

Panta rhei: The APC/C at steady state

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The anaphase-promoting complex or cyclosome (APC/C) is a conserved, multisubunit E3 ubiquitin (Ub) ligase that is active both in dividing and in postmitotic cells. Its contributions to life are especially well studied in the domain of cell division, in which the APC/C lies at the epicenter of a regulatory network that controls the directionality and timing of cell cycle events. Biochemical and structural work is shedding light on the overall organization of APC/C subunits and on the mechanism of substrate recognition and Ub chain initiation and extension as well as on the molecular mechanisms of a checkpoint that seizes control of APC/C activity during mitosis. Here, we review how these recent advancements are modifying our understanding of the APC/C.

Preamble

A conserved autocatalytic biochemical oscillator is the basic device driving the cell division cycle of eukaryotes (Ferrell et al., 2011). Protein phosphorylation, ubiquitination, and degradation, as well as their reversal through dephosphorylation, deubiquitination, and protein synthesis, are important actuators of the cell cycle oscillator. In this review, we focus on the anaphase-promoting complex or cyclosome (APC/C; King et al., 1995; Sudakin et al., 1995), an essential component of a pathway that controls the ubiquitination of crucial cell cycle regulators and their subsequent destruction by the proteasome.

Targeting of proteins to the proteasome requires the assembly of a polyubiquitin (Ub) chain onto the substrates. The activation of Ub for incorporation onto a substrate requires a three-enzyme cascade reaction (Fig. 1). A Ub-activating enzyme (named E1) activates Ub and transfers it to a Ub-conjugating enzyme (E2) in a covalent adduct. Subsequently, a Ub ligase (E3) enzyme promotes the transfer of Ub onto specific substrate, usually in repeated cycles that lead to the assembly of a poly-Ub chain.

The APC/C acts as an E3 Ub ligase. Among its numerous substrates, B-type cyclins and Securin enjoy the highest degree of notoriety. Their degradation at the metaphase–anaphase transition

promotes sister chromatid separation and mitotic exit, leading to the formation of two daughters from a mother cell (Murray et al., 1989; Cohen-Fix et al., 1996; Shirayama et al., 1999). With ≥ 13 subunits (Table 1), several of which are present in multiple copies, the APC/C displays striking molecular and regulatory complexity. Its catalytic core is related to that of Cullin-RING (really interesting new gene) Ub ligases (Yu et al., 1998; Zachariae et al., 1998a). In the APC/C, however, this catalytic core is embedded in a complex framework of structural linkers and substrate- and activator-binding subunits (Fig. 2), which enables a dynamically regulated pattern of interactions with substrates and inhibitors. Here, we present an account of this complex regulatory network and discuss unresolved questions and directions for future investigation.

General description of APC/C organization

There has been tremendous recent progress on the elucidation of the structural organization of the APC/C and of its mechanism of action and inhibition. Extensive coverage of these recent efforts is to be found in several recent reviews (Peters, 2006; Barford, 2011a,b; Pines, 2011). Progress in the structural investigation of the APC/C is being brought forward through hybrid approaches that combine biochemical reconstitution, x-ray crystallography of subdomains, negative stain and cryo-EM, mass spectrometry, nuclear magnetic resonance, and cross-linking analysis (Gieffers et al., 2001; Wendt et al., 2001; Au et al., 2002; Dube et al., 2005; Passmore et al., 2005; Thornton et al., 2006; Ohi et al., 2007; Herzog et al., 2009; Wang et al., 2009; Zhang et al., 2010a,b; Buschhorn et al., 2011; da Fonseca et al., 2011; Schreiber et al., 2011; Chao et al., 2012; Tian et al., 2012; Uzunova et al., 2012; Zhang et al., 2013). A recently proposed pseudoatomic model of the APC/C (Fig. 2, A–D), together with medium-resolution analyses of APC/C complexes with activator subunits or spindle checkpoint inhibitors, provide an invaluable new framework for understanding the molecular details of APC/C function (Dube et al., 2005; Ohi et al., 2007; Herzog et al., 2009; Buschhorn et al., 2011; da Fonseca et al., 2011; Schreiber et al., 2011).

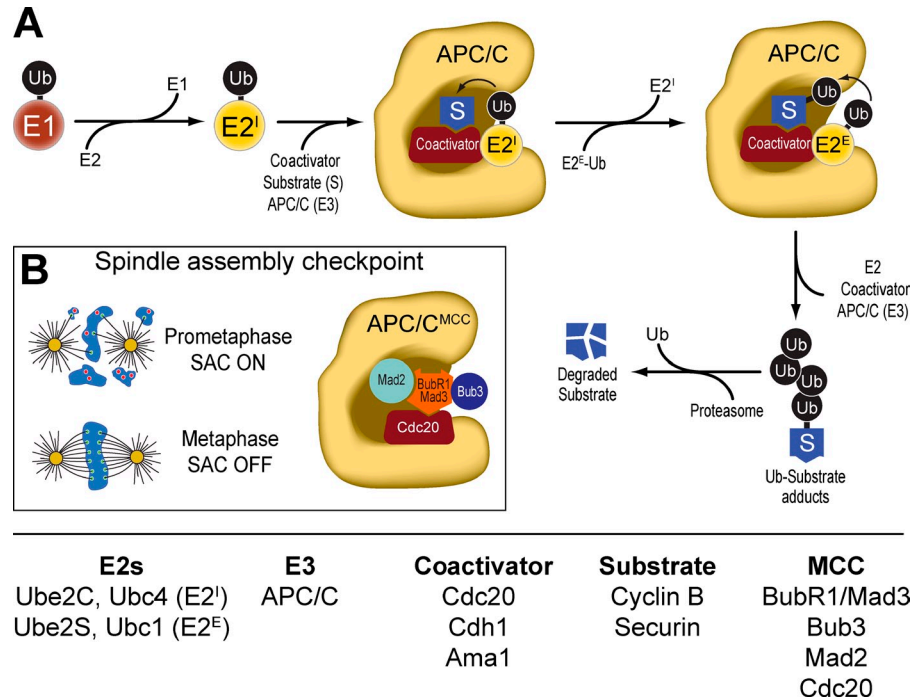
The APC/C can be structurally and functionally subdivided into three main subdomains (Fig. 2 A). A three- or four-layered sandwich of APC/C subunits that are predominantly consisting

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Abbreviations used in this paper: APC/C, anaphase-promoting complex or cyclosome; MCC, mitotic checkpoint complex; MIM, Mad2 interaction motif; SAC, spindle assembly checkpoint; Ub, ubiquitin; TPR, tetratricopeptide repeat.

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Figure 1. **Main pathways discussed in this review.** (A) Ubiquitin (Ub) is first activated by an E1 enzyme, transferred to a chain-initiating E2 (E2ⁱ), and transferred to a substrate (S). The creation of poly-Ub chains by the APC/C requires an elongating E2 (E2^e). The substrate is presented to the RING-E3 ligase APC/C by a coactivator. The main components of the pathway discussed in the main text are shown. (B) Cells in prometaphase activate the spindle assembly checkpoint (SAC ON condition) through the production of an APC/C inhibitor named the mitotic checkpoint complex (MCC). The MCC binds to the APC/C and inhibits it. Chromosomes are shown in blue, and the microtubules are shown as black fibers. Kinetochores are shown as red or green dots. Kinetochores that are not attached to microtubules (red dots) are believed to catalyze the production of the MCC. Attached kinetochores (green dots) stop producing the inhibitor. Mad2, BubR1, and Bub3 are MCC components. See Mitotic inhibition of the APC/C for details.



of 34-residue tetratricopeptide repeats (TPRs) forms the so-called arc lamp of the APC/C, which mediates coactivator binding (see next section). From top to bottom (when referring to the “classical” frontal view of the APC/C shown in Fig. 2 A), the layer of TPR subunits consists of a succession of the Cdc27/Apc3, Cdc16/Apc6, and Cdc23/Apc8 subunits, in which Cdc27/Apc3 and Cdc23/Apc8 are paralogues (Schreiber et al., 2011). All three TPR subunits are evolutionarily conserved components of the arc lamp. In higher eukaryotes, an additional TPR subunit, Apc7, is also part of it, and it interacts with Cdc27/Apc3 (Yu et al., 1998). Cdc27/Apc3, Cdc16/Apc6, and Cdc23/APC8 all form symmetric homodimers through their N-terminal regions and are therefore present in two copies in the APC/C particle (Zhang et al., 2010b). Additional subunits that are part of the arc lamp are Cdc26, which interacts with Cdc16 (Wang et al.,

2009), Apc9, which interacts with Cdc27/Apc3, and Mnd2/Apc15, which interacts with Cdc23/Apc8 (Thornton et al., 2006; Schreiber et al., 2011).

