The APC/C subunit Cdc16/Cut9 is a contiguous tetratricopeptide repeat superhelix with a homo-dimer interface similar to Cdc27

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The anaphase-promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase responsible for controlling cell cycle transitions, is a multisubunit complex assembled from 13 different proteins. Numerous APC/C subunits incorporate multiple copies of the tetratricopeptide repeat (TPR). Here, we report the crystal structure of *Schizosaccharomyces pombe* Cut9 (Cdc16/Apc6) in complex with Hcn1 (Cdc26), showing that Cdc16/Cut9 is a contiguous TPR superhelix of 14 TPR units. A C-terminal block of TPR motifs interacts with Hcn1, whereas an N-terminal TPR block mediates Cdc16/Cut9 self-association through a homotypic interface. This dimer interface is structurally related to the N-terminal dimerization domain of Cdc27, demonstrating that both Cdc16/Cut9 and Cdc27 form homo-dimers through a conserved mechanism. The acetylated N-terminal Met residue of Hcn1 is enclosed within a chamber created from the Cut9 TPR superhelix. Thus, in complex with Cdc16/Cut9, the N-acetyl-Met residue of Hcn1, a putative degron for the Doa10 E3 ubiquitin ligase, is inaccessible for Doa10 recognition, protecting Hcn1/Cdc26 from ubiquitin-dependent degradation. This finding may provide a structural explanation for a mechanism to control the stoichiometry of proteins participating in multisubunit complexes.

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Introduction

The anaphase-promoting complex/cyclosome (APC/C) is a large multisubunit E3 ubiquitin ligase that controls defined transitions of the cell cycle by coordinating the selective ubiquitin-dependent proteolysis of cell cycle regulatory proteins (Peters, 2006; Pines, 2006; Thornton and Toczyski, 2006; Sullivan and Morgan, 2007). APC/C-catalysed

ubiquitylation of securin and S-phase cyclins initiates sister chromosome segregation to promote the metaphase to anaphase transition, whereas subsequent ubiquitylation of mitotic cyclins regulates mitotic exit and prevents premature initiation of S-phase. Recognition of APC/C substrates, dependent on D-box and KEN-box destruction motifs (King *et al*, 1996; Pfleger and Kirschner, 2000), and stimulation of E3 ligase catalytic activity (Kimata *et al*, 2008), is controlled by the association of the core APC/C assembly with either of two structurally related co-activator proteins, Cdc20 and Cdh1. *In vivo*, these two regulatory subunits confer distinctive substrate specificities with Cdc20 being responsible for destruction of securin and S-phase cyclins, and Cdh1 targeting mitotic cyclins.

The core APC/C is assembled from 13 different subunits, mostly highly conserved and essential for function, generating a macromolecular machine exceeding 1.2 MDa in mass (Peters, 2006; Pines, 2006; Thornton and Toczyski, 2006; Sullivan and Morgan, 2007). The catalytic centre of the APC/C is formed from the cullin and RING subunits Apc2 and Apc11, analogous to Cul1 and Rbx1, respectively, of the SCF cullin RING ligase. In combination, Apc2 and Apc11 are competent to catalyse protein ubiquitylation, although lack substrate selectivity (Gmachl *et al*, 2000; Leversson *et al*, 2000; Tang *et al*, 2001). Single particle electron microscopy studies of yeast and vertebrate APC/C, revealing the overall shape, architecture and dimensions of the molecule (Dube *et al*, 2005; Passmore *et al*, 2005b; Ohi *et al*, 2007; Herzog *et al*, 2009), have been supplemented by crystallographic studies of a few individual APC/C subunits (Wendt *et al*, 2001; Au *et al*, 2002; Han *et al*, 2009; Wang *et al*, 2009; Zhang *et al*, 2010).

A particularly striking feature of APC/C subunits is that many incorporate multiple sequence repeats. The most prevalent structural motif is a degenerative 34-amino acid unit termed the tetratricopeptide repeat (TPR). Four conserved APC/C subunits (Cdc16/Cut9/Apc6, Cdc23/Cut23/Apc8, Cdc27/Nuc2/Apc3 and Apc5), together with Apc7 unique to vertebrate APC/C, are almost exclusively composed of multiple TPR motifs, usually arranged in contiguous arrays. In contrast, Apc1, the largest APC/C subunit, consists of nine tandem repeats of a 30–40 residue proteasome cyclosome motif (Lupas *et al*, 1997), whereas the N-terminus of Apc2 comprises three consecutive ~130 residue cullin repeats. Multiple TPR arrays, originally discovered within proteins subsequently identified as components of the APC/C (Hirano *et al*, 1990; Sikorski *et al*, 1990; Lamb *et al*, 1994; Irniger *et al*, 1995; King *et al*, 1995; Sudakin *et al*, 1995; Tugendreich *et al*, 1995), and now recognized as ubiquitous to all domains of life, form superhelical structures that function to mediate protein–protein interactions, particularly the assembly of multiprotein complexes (Das *et al*, 1998; Groves and Barford, 1999; D’Andrea and Regan, 2003).

