Understanding the structural basis for controlling chromosome division

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The process of chromosome division, termed mitosis, involves a complex sequence of events that is tightly controlled to ensure that the faithful segregation of duplicated chromosomes is coordinated with each cell division cycle. The large macromolecular complex responsible for regulating this process is the anaphase-promoting complex or cyclosome (APC/C). In humans, the APC/C is assembled from 20 subunits derived from 15 different proteins. The APC/C functions to ubiquitinate cell cycle regulatory proteins, thereby targeting them for destruction by the proteasome. This review describes our research aimed at understanding the structure and mechanism of the APC/C. We have determined the crystal structures of individual subunits and subcomplexes that provide atomic models to interpret density maps of the whole complex derived from single particle cryo-electron microscopy. With this information, we are generating pseudo-atomic models of functional states of the APC/C that provide insights into its overall architecture and mechanisms of substrate recognition, catalysis and regulation by inhibitory complexes.

1. The anaphase-promoting complex controls chromosome division

Following chromosome replication, the duplicated chromosomes remain tightly associated as sister chromatid pairs. As the cells enter mitosis, two events occur. First, the chromosomes condense to form compact flexible rods, and, second, the microtubules assemble to form the mitotic spindle, visible as an array of filaments radiating out from opposite spindle poles. The chromosomes then attach to the equator of the mitotic spindle, an association mediated by kinetochores, large
protein assemblies that link the DNA to the tubulin subunits of the spindle microtubules [1–5]. Sister chromatids are aligned with a bipolar orientation, so that each sister chromatid of the chromatid pair is attached to the opposite spindle pole. Once all 46 sister chromatids have achieved correct bipolar attachment to the mitotic spindle, the cohesion linking all sister chromatids is simultaneously disrupted. This allows the chromatids to separate and segregate to opposite poles of the cell, driven by the molecular motors associated with the mitotic spindle and kinetochores. Once chromosome segregation is complete, the cell divides perpendicular to the mitotic spindle creating two new daughter cells, each with an identical complement of chromosomes.

To ensure that these cell cycle events occur in the correct sequence, which is necessary for error-free chromosome segregation, cell cycle checkpoints have evolved. One of these is the mitotic checkpoint, also known as the spindle assembly checkpoint (SAC) [3,6–8]. The mitotic checkpoint is a surveillance mechanism which ensures that the cohesion between sister chromatids is disrupted only once all sister chromatids have achieved correct bipolar attachment to the mitotic spindle. In the event that a single unattached chromatid pair has failed to attach to the mitotic spindle, a signal is generated from the lagging chromosome to pause mitosis by activating the mitotic checkpoint. A delay in mitosis allows more time for the lagging chromosome to be captured by the mitotic spindle. On achieving successful bipolar attachment, the lagging chromosome silences the mitotic checkpoint, and the process of mitosis continues with the separation of all chromosomes occurring simultaneously, resulting in their equal distribution into each daughter cell.

It is important that chromosome segregation completes successfully, because the mis-segregation of chromosomes leads to aneuploidy, a condition that underlies genetic diseases such as cancer and developmental disorders. Some anti-cancer drugs, such as Taxol, function by blocking mitosis—through microtubule stabilization—thereby activating the mitotic checkpoint artificially [9,10].

The cell cycle is defined by a sequence of discrete phases. These are S-phase when chromosomes are replicated, mitosis when chromosomes divide, and cytokinesis when cells divide. Two gap phases—G1 and G2—separate S-phase from mitosis. The APC/C regulates ordered progression through these phases by controlling the destruction of proteins whose activities inhibit specific transitions of the cell cycle [11–13]. By selecting different proteins during different phases of the cell cycle, the APC/C coordinates the correct order of cell cycle events.