At the bottom left edge of the APC/C, Cdc23/Apc8 interacts with a platform made of three large structural subunits, Apc4, Apc5, and Apc1, which extend to the right edge of the APC/C, filling up the entire platform at the bottom of the APC/C (Fig. 2 C). The catalytic core of the APC/C is made of APC2, which shows an evolutionary relationship with the Cullin subunits of SCF (Skp1-Cullin-F-box) protein-type Ub ligases, and APC11, which features a RING-type Ub ligase domain (Yu et al., 1998; Zachariae et al., 1998a). These subunits occupy the right-hand side of the complex and are sandwiched between the Apc1-Apc4-Apc5 platform at the bottom and one of the Cdc27/Apc3 monomers at the top. Apc10 (also known as Doc1), which forms a degron coreceptor with the coactivators (as detailed in the next paragraphs), docks in front of the central cavity through interactions with Apc2 and Cdc27/Apc3 and possibly with additional APC/C subunits (Wendt et al., 2001; Thornton et al., 2006; Buschhorn et al., 2011; Schreiber et al., 2011).

The coactivators

Coactivator (often referred to simply as “activator”) proteins are required for presentation of many substrates to the catalytic apparatus of the APC/C (Schwab et al., 2001) and have additionally been implicated in APC/C activation (Kimata et al., 2008a). The best characterized mitotic coactivators are Cdc20 (also known as Fizzy or Slp1) and Cdh1 (also known as Hct1, Fizzy related, Fzr1, or Ste9; Dawson et al., 1995; Schwab et al., 1997; Visintin et al., 1997; Zachariae et al., 1998b), whereas Ama1 operates during the meiotic cycle (Cooper et al., 2000; Okazaki et al., 2012). Cdc20, Cdh1, and Ama1 are structurally related and consist primarily of a seven-bladed WD40-repeat β -propeller preceded by an unstructured tail containing important functional motifs

Table 1. **Composition of APC/C in vertebrates and in *S. cerevisiae***

Vertebrate	<i>S. cerevisiae</i>
Apc1	Apc1
Apc2	Apc2
Apc3	Cdc27
Apc4	Apc4
Apc5	Apc5
Apc6	Cdc16
Apc7	Not present
Apc8	Cdc23
Apc9	Apc9
Apc10	Doc1
Apc11	Apc11
Cdc26	Cdc26
Apc13	Swm1
Apc15	Mnd2
Apc16	Unknown

Adapted from Pines (2011).

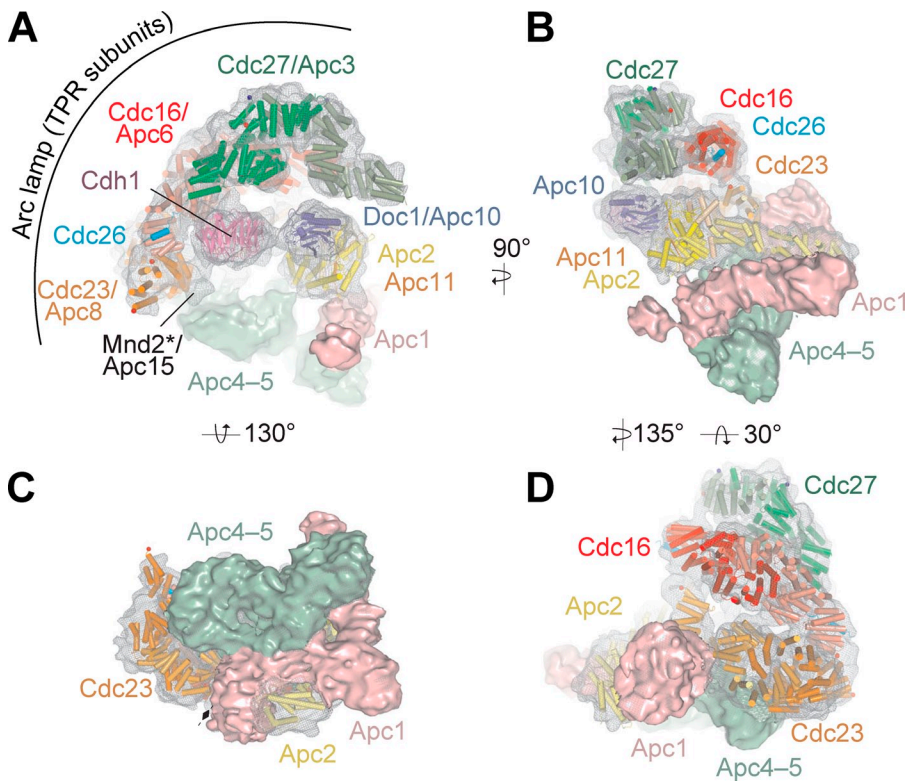


Figure 2. Structural organization of the APC/C. (A) Schematic representation of the Ub activation, conjugation, and ligation pathway and of the different players in the context of APC/C function. The asterisk on Mnd2 indicates that the indicated position of this protein in the density maps is hypothetical. (B–D) A pseudoatomic model was generated by fitting known high-resolution structures (currently available only for a subset of the APC/C subunits or for closely related sequences) into an ~ 10 -Å cryo-EM map of the APC/C^{Cdh1-D box} ternary complex from *S. cerevisiae* (Reprinted by permission from Macmillan Publishers Ltd: *Nature*, Schreiber et al., 2011). Four different orientations of the complex are shown. The subunits are labeled with both the yeast and vertebrate names. The approximate expected position of Mnd2/Apc15, for which a molecular model is currently missing, is indicated. The figure is reproduced with permission and with minor adaptations from Barford (2011b). The diamond identifies local twofold symmetry axes of Cdc27 and Cdc23. Protein Data Bank (PDB) accession numbers for deposited high-resolution models are as follows: Apc10/Doc1, 1JHJ; APC7 N-terminal domain, 3FFL; Cdc26–Apc6 complex, 3HYM; Apc10/Doc1, 1GQP; Cdc16/Cut9–Cdc26/Hcn1 complex, 2XPI; Apc8/Cdc23, 3ZN3; and Cdc27, 3KAE.

(Fig. 3 A). They interact tightly with the APC/C, and the affinity of this interaction is increased by concomitant interaction with the substrate (Vodermaier et al., 2003; Burton et al., 2005; Passmore and Barford, 2005; Matyskiela and Morgan, 2009). By EM, Cdc20 and Cdh1 appear to dock to an overlapping binding site lying at the interface of the arc lamp and the central cavity of the APC/C (as shown for Cdh1 in Fig. 2 A; Dube et al., 2005; Herzog et al., 2009; Buschhorn et al., 2011; da Fonseca et al., 2011). EM analysis of APC/C and APC/C^{Cdh1} from *Saccharomyces cerevisiae* did not reveal large conformational changes in the APC/C upon binding of Cdh1 (da Fonseca et al., 2011), unlike the large changes previously observed in vertebrate APC/C upon coactivator binding (Dube et al., 2005).