Subunit deletion experiments using modified yeast strains and biochemical studies of vertebrate APC/C have defined

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the architectural topology of APC/C subunits (Vodermaier *et al*, 2003; Thornton and Toczyski, 2006). Apc1 functions as a central scaffolding subunit that links the catalytic subcomplex formed from Apc2, Apc11 and Apc10/Doc1 with the TPR subunits Cdc16/Cut9, Cdc23 and Cdc27 via Apc4 and Apc5. The TPR subunits Cdc27, Cdc16/Cut9 and Cdc23 display a sequential binding dependency on Apc1. Thus, Cdc27 is the most peripheral TPR subunit whose contacts to the more internal Cdc23 subunit are mediated via Cdc16/Cut9 (Thornton *et al*, 2006). In yeast, the small and mainly unstructured subunits Apc9, Apc13 and Cdc26/Hcn1 function to stabilize the TPR subcomplex (Zachariae *et al*, 1998; Passmore *et al*, 2003; Thornton and Toczyski, 2006). Interactions involving Cdc16/Cut9 and Cdc26/Hcn1 were implicated from yeast two-hybrid screens (Yoon *et al*, 2006), and the genetic identification of *Schizosaccharomyces pombe* Hcn1 as a high-copy suppressor of Cut9 mutations (Yamada *et al*, 1997). Despite the essential requirement of all TPR subunits in APC/C function (Hirano *et al*, 1990; Sikorski *et al*, 1990, 1991, 1993; O'Donnell *et al*, 1991; Samejima and Yanagida, 1994), with the exception of Cdc27, the specific biological functions of TPR subunits are poorly defined. For Cdc27, it is known that its C-terminal TPR array coordinates interactions of the core APC/C with the C-terminal Ile-Arg motifs of co-activator and the intrinsic APC/C subunit Apc10/Doc1 (Wendt *et al*, 2001; Passmore *et al*, 2003; Vodermaier *et al*, 2003; Kraft *et al*, 2005; Thornton *et al*, 2006; Matyskiela and Morgan, 2009).

To understand the structure and function of the APC/C's TPR subunits, we have crystallized an *S. pombe* Cut9-Hcn1 subcomplex. In our structure, which comprises the ordered regions of both proteins, we find that Cdc16/Cut9 forms a contiguous TPR superhelix consisting of an array of 14 consecutive TPR units. Cdc16/Cut9 homo-dimerizes through its N-terminal seven TPR motifs, by means of an inter-locking TPR-TPR homotypic interaction essentially identical to the N-terminal dimerization domain recently reported for Cdc27 (Zhang *et al*, 2010). Homo-dimerization of Cut9 is consistent with the supra-stoichiometric abundance of Cdc16/Cut9, Cdc23 and Cdc27 (Passmore *et al*, 2003; Dube *et al*, 2005; Ohi *et al*, 2007) and its capacity, like Cdc23 and Cdc27, to self-associate (Lamb *et al*, 1994). A C-terminal seven-TPR motif domain confers Cdc26/Hcn1 interactions, similar to the C-terminal domain of human Apc6 (Wang *et al*, 2009). This study, demonstrating that Cdc16/Cut9 homo-dimerizes through a mechanism related to Cdc27, provides a structural framework for the self-association of TPR subunits and suggests that Cdc16/Cut9 and Cdc27 are related through divergent evolution from a common ancestor. Our structure also reveals that a post-translational N-terminal acetyl modification of Met1 of Hcn1 is buried within a chamber created by the Cdc16/Cut9 TPR-superhelix and its dimerization interface. N-terminal acetyl modifications were recently identified as a degron for the Doa10 E3 ubiquitin ligase (Hwang *et al*, 2010). We discuss the implications of this finding for defining the correct *in vivo* stoichiometry of subunits of multiprotein complexes.

Results and discussion

Structure determination

An isolated and stable complex of *S. pombe* Cut9-Hcn1 was identified when we co-expressed tagged Cut9 in the presence

of untagged Hcn1 together with other APC/C subunits using the baculovirus/insect cell expression system (see Materials and methods). The Cut9-Hcn1 complex was the major product following purification of Strep II-tagged Cut9, and an association of these two proteins is consistent with previous reports suggesting their interaction (Yamada *et al*, 1997; Yoon *et al*, 2006). Attempts to crystallize the Cut9-Hcn1 complex yielded only small crystals after a few weeks at 20°C, correlating with the spontaneous proteolytic removal of 74 C-terminal residues. Thus, a protease-resistant C-terminal boundary of *S. pombe* Cut9 was defined similar to that independently identified for human Apc6 (Wang *et al*, 2009) (Supplementary data). Consistent with these domain boundary definitions, the C-terminal 70 residues of Cut9 are poorly conserved (Figure 1A). A Cut9-Hcn1 co-expression system corresponding to residues Met1-Asn597 of Cut9 and full-length Hcn1 expressed at high levels in insect cells and crystallized readily. We determined the structure of Cut9-Hcn1 *ab initio* by means of SAD phasing from a gold derivative at 2.6 Å resolution. Data collection and refinement statistics are summarized in Supplementary Table S1.

Cut9-Hcn1 form a hetero-tetrameric assembly

Two Cut9 molecules homo-dimerize through a homotypic dimer interface generated by a two-fold non-crystallographic symmetry operation (Figure 2). As judged by several criteria, this dimer is likely to be biologically relevant. First, the dimer interface buries an extensive 6233 Å² of surface area. Second, the interface is generated from residues conserved across all orthologs of Cut9 (Figure 1A). Third, the interface shares striking structural similarity to the N-terminal dimerization interface of Cdc27, a related TPR subunit of the APC/C (Zhang *et al*, 2010), fourth, molecular size analysis of Cut9-Hcn1, using multiangle light scattering (MALS), is consistent with a Cut9-Hcn1 hetero-tetramer in solution (Supplementary Figure S1), and lastly, the X-ray structure of Cut9-Hcn1 reported here docks into the protein density defined for *Saccharomyces cerevisiae* Cdc16-Cdc26 within the APC/C molecular envelope defined by single particle electron microscopy with a high degree of correspondence (Schreiber *et al*, submitted).

