\)\(^,\)\(^17\)\(^,\)\(^25\) Protein–DNA complex was prepared by mixing a slight excess of DNA with protein complex and then purified by gel filtration before crystallization by vapour diffusion. Crystals were flash-frozen in liquid nitrogen before data collection. The structures were determined by molecular replacement using the AddAB coordinates (PDB accession number 3U4Q)\(^3\)\(^6\). Details of the final models are presented in Extended Data Table 1.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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**Supplementary Information** is available in the online version of the paper.

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METHODS

Cloning, protein expression and purification. The nuclease-dead AddAB mutant (pCOLDuet-1-AddADΔI1172ΔPΔD651A) described previously27 was modified to include a TEV protease cleavable His-tag at the amino terminus of the AddA subunit. B834 (DE3) cells harbouring the AddAB plasmid were grown at 37 °C in LB medium containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, and grown at 27 °C for 3 h. Cells were collected and frozen at −80 °C until further use. For protein purification, thawed cells were resuspended and lysed in the lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 2 SigmaFast Protease inhibitor cocktail tablets, 0.03 mg ml Dnase 1 −1, a pinch of lysozyme) using EmulsiFlex homogenizer (Avetin). After centrifugation, polyethyleneimine and ammonium sulphate (AS) precipitations were carried out essentially as described previously27, except that for the latter AS was added to a final concentrations of 60%. AS pellets were dissolved in the HisTrap binding buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, 1 mM DTT) and filtered through a 0.45-μm syringe filter. The protein was loaded onto a HisTrap FF column (GE Healthcare) and washed with HisTrap binding buffer. To remove DNA from AddAB, the bound protein was washed with high-salt (20 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM imidazole, 1 mM DTT) and low-salt (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, 1 mM DTT) wash buffers. AddAB was eluted from the column with the elution buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 250 mM imidazole, 1 mM DTT). The pooled peak fractions containing AddAB were diluted with 20 mM Tris-HCl, pH 8.0, 1 mM DTT to a conductivity of 7 mS cm−1 before loading onto the HiTrap heparin column (GE Healthcare). AddAB was eluted with a linear gradient from 50 mM to 500 mM NaCl over 30 column volumes. To pooled AddAB fractions, TEV protease was added at 1:20 (w/w) ratio, and the solution was dialysed against the dialysis buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT) and filtered through a 0.45-μm syringe filter. The protein was aliquoted, flash-frozen in liquid nitrogen and stored at −80 °C.

Crystallization. The DNA substrates used for crystallization were prepared as described previously11. Crystals of AddAB complexes were obtained by vapour diffusion in hanging drops at 12 °C. The DNA substrates used for crystallization were prepared as described previously11. Crystals of AddAB complexes were obtained by vapour diffusion in hanging drops at 12 °C. For the AddAB–023 structure, protein was mixed with the DNA substrate (5’- TTGTTTCTTAATGCGACACTGCTATTCCTAGCGAGCTCAGTTAGATTTTGTTTTTTAGCGGTTTTT-3’) at 1:1.3 molar ratio for 30 min on ice before crystallization. Crystals were obtained by mixing two volumes of protein and one volume of mother liquor consisting of 11% polyethylene glycol 4000, 0.1M Tris-HCl pH 7.5, 0.1 M magnesium acetate. Microseeding was used to improve crystal quality. Crystals were cryoprotected and flash frozen as described above.

For the AddAB–027–ADPNP structure, protein was mixed with the DNA substrate (5’-TTTTTTTCTAATGCGACACTGCTATTCCTAGCGAGCTCAGTTAGATTTTGTTTTTTAGCGGTTTTT-3’) at 1:1.3 molar ratio in the presence of 2 mM ADPNP and 5 mM magnesium chloride. The complex was incubated for 30 min on ice before crystallization. Crystals were obtained by mixing two volumes of protein and one volume of mother liquor consisting of 11% polyethylene glycol 4000, 0.1M Tris-HCl pH 7.5, 0.1 M magnesium acetate. Microseeding was used to improve crystal quality. Crystals were cryoprotected and flash frozen as described above.

Structure determination and refinement. For the AddAB–023–ADPNP structure, a 2.8 Å diffraction data set was collected at the Diamond Light Source (DLS) beamline I04 at a wavelength of 0.98 Å and a temperature of 100 K. The data were integrated and scaled using XDS and XSACE24. Intensities were converted to structure factors using CRUNCATE27. The crystals belonged to space group P21, with one AddAB–DNA complex in the asymmetric unit. The structure was determined by molecular replacement in Phaser using the AddAB–DNA complex (PDB accession 3U4Q) as a search model. To account for conformational changes, the model was first subjected to rigid-body refinement of the individual AddA and AddB subdomains. Alternating rounds of model building and refinement were carried using COOT24 and PHENIX21, respectively. The final model had good geometry with 0.1% of residues outside the allowed regions.

For the AddAB–023 structure, a 3.2 Å diffraction data set was collected at the DLS beamline I02 at a wavelength of 0.98 Å and a temperature of 100 K. The data were processed as described above. The crystals belonged to space group P21 with one AddAB–DNA complex in the unit cell. The structure was determined by molecular replacement in Phaser using the AddAB–023–ADPNP structure as a search model. Model building and refinement were carried out as described above. The final model had good geometry with 0.1% of residues outside the allowed regions.