At least three coactivator sequence motifs contribute to docking to the APC/C. Two of them, the C box and the KILR motif (single letter for the lysine-isoleucine-leucine-arginine tetrapeptide; Schwab et al., 2001; Zhang and Lees, 2001; Izawa and Pines, 2012), map to the N-terminal domain, which is predicted to be unstructured, at least in the absence of interacting proteins (Fig. 3 A). The third motif is the C-terminal dipeptide IR (isoleucine-arginine), known as the IR motif (Vodermaier et al., 2003; Thornton et al., 2006). A detailed understanding of the mechanism of APC/C binding of such motifs is at present only available for the IR tails, which have been shown to interact with the TPR subunit Cdc27/Apc3 (Vodermaier et al., 2003; Burton et al., 2005; Kraft et al., 2005; Matyskiela and Morgan, 2009). A detailed mutational analysis of the budding yeast APC/C identified evolutionarily conserved residues involved in the interaction with Cdh1 (Matyskiela and Morgan, 2009). Each of three alanine mutations, at residues Asn548^{Cdc27/Apc3}, Leu579^{Cdc27/Apc3}, and Asn405A^{Cdc23/Apc8}, strongly reduced Cdh1 or Cdc20 binding

to the APC/C (Matyskiela and Morgan, 2009; Izawa and Pines, 2011). Although individual mutations resulted in a relatively mild impairment of APC/C function and were compatible with viability, combination of the Leu579^{Cdc27/Apc3} and Asn405A^{Cdc23/Apc8} mutations resulted in cell lethality (Matyskiela and Morgan, 2009).

By studying the interaction of point mutations in Cdc27/Apc3 with deletions of the IR tail of coactivators, Matyskiela and Morgan (2009) concluded that Cdc27/Apc3 is the main IR tail receptor on the APC/C. On the other hand, mutations in the putative binding site of Cdc23/Apc8 had synergist deleterious effects on APC/C activity when combined with deletions of the IR tail of coactivators, suggesting that this binding site engages with a different coactivator motif. The recently discovered KILR motif is a plausible (but yet undemonstrated) candidate (Izawa and Pines, 2012), not least because its sequence contains a hydrophobic arginine motif reminiscent of the IR tail. The importance of Asn405^{Cdc23/Apc8} is further emphasized by the observation that mutation of the equivalent residue of human Apc8, Asn338, counteracts Cyclin A and Cyclin B destruction (Izawa and Pines, 2011).

Coactivator mutants lacking the C box also display synergist deleterious effects on APC/C activity when combined with the Asn548^{Cdc27/Apc3} or Asn405^{Cdc23/Apc8} mutations, suggesting that the C box binds to a different site on the APC/C (Matyskiela and Morgan, 2009). When devoid of the Cdc27/Apc3 subunit, the APC/C retains part of its activity, and the latter is entirely dependent on the C box of coactivator (Thornton et al., 2006). EM and biochemical analyses of the APC/C^{Cdh1} complex strongly implicate the conserved C-terminal region of Apc2 as a C box receptor, possibly with further involvement of Cdc27/Apc3 (Kraft et al., 2005; Thornton et al., 2006; da Fonseca et al., 2011).

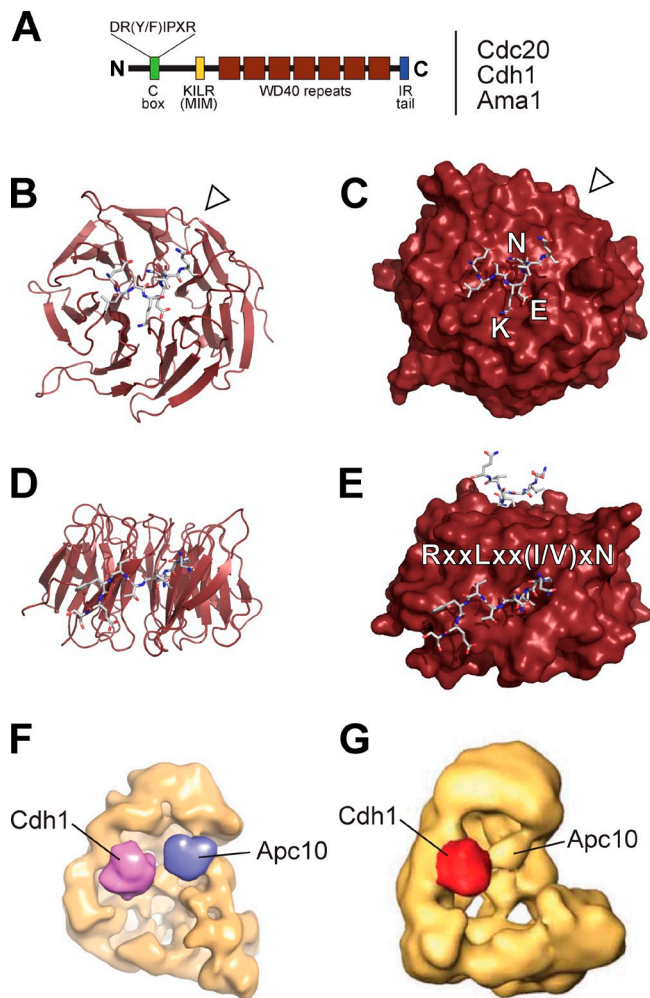


Figure 3. The coactivators. (A) Structural organization of prototypical coactivators. The N-terminal domain is predicted to be largely unstructured. It contains the C box motif, whose sequence is displayed, and the KILR motif. Both motifs are believed to contribute to APC/C binding and activation. The KILR motif partly overlaps with a larger Mad2-interacting motif (MIM), which consists of ~10 residues. The N-terminal region is followed by a seven-bladed β -propeller consisting of WD40 repeats. The short C-terminal extension terminates with the Ile-Arg (IR) motif, which also contributes to APC/C binding. N, N terminus; C, C terminus. (B) Cartoon model of the β -propeller of human Cdc20 bound to a KEN box motif shown in “sticks” mode (PDB ID: 4GGD; Tian et al., 2012). The triangle points to a region on the side of the toroid, distinct from the KEN binding site, where a putative D box binding site is positioned. (C) Molecular surface of the Cdc20-KEN motif from the same perspective as in B. The position of the three residues composing the KEN motif is indicated. (D) Cartoon model of Cdc20 from *S. pombe* bound to a BubR1 peptide mimicking a D box (Chao et al., 2012). To create this image, the atomic coordinates of the MCC complex (PDB ID: 4AEZ) were manipulated to remove Mad2 and Mad3/BubR1. The position of the KEN box of Mad3/BubR1 on the Cdc20 propeller is also shown. (E) Molecular surface of the Cdc20-D box mimic complex shown from the same perspective as in D. The consensus sequence of the D box is shown. (F) Negative stain EM reconstruction of APC/C^{Cdh1} from *S. cerevisiae* (da Fonseca et al., 2011) with densities assigned to Cdh1 and Apc10. (G) Cryo-negative stain EM reconstruction of human APC/C^{Cdh1} (Buschhorn et al., 2011). Densities assigned to Cdh1 and Apc10 are indicated.

Binding of substrates to APC/C^{coactivator} complexes

Many APC/C substrates interact directly with the coactivators via short sequence motifs (degrons). The most widespread degrons

in APC/C substrates are the D (destruction) box (Glotzer et al., 1991) and the KEN (lysine-glutamate-asparagine) box (Fig. 3; Pflieger et al., 2001), but other targeting motifs have been identified (Pines, 2011). Through biochemical and structural work, we know that the D and KEN boxes interact with two distinct pockets on the WD40 β -propellers of the coactivators (Fig. 3, B–E; Kraft et al., 2005; Passmore and Barford, 2005; Chao et al., 2012; Tian et al., 2012).