Cut9/Cdc16 is a TPR superhelix of 14 motifs that homo-dimerizes

A cartoon representation of the Cut9-Hcn1 hetero-tetramer is shown in Figure 2. The molecule's appearance is dominated by the rod-like structure of each Cut9 protomer. Cut9 is composed virtually entirely of 14 contiguous TPR motifs (including two non-canonical TPR motifs) (Figures 1 and 2; Supplementary Figure S7). Each TPR motif consists of a pair of anti-parallel α -helices, termed A and B, rotated relative to one another by $\sim 24^\circ$. Successive A and B α -helices generate a right-handed TPR superhelix with A-helices lining the inner concave groove and B-helices forming the outer convex surface (Das *et al*, 1998). In Cut9, the 14 TPR motifs together with a C-terminal capping α -helix (α_{29}) generate 29 anti-parallel α -helices that adopt a linear superhelix measuring some 110 Å in length and 60 Å in diameter.

The 29 α -helices of Cut9 are divided into two functionally and structurally distinct domains; an N-terminal domain (Cut9^{Nterm}) comprising TPR1-TPR7 (α -helices α_1 - α_{14}), and a C-terminal domain (Cut9^{Cterm}) of TPR8-TPR14 (α -helices

Figure 1 Primary structure of Cdc16/Cut9/Apc6. (A) Multiple sequence alignment of Cdc16/Cut9/Apc6 orthologs; *S. pombe*, *S. cerevisiae*, *Arabidopsis thaliana*, *Drosophila melongaster* and *H. sapiens*. Red denotes invariant residues and yellow denotes conserved residues. Positions of observed TPR motifs and secondary structural elements are shown in light blue. Residues of the homotypic Cut9 dimer interface are indicated with red arrows. Mutated conserved ridge residues are indicated with blue arrows. Helices are labelled both by their structural TPR designations and by topology. Thus, the $\alpha 1$ helix of Cut9 has the TPR designation '1A' indicating the A helix of TPR1. A pair of inter-subunit salt bridges (Lys109–Asp306 and Arg135–Asp335) are invariant across all species. (B) Schematic of Cut9 showing 14 TPR motifs, dimerization module Cut9^{Nterm} and Cdc26/Hcn1-interacting domain, Cut9^{Cterm} (A) prepared using ALSCRIPT (Barton, 1993).

Figure 2 Cut9-Hcn1 is a hetero-tetramer. Cartoon representation of Cut9-Hcn1 is shown in orthogonal views. Two copies of Cut9 are shown in magenta and green, respectively, and Hcn1 are shown in yellow and cyan, respectively. ♦ indicates the two-fold symmetry axis. Cut9^{Nterm} and Cut9^{Cterm} domains are shown in each promoter, with lighter and darker shading, respectively. Figure is generated using PyMOL (<http://www.pymol.org>).

$\alpha 15$ – $\alpha 28$) and $\alpha 29$. Cut9 subunits homo-dimerize through their Cut9^{Nterm} domains to generate a shallow ‘V’-shaped molecule with a maximum dimension of some 155 Å (Figure 2). The more globular Cut9^{Nterm} dimerization module forms the apex of the ‘V’ with the narrower C-terminal TPR superhelices of Cut9^{Cterm} projecting away from the dimer interface. Cut9^{Cterm} contributes the majority of Cut9 interactions to Hcn1.

Comparison of the conformation of TPR motifs of Cut9 with other TPR-containing proteins identifies two distinct TPR segments (Supplementary Figure S2). An N-terminal segment of four TPR motifs (TPR1–TPR4) superimposes most closely with the synthetic four-TPR superhelix developed by Regan and colleagues (Kajander *et al*, 2007; Holm *et al*, 2008). This N-terminal TPR segment is divided from an array of eight tandem TPR motifs (TPR7–TPR14) by four anti-parallel α -helices, forming the non-canonical TPR motifs TPR5 and TPR6. TPR7–TPR14 create a tandem array of eight TPR motifs. However, unlike the uniform tandem TPR motifs of the canonical TPR superhelix of *N*-acetyl glucosamine transferase (Jinek *et al*, 2004), TPR7–TPR14 of Cut9 are slightly overwound, especially for TPR7–TPR10, resulting in a shorter helix pitch (66 versus 46 Å). As discussed below, this is possibly due to their interaction with the extended N-terminal segment of Hcn1. The 82 residues N-terminal to TPR1, which are poorly conserved across species and absent from human Apc6/Cdc16 (Figure 1A), are largely disordered

except for residues 45–60, which incorporates a short α -helix ($\alpha 0$) that contributes to the Cut9 dimer interface (Figures 2 and 3A).

Comparison with human Apc6–Cdc26. The TPR block of TPR7–TPR14 and $\alpha 29$ of *S. pombe* Cut9 is analogous to the crystal structure of a protease-resistant domain of human Apc6 in complex with the N-terminal 29 residues of Cdc26 (Wang *et al*, 2009) (Supplementary Figure S3). Equivalent C α -atoms of *S. pombe* and human Cut9/Apc6 superimpose with an r.m.s.d. of 2.0 Å. The *S. pombe* Cut9^{Nterm} dimerization domain (equivalent to residues 1–263 of human Apc6) is not present in the crystallized human Apc6 protein, which accordingly does not form homo-dimers.