The APC/C becomes active in early mitosis, a period of high CDK activity, when it binds to a regulatory subunit called the Cdc20 coactivator. The CDK-dependent phosphorylation of core APC/C subunits and Cdc20 promotes formation of an APC/C<sup>Cdc20</sup> complex. To trigger the metaphase-to-anaphase transition, the APC/C<sup>Cdc20</sup> complex mediates the destruction of two proteins: securin and cyclin B. Securin and its CDK-dependent phosphorylation inhibits separase, the protease that cleaves a subunit of the cohesion complex responsible for sister chromatid cohesion. Thus, the degradation of securin and cyclin B triggers the loss of cohesion between sister chromatids allowing sister chromatids to separate. Once the sister chromatids are equally distributed to opposite poles of the spindle in late mitosis, the cell divides in a process controlled by the second coactivator subunit Cdh1 which replaces Cdc20. APC/C<sup>Cdh1</sup> mediates the destruction of the mitotic kinases (Plk1, Aurora A) that inhibit cytokinesis.

Switching of these coactivators (Cdc20 and Cdh1) changes the specificity of the APC/C for its target proteins. Both coactivators recognize their substrates through short sequence motifs called destruction motifs (also termed degrons). Most APC/C substrates contain a destruction box (D box) a nine-residue motif, and a KEN box [14–16]. The coactivators act as adaptors to recruit target proteins to the APC/C.

To avoid segregating sister chromatids before their complete attachment to the mitotic spindle, the mitotic checkpoint inhibits the APC/C to prevent it from mediating the destruction of securin and cyclin B. The mitotic checkpoint is imposed by the mitotic checkpoint complex (MCC) that binds to the APC/C and blocks its capacity to recognize securin and cyclin B through their destruction motifs.
The APC/C mediates the destruction of cell cycle proteins through the ubiquitin–proteasome system. In this system, the APC/C modifies its target proteins by attaching a ubiquitin chain which is a polymer of the small protein ubiquitin onto the target protein. This polyubiquitin chain functions as a signal for the degradation of the target protein by the proteosome [17]. To assemble a polyubiquitin chain, the APC/C acts in conjunction with two E2 enzymes (UbcH10 and Ube2S in humans) that activates the ubiquitin molecule to allow the APC/C to attach it to its target protein.

2. Anaphase-promoting complex architecture and structure

The complex functions of the APC/C are reflected in its complex organization and large size. Human APC/C is an assembly of 15 different proteins, including the coactivator subunit. Native mass spectrometry and crystallography of APC/C subunits indicated that many APC/C subunits are present in two copies per complex. Thus, the overall molecular mass of human APC/C is 1.2 MDa [18].

One striking feature of the APC/C is that only four proteins are involved in recognizing target proteins and catalysing the assembly of a polyubiquitin chain onto the protein. The catalytic centre is formed from Apc11, the RING domain subunit that forms a tight association with the cullin subunit Apc2. The catalytic centre formed from Apc2 and Apc11 is related to the large family of cullin RING ligases exemplified by the Skp1-cullin-F (SCF) box complex. D box substrate recognition is conferred by the core APC/C subunit Apc10 together with a coactivator subunit (either Cdc20 or Cdh1), although the KEN box is recognized by coactivator alone. Apc10 and coactivator have completely different three-dimensional structures, but both share a conserved C-terminal Ile-Arg (IR) motif required for binding to the tetratricopeptide repeat (TPR) subunit Cdc27/Apc3 [19–23]. In addition to the IR tail, coactivators interact with the APC/C through a C box motif within their N-terminal regions [24]. The C box has been shown to stimulate the catalytic activity of the APC/C [25].

All other subunits, accounting for 80% of the APC/C mass, provide scaffolding functions to organize the catalytic and substrate-recognition subunits. Most of these contain multiple repeat motifs. Five of the scaffolding subunits are TPR proteins and comprise between 13 and 14 TPR motifs arranged in contiguous arrays. The TPR is a 34 amino acid motif, and multiple TPR motifs are known to mediate protein–protein interactions. The four canonical TPR subunits Apc3, Apc6, Apc7 and Apc8 form similar homo-dimeric structures. Apc5 differs slightly by not forming homodimers and being composed of slightly larger TPR motifs [18]. Apc1, the largest APC/C subunit, contains 11 repeats of a 30- to 40-residue motif termed the proteasome–cyclosome (PC) repeat. These PC repeats are found in exactly the same arrangement in the proteasomal subunits Rpn1 and Rpn2 [26,27]. The structures of the N-terminus of Apc1 and the whole of Apc4 are unknown and these proteins show no clear sequence similarity to other proteins of known structure and function. In addition to the multiple motifs of the scaffolding subunits, other APC/C subunits also contain multiple repeats. For example, the coactivators are WD40 repeat proteins, and Apc2 contains three N-terminal cullin repeats.