It is believed that Cdc20 and Cdh1 have intrinsic preferences for D or KEN box-containing substrates, respectively (Pflieger et al., 2001; Burton et al., 2005; Chao et al., 2012). However, certain reciprocal arrangements of KEN and D boxes, such as those observed in the APC/C pseudosubstrate inhibitors Acml and Mes1, are compatible with concomitant, cooperative binding to coactivators (Choi et al., 2008; Enquist-Newman et al., 2008; Kimata et al., 2008b; Ostapenko et al., 2008; Chao et al., 2012). Like additional APC/C substrates, Securin also contain KEN and D boxes, and both are required for its efficient degradation (Hagting et al., 2002; Zur and Brandeis, 2002; Leismann and Lehner, 2003), but only modest cooperativity between KEN and D boxes in promoting *in vitro* ubiquitination of Securin was previously described (Tian et al., 2012).

There is strong evidence that substrate ubiquitination depends on the assembly of a tripartite complex containing the APC/C, the coactivator, and the substrate (Passmore et al., 2003; Burton et al., 2005; Matyskiela and Morgan, 2009; Matyskiela et al., 2009). The APC/C subunit Apc10 plays a crucial function in this mechanism (Wendt et al., 2001; Carroll and Morgan, 2002; Passmore et al., 2003; Carroll et al., 2005; Matyskiela and Morgan, 2009; Buschhorn et al., 2011; da Fonseca et al., 2011). Cryo-EM studies on budding yeast and human APC/C^{Cdh1} indicate that the coactivator binds in the proximity of Apc10 and that Apc10 and Cdh1 reciprocally stabilize their position on the APC/C (Fig. 3, F and G; Buschhorn et al., 2011; da Fonseca et al., 2011). In the presence of a substrate, a density bridge between Cdh1 and Apc10 becomes apparent (Buschhorn et al., 2011; da Fonseca et al., 2011). Such a bridge was also observed with an 18-residue D box peptide modeled on the Cyclin B sequence (da Fonseca et al., 2011). Density fitting predicts that the D box peptide becomes squeezed at the interface of the Cdh1 β -propeller and Apc10 (Buschhorn et al., 2011; da Fonseca et al., 2011). The involvement of Apc10 in a tripartite interaction with the D box and Cdh1 might explain the contributions of Apc10 to processive substrate ubiquitylation by the APC/C (Carroll and Morgan, 2002; Passmore et al., 2003; Carroll et al., 2005). Apc10 sits near the catalytic core of the APC/C, interacting with Cdc27/Apc3 through a C-terminal IR motif similar to those found in coactivators and through additional contacts with Apc2 (Fig. 2 A; Wendt et al., 2001; Buschhorn et al., 2011; da Fonseca et al., 2011).

There is also considerable interest in alternative mechanisms leading to substrate ubiquitination by the APC/C in the absence of classical degrons. For instance, the Cks (cyclin-dependent kinase cofactor) protein has been implicated in the recruitment of Cyclin A and Cyclin B to the APC/C (Wolthuis et al., 2008; Di Fiore and Pines, 2010; van Zon et al., 2010). Another example is provided by Nek2A and Kif18A, two APC/C substrates that interact with the APC/C via C-terminal MR (Met-Arg) or

LR (Leu-Arg) sequences similar to the C-terminal IR motifs of Apc10, Cdc20, and Cdh1 (Hayes et al., 2006; Sedgwick et al., 2013). The β -propeller of Cdc20 is not required for ubiquitination of Nek2A, whereas the C box of Cdc20 is necessary and sufficient for this process (Kimata et al., 2008a).

Mechanisms of Ub chain formation by the APC/C

The mechanism of Ub transfer catalyzed by the APC/C is complex (Fig. 1). Relevant elements of such mechanism include the following: (a) The presentation of substrates to the catalytic machinery via docking interactions of degrons in substrates (D box and KEN box) with coactivators and intrinsic APC/C subunits. In particular, Apc10 increases the processivity of the ubiquitination reaction (Carroll and Morgan, 2002; Passmore et al., 2003; Carroll et al., 2005; Matyskiela and Morgan, 2009), probably by restraining the position of the substrate in the proximity of the binding site for the initiating E2 on Apc11; (b) The usage of distinct E2s for chain initiation and elongation, resulting in the attachment of homogeneous K48 or K11 chains at multiple sites on substrates; (c) The contribution of coactivators to the overall catalytic rate of Ub transfer mediated by interactions of their motifs, such as the C box, with the APC/C.

In *S. cerevisiae*, the APC/C generates K48 (lysine 48)-linked chains (Rodrigo-Brenni and Morgan, 2007). The initiation and extension of the poly-Ub chain in *S. cerevisiae* is performed by two different E2s (Matyskiela et al., 2009; Behrends and Harper, 2011; Wickliffe et al., 2011a). Ubc4 carries out the initial modification of the substrate, whereas Ubc1 mediates chain extension (Rodrigo-Brenni and Morgan, 2007). Creation of K48-linked chains requires residues located in two loops in the vicinity of the active site cysteine of Ubc1 (Rodrigo-Brenni et al., 2010). Plausibly, these residues interact with, and orient, the acceptor Ub (i.e., the one already attached on the substrate) so that its K48 is optimally positioned for nucleophilic attack of the thioester linkage of the E2-donor Ub complex. Remarkably, the residues involved in this mechanism are not conserved in another K48-specific E2, Cdc34 (Petroski and Deshaies, 2005). Thus, two E2 enzymes sharing the same conserved scaffold have evolved different mechanisms to perform the same task, the assemblage of K48-linked poly-Ub chains. A residue on Ub, tyrosine 59 (Y59^{Ub}), contributes to the formation of K48-linked Ub moieties by the APC/C and Ubc1 (Rodrigo-Brenni et al., 2010). Interestingly, mutation of Y59^{Ub} causes a dramatic reduction in the catalytic rate of Ub transfer, with only small effects on the K_M (Michaelis constant) for Ub binding by the E2-donor complex. This observation indicates that Ub contributes to its own transfer to a target protein through substrate-induced catalysis (Rodrigo-Brenni et al., 2010).

Similar concepts apply to the APC/C of higher eukaryotes, with the remarkable difference that in these organisms, the APC/C catalyzes the formation of poly-Ub chains linked through K11 (lysine 11) of Ub, rather than K48 (Jin et al., 2008; Williamson et al., 2009; Wu et al., 2010). In humans, the E2s Ube2C or Ube2D (also known as UbcH10 and UbcH5, respectively) can carry out initial ubiquitination of the substrate, whereas another E2, Ube2S, extends K11-linked chains (Baboshina and Haas, 1996; Jin et al.,

2008; Walker et al., 2008; Garnett et al., 2009; Williamson et al., 2009; Wu et al., 2010; Dimova et al., 2012; Tischler et al., 2012).

To generate K11-linked chains, Ube2S interacts with an extended surface on Ub containing lysine 6, threonine 12, and E34 (glutamate 34) among other residues. E34 was shown to facilitate the deprotonation of K11, in turn favoring formation of an isopeptide bond with the donor Ub (Wickliffe et al., 2011b). Thus, E34 of Ub is involved in substrate-assisted catalysis and contributes, through its proximity to K11, to the specificity of Ube2S for K11 chains. Furthermore, Ube2S restrains the position of the donor Ub on the E2 for optimal transfer to the acceptor, enhancing the processivity of the reaction (Wickliffe et al., 2011b).

It has been proposed that the surface of Ub containing E34 might be sequence related and functionally equivalent to the TEK (threonine-glutamate-lysine) box, a linear motif of Securin containing a TEK tripeptide (Jin et al., 2008). The existence of related sequences on substrates and on Ub was originally proposed to explain how the same E2 could, through structurally related interactions, carry out the initial ubiquitination of a substrate and subsequent chain extension (Jin et al., 2008). Although this was an attractive model, the discovery, that initiation and elongation of K11 chains are performed by distinct E2s, casts doubts on its significance. The E34-containing distributed interface of Ub that contributes to the specificity of K11 chain formation is structurally more complex than, and most likely sequence unrelated to, the linear TEK motif of Securin. Referring to this region of Ub as the TEK box (Jin et al., 2008) is therefore a potentially confusing misnomer.