Cut9 forms a TPR-mediated homo-dimer interface similar to Cdc27

Cut9^{Nterm} forms an intimate homotypic dimer interface (Figures 2 and 3A). In essence, the concave surface of each Cut9^{Nterm} domain encircles its dimer counterpart, in an inter-locking clasp-like arrangement. This is achieved as the N-terminal TPR1 and TPR2 motifs of each Cut9 subunit interacts with residues lining the inner groove TPR α -helices (A-helices) of TPR1–TPR7 of the opposite subunit. Thus, 14 anti-parallel α -helices constitute the Cut9 dimer interface. In Cut9, the subunit interface is augmented by the N-terminal residues 45–60 that fill the gap between the TPR helices of

Figure 3 N-terminal dimerization domain of Cut9 and Cdc27 are structurally related. (A) Cartoon representation of Cut9^{Nterm} (B) Cdc27^{Nterm} after structural superimposition. ♦ indicates the two-fold symmetry axis. Superimpositions were performed using DALI (Holm *et al*, 2008), Z-score = 14, r.m.s.d. = 2.4 Å for 135 aligned residues. (C) Stereoview of a superimposition of Cut9^{Nterm} and Cdc27^{Nterm}.

opposing subunits; however, their lack of conservation in other Cut9/Cdc16/Apc6 orthologs (Figure 1A) suggests this may be a feature unique to *S. pombe* Cut9. The arrangement of TPR motifs at the Cut9 dimer interface is strikingly similar to the TPR-based N-terminal dimerization domain of Cdc27 (Cdc27^{Nterm}) (Figure 3B and C) (Zhang *et al*, 2010). Similar to Cut9^{Nterm}, TPR1-TPR4 of Cdc27 conform to an idealized TPR superhelix. Although less well aligned, TPR6 and TPR7 of Cut9 correspond to TPR5 and TPR6 of Cdc27^{Nterm}. A small difference between the two proteins is that TPR5 of Cut9^{Nterm} has no direct counterpart in Cdc27^{Nterm}, but is equivalent to α10 and a disordered loop (Figure 3).

The structural relationship between Cut9 and Cdc27 is clearly illustrated in a structure-based sequence alignment of Cut9^{Nterm} and Cdc27^{Nterm}. Marked sequence similarity comprises TPR1-TPR5A of Cut9^{Nterm} and TPR1-TPR4 and α10 of Cdc27^{Nterm} (Figure 4A). At the Cut9^{Nterm} dimer inter-

face, contacts between the two subunits are defined by two clusters of residues involving a mixture of conserved hydrophobic and hydrophilic interactions, analogous to the Cdc27 dimer interface (Zhang *et al*, 2010) (Figure 4B; Supplementary Figure S4). Specifically, in cluster 1 of Cut9, the aromatic side chain of Phe105 of TPR1B (helix B of TPR1) participates in hydrophobic packing interactions with Tyr234' of TPR5A' and Leu274' of TPR6A' of the opposite subunit (superscript denotes residues of the opposite subunit). In Cdc27^{Nterm}, Phe23 of TPR1B (structurally equivalent to Phe105 of Cut9) interacts with Phe145' and Phe147' of α10' and Tyr180' of TPR5A'. Tyr234' and Leu274' of Cut9^{Nterm} are structurally equivalent to Phe147' and Tyr180', respectively of Cdc27^{Nterm} (Figure 4). In cluster 2, Tyr154 (TPR3A) and Ser201 (TPR4A) of Cut9 contact the intra-helix turn of TPR1 of the opposite subunit, a role played by Tyr66 and Phe115 of Cdc27^{Nterm} (Figure 4).

Figure 4 Cdc16/Cut9^{Nterm} and Cdc27^{Nterm} share sequence similarity. (A) Structure-based alignment of dimerization domains of Cdc16/Cut9 and Cdc27 (TPR1–TPR4 and TPR5A of Cdc16/Cut9 and TPR1–TPR4 and α10 of Cdc27). Yellow denotes conserved residues. Positions of observed TPR motifs and secondary structural elements are shown in light blue. Blue arrows denote positions of structurally equivalent dimerization residues, with corresponding *Encephalitozoon cuniculi* Cdc27 and *S. pombe* Cut9 residue numbers indicated below. Interface cluster is indicated in parenthesis. Residues conserved between Cdc16/Cut9 and Cdc27 are not confined to TPR consensus positions: numbers below sequence correspond to consensus TPR positions: 8, 20 and 27 (in blue) are small non-residues, 4, 12, 17, 21, 28 and 30 (in red) are larger non-polar residues. (B) Stereoview of a superimposition of the Cdc16/Cut9 and Cdc27 homotypic dimer interface, showing two clusters of contacts defining the interface. Colour scheme as for Figure 3. For clarity, Leu274 and Tyr180 of Cut9 and Cdc27, respectively, are not included.

In an attempt to demonstrate that Cut9 homo-dimerization is mediated by the interface observed in the crystal structure, we replaced dimer interface residues with amino acids predicted to interfere with homo-dimerization. Specifically, changes were: M96R, Q97R, F105R and K109A (Figure 1A). These mutations severely destabilized Cut9 to such an extent that the expressed protein was aggregated, and an assessment of its oligomeric state was not possible. Such destabilization of Cut9 would be consistent with a disrupted dimer interface that exposes an uncompensated non-polar surface and, due to loss of protein stabilizing inter-molecular interactions, results in protein mis-folding and aggregation. Disruption of the structurally related Cdc27 homo-dimer interface similarly destabilizes the protein (Zhang *et al*, 2010).