The fourth structural class comprises the TPR accessory subunits. These are small subunits that stabilize the larger TPR subunits and also mediate inter-TPR interactions. In isolation, these TPR-accessory subunits are disordered and they assume a defined conformation only when associated with TPR subunits. Finally, Apc15 is a subunit that negatively regulates the MCC when bound to the APC/C [28–30]. Most APC/C subunits are conserved throughout eukaryotes, although Apc7 is unique to metazoans.

3. Generation of a pseudo-atomic model of the anaphase-promoting complex

Our research has been aimed at obtaining atomic structures of individual APC/C subunits and then to define how these subunits are organized within the whole complex as a means to understand how the APC/C recognizes its substrates, catalyses the assembly of a polyubiquitin
Table 1. APC/C subunits determined at Diamond Light Source.

<table>
<thead>
<tr>
<th>APC/C subunit</th>
<th>DLS beam-line</th>
<th>RCSB accession</th>
<th>refs</th>
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<tbody>
<tr>
<td>Apc3</td>
<td>I02</td>
<td>3ZN3</td>
<td>[31]</td>
</tr>
<tr>
<td>Apc6–Cdc26</td>
<td>I02</td>
<td>2XPI</td>
<td>[32]</td>
</tr>
<tr>
<td>Apc8</td>
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<td>MCCb</td>
<td>I02</td>
<td>4AEZ</td>
<td>[34]</td>
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<td>Cdh1–Acm1</td>
<td>I04–1</td>
<td>4BH6</td>
<td>[27]</td>
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*aHuman nomenclature.

*bS. pombe mitotic checkpoint complex (Cdc20, Mad2, Mad3).

chain, and is controlled by regulatory factors such as the MCC and Emi1. To address these questions, we have generated pseudo-atomic models of the APC/C in various functional states. We used a hybrid approach of combining X-ray crystallography of individual subunits and single particle electron microscopy of the whole complex. We have crystallized a number of APC/C subunits and subcomplexes and have collected data on these crystals at the macromolecular beam-lines at the Diamond Light Source, and subsequently determined their three-dimensional structures (table 1).

An example of one of the TPR subunits—Apc6 associated with its accessory subunit Cdc26 is shown in figure 1. Two Apc6 molecules self-associate to form a ‘V’-shaped homo-dimer [32]. Each Apc6 molecule consists of 14 contiguous TPR motifs, and these form an array of 28 anti-parallel α-helices. Because these α-helices rotate relative to their neighbour by a uniform angle, they generate a right-handed superhelix. The 14 TPR motifs of Apc6 form two complete turns of superhelix. Apc6 is one of the scaffolding subunits and its helical structure and large exposed surface accessible area are ideally suited to mediate protein–protein interactions and the assembly of multi-protein complexes. Cdc26 associates with the TPR superhelix by interacting with the inner groove of the TPR superhelix and also by forming an α-helix that caps the C-terminus of the Apc6 TPR superhelix. We can derive homology models for other APC/C subunits. Apc2 and Apc11 can be modelled on Cul1 and Rbx1 of the SCF [36], whereas the PC domain of Apc1 can be modelled on the PC domain of Rpn2 [27].

By combining X-ray crystallography of individual subunits, with a few homology-derived models, we have generated atomic models for most of the APC/C subunits. The other canonical TPR subunits (Apc3 and Apc8) have related homo-dimeric structures to Apc6. We determined the structures of their N-terminal dimerization domains that promote their self-association [31,33]. Apc1 contains a PC domain composed of 11 PC repeats which create a closed toroidal structure comprising two concentric rings of α-helices surrounding a central anti-parallel α-helical hairpin. The coactivators Cdc20 and Cdh1 have seven WD40 repeats that form a β-propeller. Apc10, the subunit that mediates high-affinity D box association through a bipartite D box co-receptor with coactivator [37–40], has a β-sandwich jelly roll structure [19,35].