In the meantime, a larger positively charged linear motif in Securin, also encompassing the TEK box (Jin et al., 2008), was identified for its ability to facilitate the initial attachment of Ub and named accordingly as initiation motif (Williamson et al., 2011). In a relatively poorly conserved form, initiation motifs are also found in additional APC/C substrates (e.g., Geminin, Cyclin B, and Plk1). They contain positively charged residues, but not necessarily lysines, and are usually located in the proximity of the D box of substrates. Exactly how they facilitate initiation is currently unclear. Although uncertainties remain as to whether both Ube2C and Ube2S bind to the RING domain of Apc11 (Summers et al., 2008; Williamson et al., 2009, 2011), this is plausible in light of evidence that the interaction with the RING domain primes the E2-Ub conjugate for catalysis (Dou et al., 2012; Plechanová et al., 2012; Pruneda et al., 2012).

Mitotic inhibition of the APC/C

Cdc20 is the target of the spindle assembly checkpoint (SAC), a feedback control mechanism operating in mitosis. The SAC has been thoroughly discussed in recent reviews (Lara-Gonzalez et al., 2012; Foley and Kapoor, 2013). In brief, the SAC is a molecular pathway, originating at kinetochores, which restrains mitotic exit to cells that have achieved biorientation of all their chromosomes, thus ensuring that chromosome segregation at anaphase progresses without chromosome loss or gain. To prevent mitotic exit, the SAC proteins bind to Cdc20 and lock it into a complex that binds tightly to the APC/C, inhibiting its ability to ubiquitinate Cyclin B and Securin (Fig. 1). Stabilization by the SAC of these two crucial APC/C substrates, whose

degradation is required for mitotic exit, prevents mitotic exit. The SAC brake to APC/C activity is released after chromosomes have attained bipolar attachment on the mitotic spindle. Here, we concentrate on how the SAC proteins target Cdc20 and how the complex of SAC proteins with Cdc20 targets the APC/C.

The complex of the SAC proteins Mad2, BubR1/Mad3, and Bub3 (the latter being a constitutive regulatory subunit of BubR1/Mad3 in most but not all species—e.g., *Schizosaccharomyces pombe*) with Cdc20 is known as the mitotic checkpoint complex (MCC) and was identified as the checkpoint effector (Hardwick et al., 2000; Fraschini et al., 2001; Sudakin et al., 2001). Within the MCC, Mad2 and BubR1/Mad3 bind directly to distinct regions of Cdc20. Mad2 binds to a short linear motif in the N-terminal region of Cdc20 that encompasses, but is not limited to, the KILR box (Hwang et al., 1998; Kim et al., 1998; Luo et al., 2002; Sironi et al., 2002). This ~10-residue motif has been named the Mad2 interaction motif (MIM; Fig. 4 A). BubR1/Mad3, on the other hand, contains two KEN boxes (KEN1 and KEN2) that are both essential for SAC function (Fig. 4 B; Burton and Solomon, 2007; King et al., 2007; Sczaniecka et al., 2008; Malureanu et al., 2009; Elowe et al., 2010; Lara-Gonzalez et al., 2011).

Crystal structure determination of a ternary complex containing Cdc20, Mad2, and a fragment containing the N-terminal region of BubR1/Mad3 but lacking KEN2 (Chao et al., 2012) delivered precious insights into the organization of the MCC. In the ternary complex, the MIM engages the safety belt of the so-called closed conformation of Mad2 (C-Mad2), the bound conformation of Mad2 (as opposed to open Mad2 [O-Mad2], the unliganded conformation [Mapelli and Musacchio, 2007]). KEN1, which is embedded in the folded environment of a helix-loop-helix extension that precedes the three TPRs of BubR1/Mad3, interacts with an exposed surface pocket of the Cdc20 β -propeller (Fig. 4, C and D; Chao et al., 2012; Tian et al., 2012). Additional extensive interactions between Mad2 and Mad3/BubR1 indicate that MCC is a cooperative assemblage (Fig. 4 F; Chao et al., 2012), a feature that might have important implications for MCC disassembly (discussed in the next paragraphs).

Overall, the structure of MCC clarifies why KEN1 is required for the incorporation of BubR1/Mad3 in a complex with Mad2 and Cdc20 and for its binding to the APC/C (Lara-Gonzalez et al., 2011). By showing that KEN1 occupies the KEN box binding pocket of Cdc20, the structure illustrates the proposal that BubR1/Mad3 is a pseudosubstrate inhibitor of the APC that directly competes with Cdc20 substrates (Burton and Solomon, 2007; King et al., 2007; Sczaniecka et al., 2008). Incidentally, this might not be an isolated example: additional APC/C inhibitors, including Acml, Mes1, and Emi1/Rca1 (Dong et al., 1997; Reimann et al., 2001; Miller et al., 2006), are also believed to inhibit Cdh1 or Cdc20 as pseudosubstrates (Choi et al., 2008; Enquist-Newman et al., 2008; Kimata et al., 2008b; Ostapenko et al., 2008; Chao et al., 2012).

Several crucial questions, however, remain unanswered. First and foremost, the role of KEN2 in the mechanism of APC/C inhibition by the MCC remains incompletely understood. KEN2 is not required for the association of the MCC with the APC/C but is required for mitotic arrest (Burton and Solomon, 2007;

King et al., 2007; Malureanu et al., 2009; Elowe et al., 2010; Lara-Gonzalez et al., 2011). In vitro, the MCC prevents the D box- and Cdc20-dependent binding of Cyclin B to the APC/C (Herzog et al., 2009; Kulukian et al., 2009; Lara-Gonzalez et al., 2011), and this is exquisitely dependent on KEN2 (Lara-Gonzalez et al., 2011). Thus, a thorough understanding of the function of KEN2 is necessary to unlock the secrets of MCC function. The available structural and biochemical evidence seems to exclude that KEN2 binds to a pocket of Cdc20 different from the one engaged by KEN1 (Chao et al., 2012; Tian et al., 2012). Thus, what is its target? A most economical, as much as speculative, hypothesis is that it binds to the KEN box binding site of a second Cdc20 molecule that might also be part of the MCC (this hypothesis is explored more thoroughly in the last section).

Second, the molecular details of the interaction of MCC with the APC/C remain unclear. An initial hint comes from the EM analysis of the APC/C^{MCC} (Herzog et al., 2009; Buschhorn et al., 2011) and from fitting of the MCC structure into the EM density (Chao et al., 2012). This revealed that Mad2 might contact Apc5 and Cdc23, and BubR1/Mad3 might contact Apc1. The position of Cdc20 in APC/C^{MCC} is different from that observed in the APC/C^{Cdc20} and APC/C^{Cdh1} complexes, displaced away from Cdc27/Apc3 toward Cdc23/Apc8 (Fig. 4 G). In this position, Cdc20 could be prevented from forming a tripartite D box coreceptor with Apc10 (Herzog et al., 2009; Chao et al., 2012), and indeed, Apc10 is not necessary for robust association of the MCC with the APC/C (Foster and Morgan, 2012). The displacement of Cdc20 in the APC/C^{MCC} structure is consistent with the observation that Cdc27/Apc3, which is required for robust Cdc20 binding at metaphase (i.e., after checkpoint silencing), might not be required for MCC binding to the APC/C in prometaphase (Izawa and Pines, 2011, 2012). Consistently, the IR tail and the C box of Cdc20, which are disordered in the structure of MCC (Chao et al., 2012), are not strictly required for MCC loading onto the APC/C (Izawa and Pines, 2012). Conversely, the KILR of the Cdc20 motif is necessary for the interaction of Cdc20 with the APC/C in mitosis, but this might reflect a requirement of this motif for the interaction with Mad2 (Izawa and Pines, 2012). Mad2 and Cdc20 cannot associate stably with the APC/C in the absence of BubR1/Mad3 (Foster and Morgan, 2012; Lau and Murray, 2012). Forcing the interaction of Mad2 with Cdc20 through an artificial dimerizer creates a potent anaphase inhibitor that sequesters Cdc20 from the APC/C and that operates in the absence of upstream checkpoint components (Izawa and Pines, 2012; Lau and Murray, 2012). Thus, Mad2 is an inhibitor of Cdc20 in its own right: it inhibits Cdc20 by engaging a motif (the KILR motif) that is crucially required for the productive interaction of Cdc20 with the APC/C (Izawa and Pines, 2012). The compounded effects of such inhibitory interaction with pseudosubstrate inhibition and APC/C targeting provided by the BubR1/Mad3–Cdc20 complex probably explain the extraordinary APC/C inhibitory power of the MCC.