The N-terminal acetyl-Met residue of Hcn1 is buried within Cut9

Full-length Hcn1 was co-crystallized with Cut9; however, only residues 1–24 are clearly visible in the electron density map, and thus the lack of density for residues 25–80 indicates structural disorder. Notably, these latter residues share little sequence similarity to related Hcn1 orthologs. Similar to the human Apc6–Cdc26 structure (Wang *et al*, 2009), the N-terminal 13 residues of Cdc26 form an extended conformation running parallel with, and lining the inner groove of the Cut9/Apc6 TPR superhelix contacting, primarily, although not exclusively, A-helices of Cut9^{Cterm} (specifically TPR7–TPR14) (Figure 5). Residues 14–21 of Hcn1 fold into an α-helix that packs parallel with α29 of Cut9, augmenting the array of Cut9 α-helices. As judged by increased α-helical

Figure 5 The N-terminal Met of Hcn1 is acetylated and enclosed within a chamber formed from the Cut9 TPR superhelix and homo-dimer interface. **(A)** $2F_o - F_c$ electron density map showing Hcn1. The N-terminus of Hcn1 is well ordered, and the *N*-acetyl-Met group is well defined. **(B)** N-terminal region of Hcn1 is shown in cyan with Cut9 shown as a surface representation, with secondary structure indicated.

content and thermal denaturation temperatures, Cdc26 interactions with Apc6 confer structural stability upon Apc6 (Wang *et al*, 2009). Hcn1 is an essential gene in *S. pombe* (Yoon *et al*, 2006), whereas its deletion confers a *ts* phenotype in *S. cerevisiae* (Passmore *et al*, 2003), however, consistent with both *S. pombe* and human Cut9–Hcn1 structures, only the conserved N-terminal 31 residues are required for budding yeast viability at the restrictive temperature (Wang *et al*, 2009).

One significant difference between the *S. pombe* and human Cut9–Hcn1 structures is the N-terminal acetylation of Met of *S. pombe* Hcn1. This is most likely attributable to our expression of *S. pombe* Cut9–Hcn1 in insect cells, whereas human Apc6–Cdc26 was expressed in *E. coli*. Electron density for the acetyl modification of Met1 of Hcn1 is clearly visible in the $2F_o - F_c$ map (Figure 5A). The N-terminal acetylated-Met residue is located within an enclosed chamber formed from the inner groove of the

Cut9 TPR superhelix (Figure 5B). Because of a combination of the tighter winding of the Cut9 TPR helix relative to canonical TPR superhelices, and the extended α -helices of TPR12B and TPR13A, acting to close the groove of the TPR superhelix, the N-terminus of Hcn1 is completely surrounded by its interacting Cut9 subunit. The upper extent of the chamber is capped by the N-terminus of the opposing Cut9 subunit (Figure 5B), although Hcn1 does not participate at the dimer interface. Thus, the acetyl group of the N-terminal Met of Hcn1 is completely inaccessible to bulk solvent.

LC–MS/MS confirmed that recombinant *S. pombe* Hcn1 was N-terminally acetylated on Met 1 (Figure 6A; Supplementary Figure S8). Importantly, we did not detect any non-acetylated N-terminal peptide. To investigate whether *S. cerevisiae* Cdc26 is N-terminally acetylated *in vivo*, we purified endogenous *S. cerevisiae* APC/C (Passmore *et al*, 2003, 2005a), and analysed coomassie-stained gel bands corresponding to Cdc26, Apc11 and Apc13/Swm1. Although peptides corresponding to Cdc26 were identified, we could not detect an N-terminal peptide, and thus have been unable to establish whether Cdc26 is acetylated *in vivo*. However, an unexpected peptide corresponding to N-terminally acetylated Cdc27 was detected and validated (Figure 6B). This peptide probably derived from a truncated N-terminal domain of the protein as a contaminant of the excised Apc11 band. An identical N-terminal acetylated peptide was identified from purified recombinant *S. cerevisiae* Cdc27 produced using the insect cell system (Supplementary Figure S5). The correlation between recombinant and endogenous *S. cerevisiae* Cdc27 N-terminal acetylation would suggest that, as recombinant *S. pombe* Hcn1 is acetylated, endogenous Cdc26/Hcn1 is also likely to be similarly modified. We did not perform a systematic analysis of all APC/C subunits but we note that acetylated N-terminal peptides were detected for recombinant *S. pombe* Apc14, *S. cerevisiae* Cdc23, and *Homo sapiens* Apc13 and Apc16 (data not shown).

Recently, N-terminal acetylation was identified as a secondary degradation signal (degron) for the Doa10 E3 ubiquitin ligase (Hwang *et al*, 2010). One suggested role for N-terminal acetylation, which is present in 80% of human proteins, was to control the relative stoichiometry of subunits constituting a multisubunit complex. Our findings support this model as the burial of *N*-acetyl-Met of Hcn1 in complex with Cut9/Cdc16 would prevent its recognition and degradation via the Doa10-proteasome pathway, whereas excess levels of Hcn1 not in complex with Cut9/Cdc16, and therefore with an exposed *N*-acetyl-Met residue, would be available for potential recognition by the Doa10 E3 ubiquitin ligase and therefore subject to degradation via the ubiquitin proteasome system. To our knowledge, this is the first structure of a protein complex showing structural details of an N-terminal acetylated residue.