For the AddAB–027–ADPNP structure, a 3.0 Å diffraction data set was collected at the DLS beamline I24 at a wavelength of 0.969 Å and a temperature of 100 K. The crystals belonged to space group P21 with one AddAB–DNA complex in the asymmetric unit. The structure was determined by molecular replacement in Phaser using the AddAB–023–ADPNP structure as a search model. Model building and refinement were carried out as described above. The structures were validated using MOLPROBITY27 as implemented in PHENIX. The final model had good geometry with 0.2% of residues outside the allowed regions.

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Extended Data Figure 1 | DNA substrates used in the structure determinations. We have previously determined the crystal structure of an initiation complex of AddAB using the DNA substrate shown at the top. The substrates used in the current study differ by the additions shown in blue below. Five T residues were added to make a 5’ tail for the fork. The 3’ tail was extended by the addition of an AddAB χ sequence (AGCGG), preceded by a ‘spacer’ sequence of between 1 and 7 T residues to extend the distance between the fork junction and the χ sequence along the 3’ tail to mimic the products of sequential unwinding of a DNA fork. Oligo 023 used in this study contained a six-base T spacer. Oligo 027 contained a seven-T spacer plus an additional four T residues beyond the χ sequence as shown above. AddAB failed to recognize χ in substrates with six or less residues in the spacer but was able to bind to χ with seven T residues in the spacer region.
Extended Data Figure 2 | Cartoon representation of the DNA-binding site in the two complexes. Residues from AddA in orange, AddB in cyan, hydrogen bonds and charged interactions as arrows, hydrophobic and stacked interactions as solid lines.
Extended Data Figure 3 | Nucleotide-binding sites. Difference electron density (F_o – F_c, contoured at 2.5σ) for bound nucleotide at the ATP-binding sites in the AddA and AddB subunits in the ADPNP and χ complexes.
Extended Data Figure 4 | Conformational variability of domain 2A of AddA in different γ-bound structures. The two extreme variants are shown for illustration. Although domain 1A (green and light green) of the AddA protein (orange) remains in an essentially constant position, the position of the 2A domain (red and light red) varies from one that is almost superimposable with the ‘apo’ complex that lacks ADPNP, to one that is midway between the apo and ADPNP-bound conformations plus a variety of conformations between these two extreme cases. The extreme cases differ by a rotation of 5° compared to 7° for the difference between the ADPNP and apo structures. By contrast, the conformation of the 2A domain of AddA adopts an almost invariable conformation in different structures of the AddAB–DNA complex grown with ADPNP with a variety of DNA substrates in which γ is not bound.
Extended Data Figure 5 | Cartoon summarizing our current knowledge of the molecular structures of different functional states along the AddAB reaction pathway. We now have crystal structures for the first three functional states as shown in the figure. These show how the protein complex interacts with DNA (initiation complex), what happens when ATP binds and how this leads to DNA unwinding (translocation complex) and what happens when this complex encounters a χ sequence (χ recognition complex). At least two further states have yet to be visualized: (1) what happens after the pause at χ (χ reactivation complex) and (2) how the protein interacts with and loads RecA (RecA-loading complex).
Extended Data Table 1 | X-ray data and refinement statistics

| Data collection | DD027ANP (Chi) | DD023ANP | DD023apo |
|-----------------|----------------|-----------|----------|
| Beamline        | 124            | 104       | 102      |
| Wavelength      | 0.969          | 0.980     | 0.980    |
| Resolution (Å)  | 35.6-3.0       | 47.9-2.8  | 39.5-3.2 |
| \(R_{	ext{free}}\) (%) | 4.8 (41.5)    | 5.8 (35.9) | 4.7 (33.4) |
| \(I/\sigma\)    | 18.4 (2.9)     | 15.9 (3.8) | 13.2 (2.3) |
| Completeness %  | 99.7 (89.7)    | 99.9 (99.9)| 98.5 (97.7)|
| Multiplicity    | 3.8 (3.9)      | 6.4 (6.5)  | 2.9 (2.9) |
| Space Group     | \(P\ 2_1\)    | \(P\ 2_1\) | \(P\ 1\) |
| \(a, b, c\) (Å) | 77.4, 152.9, 125.2 | 75.9, 151.3, 124.6 | 77.4, 96.8, 109.7 |
| \(A, B, C\) (°) | 90.0, 94.3, 90.0 | 90.0, 96.0, 90.0 | 104.4, 99.1, 90.0 |

**Data refinement**

|                  | DD027ANP (Chi) | DD023ANP | DD023apo |
|------------------|----------------|-----------|----------|
| \(R\) factor (%) | 20.2           | 20.2      | 24.9     |
| \(R_{\text{free}}\) (5% of data) | 24.0          | 24.1      | 27.8     |
| Reflections (All/test) | 57963/2924   | 68664/3459 | 47941/2427 |
| rmsd bond length (Å) | 0.004        | 0.004     | 0.003    |
| rmsd bond angle (°) | 0.921         | 0.884     | 0.650    |
| Rama outliers (%) | 0.2           | 0.1       | 0.1      |

*values in parentheses represent the highest resolution shell*