Third, the inability of D box-containing substrates such as Cyclin B to interact with APC/C^{MCC} argues that the D box binding site is unavailable to D boxes in substrates when Cdc20 is part of the MCC. Whether the aforementioned displacement of Cdc20 in the APC/C^{MCC} structure is sufficient to account for

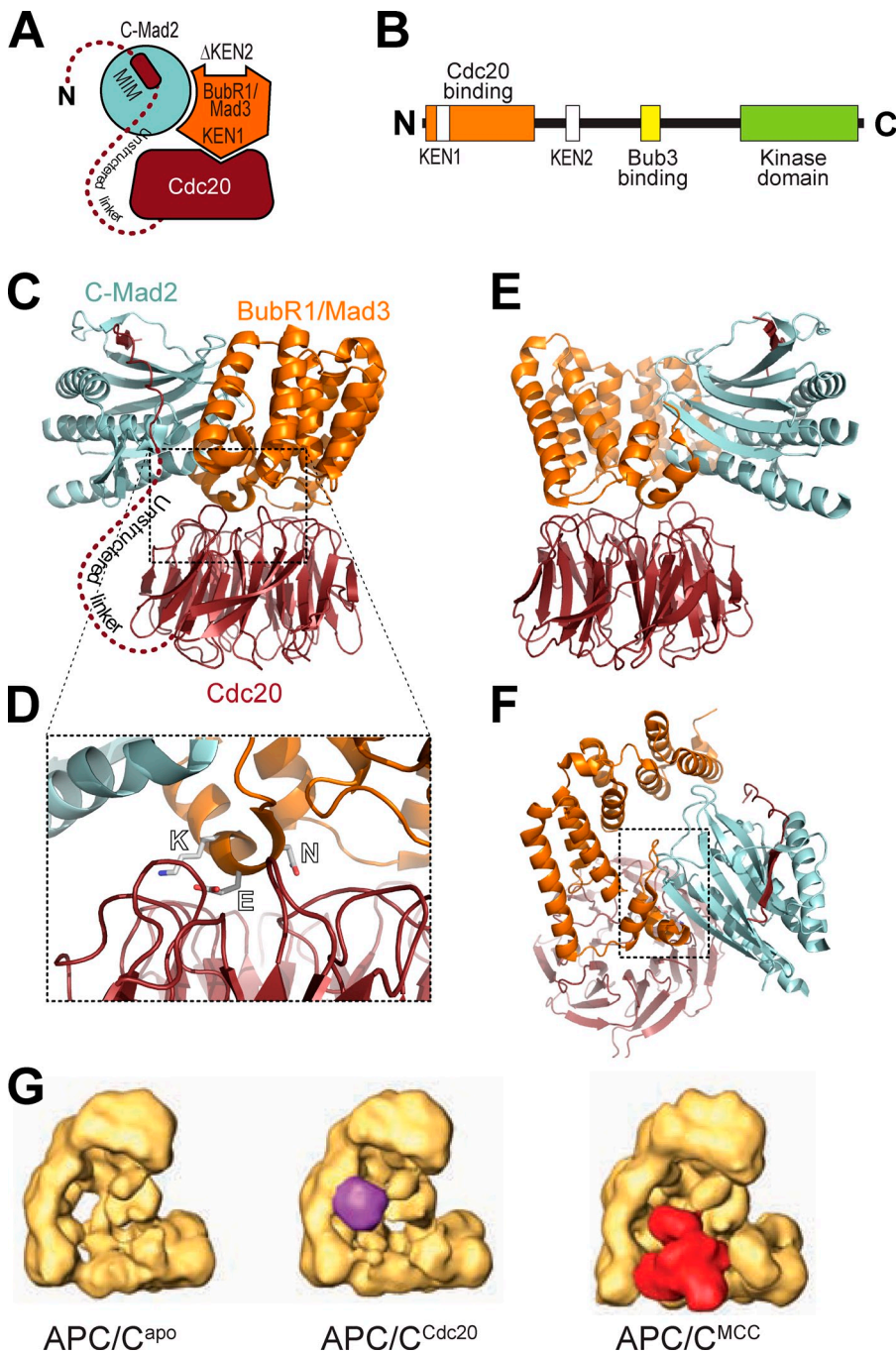


Figure 4. Organization of the MCC. (A) Schematic organization of the Mad2-BubR1/Mad3-Cdc20 complex (PDB ID: 4AEZ; Chao et al., 2012). The KEN1 motif of BubR1/Mad3 binds on the Cdc20 propeller. Mad2 binds to the MIM motif and makes extensive contacts with BubR1/Mad3. The Cdc20 linker between the MIM and the entry point in the propeller was disordered in the crystal structure (Chao et al., 2012). The KEN2 motif of BubR1/Mad3 was not included in the crystallized construct. (B) Domain organization of BubR1/Mad3. The Cdc20-binding domain in the N-terminal region consists of three TPR repeats. The TPR motif is preceded by a helix-loop-helix domain that embeds the KEN1 motif. The KEN2 motif is C-terminal to the TPR repeats. N, N terminus; C, C terminus. (C) Cartoon model of the MCC (PDB ID: 4AEZ). (D) An enlargement of the area boxed in C and showing the interaction of the KEN1 motif with Cdc20. The KEN motif lies within a folded region. (E) A view of the MCC rotated about a vertical axis $\sim 180^\circ$ with respect to C shows that Mad2 does not make direct contacts with the Cdc20 propeller. (F) After an additional 90° rotation about a horizontal axis, the extensive interactions between Mad2 and BubR1/Mad3 become evident. The Mad2 interface engaged in the interaction with BubR1/Mad3 overlaps significantly with the interface required to interact with O-Mad2 and p31^{comet} (Mapelli et al., 2007; Yang et al., 2007). The box encloses a region of the MCC complex in which extensive contacts between Mad2 and Mad3/BubR1 are formed. (G) A series of EM reconstructions showing APC/C devoid of coactivator or MCC subunits (APC/C^{apo}), APC/C^{Cdc20}, and APC/C^{MCC} (Herzog et al., 2009). The position and orientation of Cdc20 varies significantly in the presence of the MCC.

inhibition of D box-dependent interactions is uncertain. It seems unlikely in light of the observation that MCC created with a BubR1/Mad3 version lacking KEN2 binds to the APC/C but is unable to prevent D box-dependent binding of Cyclin B to the APC/C, contrarily to the MCC containing wild-type BubR1/Mad3 (Lara-Gonzalez et al., 2011). Thus, it is possible that additional interactions involving parts that were not included in the crystallized MCC might account for inhibition of the D box-binding pocket of Cdc20 in MCC.

The role of mitotic phosphorylation

Additionally, to direct inhibition by the SAC, phosphorylation is also important for the mitotic regulation of APC/C.