A conserved ridge transversing the outer convex surface of the Cdc16/Cut9 TPR superhelix mediates interactions to Cdc23 and Cdc27

Mapping the position of conserved residues onto the molecular surface of Cut9/Cdc16 reveals a striking cluster of sequence conservation defining a ridge that transverses one

Figure 6 MS/MS spectra corresponding to fragmentation of the N-terminally acetylated peptides. (A) Ac-¹MLRRNPTAIQITAEDVLAYDEE²².K (precursor ion 864.0981³⁺, mass error -0.82 p.p.m., ion score 92) from *S. pombe* Hcn1 digested with Glu-C. The fragmented mass series are consistent with N-acetylation (42 Da) of Met1, as the B2 mass of 287 Da corresponds to Met-Leu + (42 Da). (B) Ac-²AVNPELAPFTLSR¹⁴.G (precursor ion 728.8927²⁺, mass error -1.0 p.p.m., ion score 36.9) from endogenous *S. cerevisiae* Cdc27 digested with trypsin. LC-MS/MS confidently identified acetylated N-terminal peptides, with high mass accuracy and comprehensive fragmentation data for both *S. pombe* Hcn1 and *S. cerevisiae* Cdc27.

face of the outer convex surface of Cut9/Cdc16, primarily localized to TPR9–TPR11 (Figures 1A and 7). The conserved ridge features adjacent positively and negatively charged regions, with a cluster of solvent exposed basic residues on TPR9B adjacent to acidic residues of TPR10B. These evolutionarily conserved residues may mediate Cdc16/Cut9 interactions with other APC/C subunits, APC/C substrates and/or regulators. In the context of the holo APC/C, Cdc16/Cut9 mediates interactions between Cdc23 and Cdc27 (Thornton *et al*, 2006). To test whether the conserved residues mediate assembly of Cdc16/Cut9 with either Cdc27 or Cdc23, we determined the effects of ridge mutations on the capacity of

Cdc16/Cut9 to bind Cdc23 and Cdc27. Wild-type *S. cerevisiae* Cdc16–Cdc26 forms stable quaternary complexes with both Cdc23–Apc13 and Cdc27–Apc9 (Supplementary Figure S6; Supplementary Table S2). However, when we mutated conserved ridge residues (mainly through charge reversal) (Figure 1A), we completely abolished the capacity of Strep-tagged Cdc16 to pull-down Cdc23 and Apc13, and severely impaired interactions with Cdc27, but not Apc9 (Supplementary Figure S6). These results provide strong evidence that conserved residues on the convex face of the Cdc16/Cut9 TPR superhelix mediate its association with Cdc23–Apc13, and to a less extent Cdc27.

Figure 7 A structurally conserved ridge defines one face of Cdc16/Cut9. (A) Two orthogonal views showing a surface representation of Cdc16/Cut9 colour coded according to structural conservation ramped from red to blue for low-to-high sequence conservation, based on sequences included in Figure 1. One Cut9-Hcn1 dimer of the tetramer is shown as a cartoon, with the surface of the symmetry-related molecule displayed. (B) Close-up view with mutated residues indicated. Conservation score is defined using ConSurf (Landau *et al*, 2005).

Structural similarities between Cdc16/Cut9 and Cdc27 suggest a common evolutionary ancestor

Our structure of the Cut9-Hcn1 hetero-tetramer described here, together with our previous structure of the Cdc27^{Nterm} dimerization domain (Zhang *et al*, 2010), strongly suggests that Cdc16/Cut9 and Cdc27 evolved from a common ancestor. Both Cdc16/Cut9 and Cdc27 homo-dimerize through an N-terminal domain composed of multiple TPR motifs that engage through an intimate clasp-like interaction (Figure 3). Conventional sequence alignment programs failed to detect clear sequence similarities between the N-terminal regions of Cdc16/Cut9 and Cdc27; however, a structure-based alignment reveals a significant sequence similarity of 18% (Figure 4A). Residues that mediate dimer contacts within

each protein are structurally equivalent, and in many instances, conserved (Figure 4). The TPR motifs of Cut9^{Nterm} form an array of TPR motifs that are contiguous with seven TPR motifs of Cut9^{Cterm} (TPR8-TPR14 with α 29). TPR7-TPR14 of Cut9^{Cterm} were correctly defined by TPR prediction programs, whereas TPR motifs of Cut9^{Nterm} were underpredicted such that only TPR2-TPR4 were assigned. Cut9^{Cterm} extends from the Cut9^{Nterm} dimerization domain, forming over one turn of isolated TPR superhelix, and generating a highly elongated molecule (Figure 2). Similar to Cdc16/Cut9, the region C-terminal of Cdc27^{Nterm} is predicted to be composed of a contiguous array of seven TPR motifs that would form a TPR superhelix to extend out from Cdc27^{Nterm} creating a 'V'-shaped molecule (Zhang *et al*, 2010). Thus, Cdc16/Cut9

and Cdc27 share the common structural features of an N-terminal TPR-mediated dimerization domains of ~seven TPR motifs connected to a C-terminal TPR superhelix formed from an additional seven contiguous TPR motifs.