To determine the structure of the whole APC/C, we have used single particle electron microscopy. This technique is well suited to large complexes such as the APC/C, although the structures obtained are at generally lower resolution than those achieved by X-ray crystallography. This technique also provides very accurate information on how atomic models of APC/C subunits are associated in the whole complex. A cryo-electron micrograph of Schizosaccharomyces pombe APC/C generated from a recombinant overexpression system recorded using a transmission electron microscope is shown in figure 2. We visualized APC/C particles as two-dimensional projections embedded in vitreous ice. By collecting sufficient two-dimensional projections, we can determine three-dimensional reconstructions of the entire complex. Figure 3 shows two views of the structure of endogenous S. cerevisiae APC/C that represents the molecular envelop of the complex [18,41].
Figure 1. Crystal structures of APC/C subunits. Shown are (a) *S. cerevisiae* Apc10/Doc1p PDB code: 1GQP [35]; (b) a model for the Apc3 homo-dimer based on crystal structures of the dimerization domains of Apc3 (PDB code: 3KAE [31]). Apc7 and Apc8 form structurally related homo-dimers. (c) The Apc6–Cdc26 heterotetramer (PDB code: 2XPI [32]). Apc6 and Cdc26 subunits are labelled. (d) Cdc20 from the MCC structure (PDB code: 4AEZ [34]); (e) model of the proteasome–cyclosome (PC) domain of Apc1 based on Rpn2 (PDB code: 4ADY [27]) and (f) a model of Apc2–Apc11 based on the Cul1–Rbx structure (PDB code: 1LDK [36]). TPR, tetratricopeptide repeat.

Figure 2. Cryo-electron micrograph of recombinant *S. pombe* APC/C. Example of a CCD image of the cryo-electron of recombinant *S. pombe* APC/C. Individual APC/C particles (encircled in red) are viewed in two-dimensional projection.
Overall, the APC/C adopts a triangular structure measuring 250 Å in its longest dimension. The molecule has an open lattice-like appearance, and there are clear indications of rod-like and curved tubular densities, some of which correspond to TPR superhelices. To interpret this structure and to generate a pseudo-atomic model of the APC/C, we have to locate the positions of atomic models of APC/C subunits within the molecular envelope. To locate individual subunits, we compared the structures of pairs of APC/C subcomplexes that differ in their composition by a specific subunit [18]. This is illustrated in figure 4, where we compared two APC/C subcomplexes where only one complex incorporates Apc6–Cdc26. The Apc6 difference density in mesh exactly matches the atomic model of the Apc6 dimer [32], and this allows us to position Apc6 within the APC/C molecular envelope [18]. We could apply a similar approach to Apc3 by comparing the holo APC/C with APC/C lacking the Apc3 subunit. The Apc3 difference density, at the top of the TPR lobe, has a twofold symmetrical structure that matches the twofold symmetry of the Apc3 homo-dimer atomic model [18, 31].

We applied the subunit deletion approach to four APC/C subunits. A disadvantage of this subunit deletion method is that it is restricted to cases where the resultant subcomplexes are large enough and stable enough to be analysed by single particle electron microscopy. Some subunits play critical scaffolding functions and their deletion prevents the assembly of usably sized subcomplexes. For example, deletion of Apc8 results in the failure to generate APC/C subcomplexes of reasonable size. However, we could define the position and molecular boundaries of Apc8 by superimposing the subcomplexes (TPR6 and SC8) onto the intact holo APC/C structure [18]. Because these two subcomplexes only share Apc8 in common, their overlapping densities can be assigned to Apc8. The Apc8-assigned density has an almost identical structure to that assigned for Apc3, related by an approximate twofold rotation about Apc6.