Cdk1-dependent phosphorylation of the APC/C, which is directed predominantly, but not exclusively, to the TPR subunits (Kramer et al., 2000; Rudner and Murray, 2000; Kraft et al., 2003; Steen et al., 2008; Hegemann et al., 2011), stimulates Cdc20 binding and activation of the APC/C, thus promoting selective binding of Cdc20 to mitotic (rather than interphase) APC/C. Cdk1-dependent phosphorylation of Cdh1, on the other hand, inhibits its association with the APC/C (Zachariae et al., 1998b; Jaspersen et al., 1999; Kramer et al., 2000). Several Cdk1 sites are disseminated in the N-terminal region of Cdh1 and include a SPKR (Ser-Pro-Lys-Arg) substrate that flanks the sequence KLLR, equivalent to the KILR motif of Cdc20 (Sironi et al., 2002). Whether the KLLR motif of Cdh1 contributes to APC/C binding and activation

is unknown, but plausible, based on sequence similarity with Cdc20. Were this the case, phosphorylation on the SPKR motif might be expected to contribute to mitotic inactivation of Cdh1.

The extensive mitotic phosphorylation of the N-terminal region of Cdc20 is not required for activation of the APC/C, and rather, it might be reducing the ability of the Cdc20 C box to stimulate the catalytic activity of the APC/C (Kramer et al., 2000; Yudkovsky et al., 2000; Labit et al., 2012). Cdc20 phosphorylation has been proposed to stimulate its inhibition by the SAC (Chung and Chen, 2003; D'Angiolella et al., 2003; Tang et al., 2004). However, as discussed in the next paragraph, a recent study implicated Cdc20 phosphorylation by Cdk1–Cyclin B in the disassembly of the checkpoint effector, the MCC (Miniowitz-Shemtov et al., 2012).

Turnover of Cdc20 and checkpoint silencing

Cdc20 is an unstable protein throughout the cell cycle (Prinz et al., 1998; Shirayama et al., 1998; Robbins and Cross, 2010), and the APC/C is probably entirely responsible for Cdc20 turnover. The mechanism and significance of APC/C targeting of Cdc20 vary between interphase and mitosis (Prinz et al., 1998; Foe et al., 2011; Foster and Morgan, 2012). The main physiological significance of the destabilization of Cdc20 in interphase is to prevent SAC override in the presence of high levels of Cdc20 in complex with substrate and APC/C (Foster and Morgan, 2012; Musacchio and Ciliberto, 2012). Excess Cdc20 at mitotic entry counteracts the establishment of a robust SAC arrest in prometaphase without major cell cycle defects (Pan and Chen, 2004; Foster and Morgan, 2012).

The significance of proteolysis of Cdc20 and/or of additional factors (Visconti et al., 2010) during mitosis is more controversial, but several lines of evidence indicate that it counteracts the extremely robust inhibition imposed by the MCC on the APC/C, thus facilitating SAC silencing (Musacchio and Ciliberto, 2012). Cdc20 turnover in mitosis depends on its association with the SAC proteins and requires the APC/C subunit Mnd2/Apc15 (Pan and Chen, 2004; King et al., 2007; Nilsson et al., 2008; Ge et al., 2009; Ma and Poon, 2011; Mansfeld et al., 2011; Varetta et al., 2011; Foster and Morgan, 2012; Uzunova et al., 2012). Inhibition of this pathway leads to an increase in the levels of MCC bound to the APC/C and to a delay in mitotic exit. The latter conditions are significantly worsened in cells that have undergone a robust checkpoint arrest (e.g., because treated with spindle poisons) and in which MCC accumulates (Garnett et al., 2009; Herzog et al., 2009; Zeng et al., 2010; Ma and Poon, 2011; Mansfeld et al., 2011; Varetta et al., 2011; Foster and Morgan, 2012; Uzunova et al., 2012). Similar observations are made upon ablation of the Mad2 binding partner p31^{comet} or upon inhibition of APC/C with a small-molecule inhibitor (Zeng et al., 2010; Varetta et al., 2011).

The mechanism through which Mnd2/Apc15 and p31^{comet} promote SAC- and APC/C-dependent polyubiquitination of Cdc20 during mitosis is currently unclear. Mnd2 occupies a position near the C-terminal region of Cdc23/Apc8, at the interface with Apc4, Apc5, and Apc1 (Hall et al., 2003; Schreiber et al., 2011; Uzunova et al., 2012; Zeng and King, 2012), and is therefore

in close proximity of the MCC (see Mitotic inhibition of the APC/C). Incidentally, there appears to be a single copy of Mnd2/Apc15 (Schreiber et al., 2011). As Cdc23/Apc8 is a dimer and is expected to bind two copies of Mnd2, it is possible that Apc4 or Apc5 contributes to the creation of an asymmetric Mnd2/Apc15 binding site. Interestingly, Mnd2 is not required for Cdc20 turnover in interphase, pointing to an important mechanistic difference between mitotic and interphase instability of Cdc20 (Foster and Morgan, 2012). Furthermore, Apc15 is dispensable for APC/C^{Cdc20} and APC/C^{Cdh1} activity directed against common APC/C targets (Mansfeld et al., 2011; Foster and Morgan, 2012; Uzunova et al., 2012). p31^{comet}, which is only identifiable in higher eukaryotes, binds selectively to the C-Mad2 conformer of Mad2 contained in the MCC (Xia et al., 2004; Mapelli et al., 2006; Vink et al., 2006; Yang et al., 2007). In doing so, it might expose a degron of Cdc20, facilitating its Mnd2/Apc15-dependent ubiquitination (as discussed in the context of Fig. 5).

Thus, Cdc20 proteolysis sets the correct timing of mitotic exit, which in turn is believed to protect cells from “cohesion fatigue” (Daum et al., 2009; Stevens et al., 2011; Lara-Gonzalez and Taylor, 2012), the uncoordinated loss of sister chromatid cohesion arising from a prolonged arrest in metaphase. Cdc20 proteolysis, however, is not strictly speaking necessary for mitotic exit, and other means of disassembly of the MCC or its subcomplexes clearly exist. For instance, reflecting the importance of the Mps1 and Aurora B checkpoint kinases for MCC assembly, inhibition of these kinases drives rapid MCC disassembly and mitotic exit even when proteolysis is suppressed (Herzog et al., 2009; Jia et al., 2011; Mansfeld et al., 2011; Varetta et al., 2011). Among additional mechanisms that might play a role in MCC disassembly, we count nonproteolytic ubiquitination (Miniowitz-Shemtov et al., 2010; Hörmanseder et al., 2011; Lara-Gonzalez et al., 2011; Mansfeld et al., 2011), an unknown reaction that requires hydrolysis of the bond between the β - and γ -phosphate of ATP (Teichner et al., 2011), and Cdc20 phosphorylation (Miniowitz-Shemtov et al., 2012). Besides controlling the stability of Cdc20 through proteolysis, p31^{comet} has also been implicated in the nonproteolytic disassembly of the MCC and, in particular, of a soluble pool of MCC not bound to the APC/C (Reddy et al., 2007; Hagan et al., 2011; Jia et al., 2011; Teichner et al., 2011; Westhorpe et al., 2011).

What is the real composition of the SAC effector?