The homo-dimers of Cdc16/Cut9 and Cdc27 (Zhang *et al*, 2010) are consistent with previous data suggesting that Cdc16/Cut9, Cdc23 and Cdc27 self-associate (Lamb *et al*, 1994), and previous indications of supra-stoichiometric quantities of these TPR subunits (Dube *et al*, 2005; Passmore *et al*, 2005b; Ohi *et al*, 2007; Herzog *et al*, 2009). Ohi *et al* (2007) predicted that Cdc16/Cut9 and Cdc27 (and Cdc23) are present in two copies per APC/C molecule, consistent with our crystallographic structures of Cdc16/Cut9 and Cdc27, and molecular mass estimates derived from solution-based MALS (Supplementary Figure S1 for full-length Cut9-Hcn1 and (Zhang *et al*, 2010) for Cdc27). Cdc23 is also a homo-dimer (ZZ and DB, unpublished data), and it is tempting to suggest that Cdc23 homo-dimers may share some of the structural features of Cdc16/Cut9 and Cdc27. Similar to Cdc16/Cut9 and Cdc27, there is a convincing prediction for nine contiguous TPR motifs located within the C-terminus of the protein, separated from a single predicted TPR motif towards the N-terminus. Predictions of TPR motifs tend to underestimate TPR motifs, and TPR1 neither of Cdc16/Cut9 nor Cdc27 were predicted. Thus, similar to Cdc16/Cut9 and Cdc27, Cdc23 may homo-dimerize via an N-terminal TPR-domain connected to a C-terminal TPR superhelix.

Concluding remarks

Our structure of the Cut9-Hcn1 hetero-tetramer, the largest APC/C subcomplex to be crystallized to date, representing some 12% of the APC/C mass, reveals significant structural similarities to Cdc27, suggesting divergence from a common evolutionary ancestor. We suggest Cdc23 may adopt a related homo-dimeric structure, and we have identified an evolutionarily conserved ridge of Cdc16/Cut9 that mediates interactions with Cdc23-Apc13 and Cdc27. A role for a conserved ridge to contact both Cdc23 and Cdc27 simultaneously is compatible with a dimeric species of Cdc16/Cut9 and consistent with its role in mediating Cdc27 and Cdc23 interactions (Thornton *et al*, 2006). In a separate study, we have docked the crystal structure of Cdc16/Cut9 into a recently determined 10 Å resolution cryo-electron microscopy map of the APC/C (Schreiber *et al*, submitted). The excellent correspondence between the Cdc16/Cut9 coordinates and the APC/C molecular envelope confirms the biological relevance of the Cut9-Hcn1 hetero-tetramer and the accuracy of the EM map.

Materials and methods

Cloning, expression and purification of the Cut9-Hcn1 complex

Schizosaccharomyces pombe Cut9 (Samejima and Yanagida, 1994) and Hcn1 (Yamada *et al*, 1997) genes were amplified by PCR using an *S. pombe* cDNA library pTN-TH7 (a gift from T Nakamura, NBRP, Japan) as a template and cloned into the MultiBac vector pFBDM (Berger *et al*, 2004). A double Strep II tag together with a TEV cleavage site were attached to the C-terminus of Cut9. Cut23, Nuc2, Apc13, Apc14 and Apc15 were added to the Cut9-Hcn1 plasmid resulting in a plasmid containing a seven gene cassette. An *S. pombe* TPR-7 complex (Cut9-[double Strep II tag], Cut23, Nuc2, Hcn1, Apc13, Apc14 and Apc15) was expressed using the baculovirus/insect cell (high 5 cells) system. The majority of

the protein purified was identified as a Cut9-Hcn1 complex by mass spectrometry and peptide sequencing. The Cut9 C-terminal truncation-Hcn1 plasmids were modified from the full-length Cut9-Hcn1 plasmid. The resulted protein expression cassettes were recombined with DH10MultiBac by transposition. The Cut9-Hcn1 complexes were purified by a combination of Strep-Tactin (Qiagen), anion exchange chromatography Mono Q and Superose 6 size-exclusion chromatography (GE Healthcare).

For *S. pombe* Cut9 dimerization interface mutagenesis, Cut9-[double Strep II tag] was mutated: (M96R, Q97R, F105R, K109) in the Cut9-Hcn1-expressing cassette in pFBDM. For conserved ridge mutations, *S. cerevisiae* Cdc16 and Cdc26 were cloned in pFBDM with a double Strep II tag added to Cdc16 C-terminus. The resultant ScCdc16-[double Strep II tag]-Cdc26 in pFBDM was mutated to ScCdc16^{rmut}(R546E/K552E/K556E/D582K/Q583K)-[double Strep II tag]-Cdc26. These residues are equivalent to *S. pombe* Cut9 K388, R394, K398, D424, Q425 (Figure 1A). The *S. cerevisiae* Cdc23-Apc13 and Cdc27-Apc9-expressing cassettes were cloned into pUCDM. The ScCdc16-[double Strep II tag]-Cdc26-Cdc23-Apc13, ScCdc16^{rmut}(R546E/K552E/K556E/D582K/Q583K)-[double Strep II tag]-Cdc26-Cdc23-Apc13, ScCdc16-[double Strep II tag]-Cdc26-Cdc27-Apc9, ScCdc16^{rmut}(R546E/K552E/K556E/D582K/Q583K)-[double Strep II tag]-Cdc26/Cdc27-Apc9 bacmids were combined by transposition as described (Berger *et al*, 2004). The protein samples were purified by Strep-Tactin (Qiagen) from high 5 cells expression and analysed by SDS-PAGE, western blot and mass spectrometry analysis. USER methodology was used for cloning (Bitinaite *et al*, 2007).