Using this hybrid approach, we generated a pseudo-atomic model of the APC/C. The procedure we applied was to take the electron density map of the whole APC/C using single particle electron microscopy and then based on subunit assignments docked atomic models of all known APC/C subunits into the map. The pseudo-atomic model showed that three canonical TPR subunits Apc3, Apc6 and Apc8, which are all homo-dimers and structurally related, stack in parallel on one side of the complex. Together, they form a quasi-two-fold symmetrical structure.
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Figure 4. Three-dimensional localization of Apc6–Cdc26. The difference density is in grey mesh was calculated by comparing two subcomplexes of the APC/C, one lacking Apc6–Cdc26. The atomic coordinates of the S. pombe Apc6–Cdc26 heterotetramer (PDB code: 2XPI [32]) were used for rigid body docking. The two Cdc16 subunits within the heterotetramer are shown in red and light red, and the Cdc26 N terminus is shown in cyan. From Schreiber et al. [18].

4. Mechanism of D box and KEN box degron recognition

We have obtained high-resolution information on mechanisms of coactivators–degron recognition from crystal structures of coactivators in the context of inhibitory complexes that incorporate pseudo-atomic inhibitory sequences. The rationale being that these pseudo-substrate inhibitory sequences mimic substrate degrons and bind to degron receptors on coactivators. These inhibitors bind to coactivators more tightly than substrates and are therefore easier to isolate, purify and crystallize. The budding yeast protein Acm1 is a specific inhibitor of Cdh1 and incorporates KEN and D box sequences within a central inhibitory region (CIR) of 70 amino acids [42–45]. Both the KEN box and the D box are required to mediate Acm1-dependent inhibition of Cdh1, suggesting that both function as pseudo-substrate inhibitors. A region N-terminal to the KEN box, termed the A motif, is also required for optimal inhibition and Cdh1 binding [45]. We crystallized a complex of the Acm1 CIR (Acm1CIR) with Cdh1 and the structure was determined using data collected at DLS I04-1 [46] (figure 5). The structure showed that these three regions of Acm1 bind to three distinct sites on the Cdh1 WD40 domain, namely the D box-recognition site at the edge of the β-propeller, the KEN box site on the top of the β-propeller, and the A motif interaction site below the β-propeller. Acm1 inhibits Cdh1 by sterically occluding both D box and KEN box-recognition sites to block substrate binding.

The D box binds to a channel at the edge of the β-propeller between blades 1 and 7. The conventional D box is a nine-residue motif defined by an Arg at position 1 (P1) and a Leu at P4. The first seven of the nine D box residues are ordered and well defined in the crystal structure and these adopt an essentially extended conformation. The Leu at P4 is anchored within a deep hydrophobic pocket. The Arg of the D box (P1) interacts with two acidic residues at the top of the channel. We tested whether this site binds to D box degrons in substrates by mutating the Leu-binding pocket and Arg site in Cdh1 [34]. Mutating of the Leu pocket by substituting a bulky methionine for a valine situated at the base of the pocket attenuates the ubiquitination activity of APC/C Cdh1. Similarly, mutating the Arg site residue in Cdh1 virtually abolished APC/C Cdh1 activity.

The structure allows us to rationalize the D box consensus motif, derived from 68 validated substrates [46]. This analysis showed that the D box is an 11-residue motif, because there is a low degree of sequence conservation within the two residues immediately flanking the conventional
A motif

Figure 5. Model for the *S. cerevisiae* Acm1–Cdh1 heterodimer. A 13 residue linker was modelled between P103 (KEN) and R119 (D box; separated by 15 residues), indicating that it is stereochemically possible for the KEN box and D box motif of Acm1 to interact with their respective sites on the same Cdh1 molecule [27].

nine-residue motif. Position 7 is commonly an aliphatic residue, and in the Cdh1–Acm1 complex, a leucine at position 7 interacts with a non-polar patch on the surface of Cdh1. Asp and Glu are the most frequent residues at position 6, and in the Acm1–Cdh1 structure, an Asp at P6 interacts with a conserved arginine of Cdh1 and Cdc20. We do not observe residues P8–P10 of the D box bound to Cdh1. One reason for this might be that the C-terminus of the D box binds to Apc10, which creates the bipartite D box co-receptor with Cdh1 [41].