The apparent complexity of the mechanism of MCC disassembly is contributing to an ongoing controversy on the actual composition of the APC/C inhibitor generated by the SAC (Kops and Shah, 2012; Musacchio and Ciliberto, 2012). Inhibition of proteasome activity (e.g., with the proteasome inhibitor MG132) or specific inhibition of Cdc20 proteolysis (e.g., depletion of p31^{comet} or of Mnd2/Apc15) during mitosis results in the accumulation of MCC on the APC/C (Garnett et al., 2009; Williamson et al., 2009; Miniowitz-Shemtov et al., 2010; Visconti et al., 2010; Zeng et al., 2010; Jia et al., 2011; Ma and Poon, 2011; Teichner et al., 2011; Varetta et al., 2011). In the absence of such perturbations, the predominant inhibitory species identified on the APC/C of checkpoint-arrested cells is a complex of BubR1/Mad3

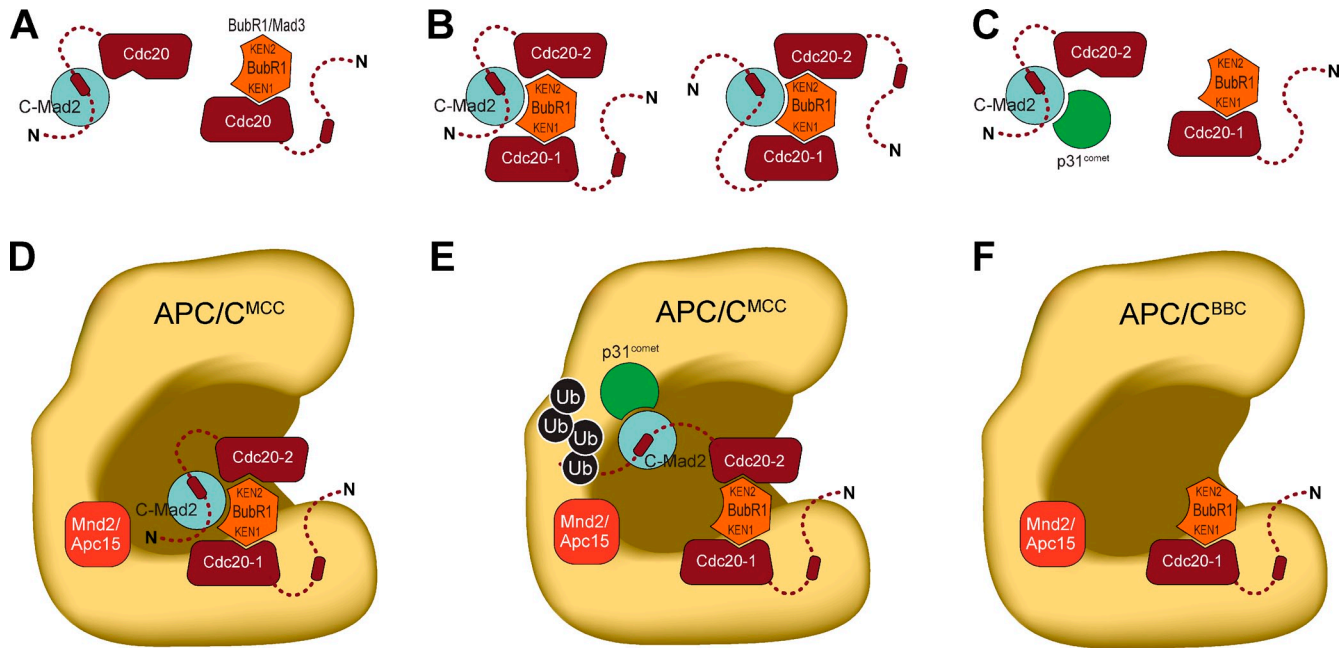


Figure 5. Hypothetical organization of the MCC with two Cdc20 protomers. (A) The MCC might be assembled from two subcomplexes, Mad2–Cdc20 and BubR1/Mad3–Bub3–Cdc20 (Bub3 is not shown to improve clarity). Each of these potential inhibitors of the APC/C might be generated with different rates and mechanisms at the kinetochore, only afterward becoming assembled into one complex. (B) Two hypothetical complexes resulting from the encounter of the Mad2–Cdc20 and BubR1/Mad3–Cdc20 subcomplexes. (left) Cdc20 bound to Mad2 (Cdc20-2) binds BubR1 through the KEN2 motif. The direct interaction of Cdc20 with KEN2 is probably low affinity, but complex formation is driven by cooperativity: BubR1 is in contact with both Mad2 and Cdc20, which in turn interact via the MIM. Cdc20-1 bound at the KEN1 motif is in principle available for APC/C binding via its N-terminal (N) tail. (right) The core of this alternative configuration is similar to that of the crystallized MCC. In this alternative configuration, Cdc20-2 binds to KEN2. In the absence of additional hypothetical contacts, this configuration is less likely because KEN2 is not required for the interaction of BubR1/Mad3 with Cdc20, suggesting that its direct contribution to the overall binding affinity of the interaction is modest. (C) In solution, p31^{comet} may promote the release of a Cdc20–Mad2 complex from BubR1/Mad3–Cdc20 by direct competition with BubR1/Mad3 for Mad2 binding. In turn, BubR1/Mad3–Cdc20 might dissociate via additional mechanisms (e.g., phosphorylation, dephosphorylation, and ATP-dependent processes). (D) One of the two MCC configurations shown in B bound to the APC/C. (E) p31^{comet} might detach Mad2 from its interaction with BubR1/Mad3, initially without disrupting MCC, which is stabilized by additional interactions with the APC/C. Cdc20-2, which is bound to Mad2-p31^{comet}, is directed to the catalytic machinery of the APC/C for ubiquitination. This reaction requires Mnd2/Apc15 and ultimately results in the destruction of Cdc20-2. (F) A BubR1/Mad3–Cdc20-1 complex is left behind. This complex can be removed from the APC/C through similar mechanisms to those discussed in C.

and Bub3 with Cdc20, accordingly referred to as BBC (Nilsson et al., 2008; Kulukian et al., 2009; Lara-Gonzalez et al., 2011; Westhorpe et al., 2011).

What is the relationship between the MCC and the BBC? It has been proposed that Mad2 might play a merely catalytic role in the assembly of the BBC and that the latter is the crucial SAC effector (Nilsson et al., 2008; Kulukian et al., 2009). As already discussed, however, many observations indicate that Mad2 is a bona fide inhibitor of the APC/C. Furthermore, there is strong evidence that Mad2 and BubR1 have synergistic inhibitory effects on APC/C activity in vitro (Tang et al., 2001; Fang, 2002; Kulukian et al., 2009; Lara-Gonzalez et al., 2011; Foster and Morgan, 2012) and that Mad2 positively contributes to the accumulation of a BubR1/Mad3–Bub3–Cdc20 complex (Hwang et al., 1998). Thus, Mad2 is probably a stoichiometric component of the MCC, which, at least when the MCC is bound to the APC/C, becomes released concomitantly with Apc15/Mnd2-dependent proteolysis of Cdc20 to generate BBC. The latter might be significantly stabilized by interaction with the APC/C, remaining bound to it, and becoming destabilized when the signal from kinetochores fades out, possibly by reversal of the effects of phosphorylation. Indeed, the latter step might in principle be also sufficient to destabilize the entire MCC even when

proteolysis is inhibited, as specified in the previous section. Such nonproteolytic pathways might be in principle responsible also for the disassembly of MCCs or BBCs that are not bound to the APC/C.

Hypothesis: Two Cdc20 protomers in the MCC?

A serious conceptual weakness of the model that the BBC is generated from the MCC through Cdc20 proteolysis is that it does not clarify what is the source of Cdc20 in the BBC that remains associated with the APC/C. A purely speculative solution to this problem is that MCC contains two Cdc20 protomers, rather than only one (Fig. 5 A). For instance, the first Cdc20 protomer may bind BubR1/Mad3 via KEN1, and the second one may bind Mad2 via the KILR motif and BubR1/Mad3–Bub3–Cdc20 via KEN2. In this case, a cooperative assemblage mechanism is expected (Fig. 5 B). How this assembly would inhibit Cdc20-dependent binding of D box-containing substrates to the APC/C (Lara-Gonzalez et al., 2011) is impossible to visualize at present. An attractive corollary of the “two Cdc20 in the MCC” hypothesis is that it depicts MCC as the assembly product of two distinct Cdc20-based inhibitors of the APC/C, Mad2–Cdc20 and BubR1–Bub3–Cdc20, each coming with its

mechanism of inactivation (Fig. 5, D–F). In this “two-pillar” model for the pathway of MCC assembly, the site and rate of production of the two Cdc20 complexes at kinetochores might be differentially regulated during spindle checkpoint activation, a hypothesis, which builds on important previous work (Skoufias et al., 2001; Essex et al., 2009).

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