Crystallization

Crystallization screening was performed at 4°C, and 20°C under oil. Small crystals were observed in a few conditions at 20°C after 2–3 weeks. The long lag period before the appearance of these crystals suggested they resulted from proteolysis of one or both proteins. To assess this possibility, harvested crystals were analysed by SDS-PAGE. This revealed a truncation of Cut9 of ~10 kDa, and full-length Hcn1. N-terminal sequencing of truncated Cut9 revealed an intact N-terminus and therefore demonstrating a C-terminal truncation. On the basis of multiple sequence conservation and disorder prediction analysis, we predicted that the C-terminal domain boundary would be located at ~residue Asn597. Cut9^{N597}-Hcn1 formed a complex and expressed at high level. A shorter construct Cut9^{N513}-Hcn1 did not form a complex with Hcn1, and purified Cut9^{N513} was aggregated. Crystals of Cut9^{N597}-Hcn1 were obtained in a buffer containing 100 mM Hepes (pH 7.5), 100 mM sodium acetate and 12.5% (w/v) PEG 3350. For cryo-freezing, the crystals were transferred to a buffer supplemented with 20% (v/v) ethylene glycol.

Structure determination

Native crystals diffract weakly to 3.1 Å resolution; however, we found that soaking crystals for 2 h in 10 mM gold cyanide increased resolution to 2.6 Å. Single-wavelength anomalous data for the Cut9-Hcn1 crystal, soaked with 10 mM potassium gold cyanide for 2 h, were collected at the Diamond Light Source beam line I02. All data were processed using iMosflm and scaled using SCALA (CCP4, 1991). Initial phases and backbone fragments were obtained using *auto solve* module of PHENIX (Adams *et al*, 2002). Iterative cycles of manual building were performed with COOT (Emsley and Cowtan, 2004) and refined with PHENIX (Adams *et al*, 2002). TLS refinement in PHENIX program was also used in the refinement of Cut9. Water molecules and N-linked acetyl group were added towards the end of the refinement. The structure was validated with PROCHECK (Laskowski *et al*, 1993) and MolProbity (Davis *et al*, 2007). There are two molecules of Cut9-Hcn1 per asymmetric unit. Breaks in the polypeptide of Cut9 occur in the residue range 61–82 and 181–191.

Multiangle light scattering

MALS were performed using a Wyatt MALS system. Full-length Cut9-Hcn1 complex was injected onto an analytical S200 gel filtration column pre-equilibrated in 20 mM Tris (pH 8.0), 150 mM NaCl, 2 mM DTT, 3 mM NaN₃. Data were analysed using Wyatt Technology software.

Liquid chromatography–mass spectrometry of 1D SDS–PAGE gel bands

Selected gel bands were excised, diced, reduced with 5 mM Tris(2-carboxyethyl)phosphine, free cysteines were alkylated with 55 mM iodoacetamide, and the proteins digested with either trypsin (Promega) or endoproteinase Glu-C. After digestion, the proteolysed peptides were extracted with acetonitrile (Rathburn) and triethylammonium bicarbonate washes, the solution dried *in vacuo* and reconstituted in 0.1% formic acid for LC–MS/MS analysis. Reversed-phase chromatography was performed using an HP1200 platform (Agilent). Peptides were resolved on a 75-mm I.D. C18 Pepmap column (3 µm particle size; LC Packings/Dionex) over 30 min using a linear gradient of 95:5–50:50 buffer A:B (buffer A: 2% acetonitrile/0.1% formic acid; buffer B: 80% acetonitrile/0.1% formic acid) at 300 nl/min. Peptides were ionized by electrospray ionization using 1.4 kV applied directly to the post-column LC eluent via a microtee built into the nanospray source. Sample was infused into an LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific) using a non-coated SilicaTip emitter (20 µm I.D., 10 µm tapered tip; New Objectives). The ion transfer tube was heated to 200°C and the S-lens set to 60%. MS/MS were acquired using data-dependent acquisition to sequence the top 10 most intense ions using enhanced ion trap scans. Automatic gain control was set to 1 000 000 for FT-MS and 30 000 for IT-MS/MS, full FT-MS maximum inject time was 500 ms and normalized collision energy was set to 30% with an activation time of 10 ms. Wideband activation was used to co-fragment precursor ions undergoing neutral loss of up to -20 m/z from the parent ion, including loss of water/ammonia. MS/MS was acquired for selected precursor ions with a single repeat count followed by dynamic exclusion with a 10 p.p.m. mass window for 15 s based on a maximal exclusion list of 500 entries.

Data analysis and protein identification

Raw MS/MS data were compiled into peaklists using Proteome Discoverer v1.2 without background subtraction. Peaklists were

interrogated against the SwissProt database v57.14 (514 789 sequences) using Mascot v2.2 (<http://www.matrixscience.com>) without taxonomy filters and assuming tryptic or Glu-C enzyme specificity with up to two missed cleavages. A precursor ion tolerance of 10 p.p.m. and fragment ion tolerance of 0.6 Da was applied and the following variable modifications were accounted for: acetylation of the protein N-terminus, carbamidomethylation of cysteine, oxidation of methionine and pyroglutamination of peptide N-terminal glutamine. The Mascot peptide and protein identification results were grouped and validated using Scaffold v3.0 (Proteome Software Inc). Protein identifications were automatically accepted if they contained at least two unique peptides assigned with at least 95% confidence by Peptide Prophet (Keller *et al*, 2002).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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