The Acm1–Cdh1 structure and MCC structure (see below) also revealed how the KEN box interacts with coactivators. The KEN box binds at the centre and top of the Cdh1 β-propeller. All three residues of the KEN box, Lys–Glu–Asn, situated on an under-wound α-helix face in the same direction to interact with Cdh1. Nearly, all the interactions between the KEN box and coactivator involve polar and charged residues of Cdh1 that are invariant in Cdc20. Residues immediately flanking the KEN box do not interact with Cdh1; however, a conserved proline three residues C-terminal to the KEN residues functions to direct the polypeptide chain away from the surface of Cdh1.

A consensus KEN box motif, based on 46 sequences, showed that residues immediately flanking the KEN box are quite well conserved, including the proline at P + 3 and either an Asp or Asn immediately N-terminal to the KEN motif. An Asp or Asn at P-1 does not interact with coactivator but helps to define the conformation of the KEN box by accepting hydrogen bonds from the side chain of the Asn of the KEN motif.

5. Mechanism of anaphase-promoting complex inhibition by the mitotic checkpoint

The activity of the APC/C is inhibited by the MCC that is the protein effector complex that imposes the SAC. The SAC is a monitoring system that detects incorrectly attached chromosomes, and this generates a diffusible ‘wait anaphase’ signal to inhibit the APC/C. The MCC is composed of four conserved subunits, Mad2, Mad3/BubR1, Cdc20 and Bub3 whose assembly is promoted by unattached kinetochores. (The name MAD comes from the phenotype caused by mutations in genes encoding these proteins that cause a mitotic arrest deficient phenotype.) In the complex, Mad2 and Mad3 synergize to bind Cdc20 to inhibit the APC/C. Cdc20 binds to the closed
To understand how MCC inhibits the APC/C, we determined the crystal structure of *S. pombe* MCC (in *S. pombe*, the essential subunits of the MCC are Cdc20, Mad2 and Mad3) to 2.3 Å resolution using data collected at beam line I02 at the Diamond Light Source [34]. Mad2, Mad3 and Cdc20 assemble into a triangular-shaped heterotrimer (figure 6). Mad3 coordinates the overall assembly of the complex by forming extensive interactions with Cdc20 and Mad2, whereas Mad2 and Cdc20 interact primarily with the Mad2-binding motif of Cdc20 bound to the safety belt of Mad2 which is generated in the closed state of Mad2.

A region of Mad3 that functions as a pseudo-substrate and structurally mimics the KEN box degron of an APC/C target protein interacts with the KEN box recognition site of Cdc20 and this blocks the ability of Cdc20 to bind its target proteins and therefore prevents target protein ubiquitination by the APC/C. One role of Mad2 is to order the N-terminal region of Mad3, incorporating the KEN motif, and to correctly position Mad3 onto Cdc20.
To understand how the MCC regulates the APC/C through blocking D box and KEN box recognition, we docked the MCC structure [34] and pseudo-atomic model of the APC/C [18] into the cryo-EM map of the human APC/C–MCC complex [54]. This map is at around 25–20 Å resolution. We can dock the MCC structure into density assigned to MCC located close to the platform region of the APC/C. Some unassigned density would correspond to the pseudo-kinase domain of BubR1 and also the Bub3 subunit that are absent from S. pombe MCC. Relative to the APC/C ternary complex, in the context of the MCC, Cdc20 is shifted downwards, away from Apc10, and towards the platform region. This suggests that the MCC inhibits the APC/C coactivator from recognizing substrate through two mechanisms. First, the KEN box motif of Mad3 directly obstructs the KEN box site on Cdc20 and, second, the shifted Cdc20 disrupts formation of the bipartite D box co-receptor formed between Cdc20 and Apc10.

6. Conclusion

Using protein crystallography of individual APC/C subunits and small subcomplexes combined with single particle electron microscopy of defined reconstituted APC/C complexes, we are generating pseudo-atomic models of a large macromolecular machine. This information provides insights into mechanisms of its overall architecture, catalysis, substrate recognition and regulation.

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References


