Molecular Mechanisms of Spindle Assembly Checkpoint Activation and Silencing

Kevin D. Corbett

Abstract In eukaryotic cell division, the Spindle Assembly Checkpoint (SAC) plays a key regulatory role by monitoring the status of chromosome-microtubule attachments and allowing chromosome segregation only after all chromosomes are properly attached to spindle microtubules. While the identities of SAC components have been known, in some cases, for over two decades, the molecular mechanisms of the SAC have remained mostly mysterious until very recently. In the past few years, advances in biochemical reconstitution, structural biology, and bioinformatics have fueled an explosion in the molecular understanding of the SAC. This chapter seeks to synthesize these recent advances and place them in a biological context, in order to explain the mechanisms of SAC activation and silencing at a molecular level.

1 Introduction

A critical decision point in the life of a eukaryotic cell is the mitotic metaphase-to-anaphase transition, when replicated chromosomes are segregated to opposite spindle poles prior to cell division. Before committing to anaphase, the cell must ensure that all chromosomes are attached to spindle microtubules, and that sister chromosomes (or homologs, in meiosis I) are bi-oriented; that is, attached to microtubules extending from opposite spindle poles. Failure to properly sense and respond to errors in microtubule attachment can lead to aneuploidy, a hallmark of cancer and (when it occurs in meiosis) a major cause of miscarriage and developmental disorders like Down Syndrome.

K.D. Corbett (✉)
Ludwig Institute for Cancer Research, San Diego Branch, La Jolla, CA, USA
e-mail: kcorbett@ucsd.edu

K.D. Corbett
Departments of Cellular & Molecular Medicine and Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093 USA

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The metaphase-to-anaphase transition is controlled by the activity of a ubiquitin E3 ligase, the Anaphase-Promoting Complex/Cyclosome (APC/C) (Sudakin et al. 1995; King et al. 1995), which ubiquitinates and promotes the degradation of a number of substrates, most notably B-type cyclins and securin (Murray et al. 1989; Glotzer et al. 1991; Cohen-Fix et al. 1996; Morgan 1997; Shirayama et al. 1999). Securin is an inhibitor of a protease, separase, that when activated cleaves the Scc1 subunit of the cohesin complexes holding bi-oriented sister chromosomes together; this cleavage is the critical step initiating chromosome segregation in anaphase (Ciosk et al. 1998; Kamenz and Hauf 2016).

Prior to anaphase onset, the activity of the APC/C is inhibited by the spindle assembly checkpoint (SAC), which monitors the state of chromosome-microtubule attachment in the cell (reviewed in Musacchio and Salmon 2007; Lara-Gonzalez et al. 2012; Musacchio 2015; Zhang et al. 2016b; Etemad and Kops 2016). Microtubule attachment is mediated by kinetochores, complex protein assemblies with both DNA-binding and microtubule-binding subunits (reviewed in Pesenti et al. 2016; Nagpal and Fukagawa 2016). When kinetochores are not properly attached to microtubules, they mediate assembly of a soluble "wait anaphase" signal in the form of the four-protein Mitotic Checkpoint Complex (MCC), which directly binds and inhibits the APC/C. In this manner, a single unattached kinetochore is in most cases able to delay anaphase onset (Rieder et al. 1995).

Seminal work published in 1991 initiated study of the SAC by isolating the first mutants defective in this pathway, termed *mad* (mitotic arrest deficient) (Li and Murray 1991) and *bub* (budding uninhibited by benzimidazole) (Hoyt et al. 1991). Only now, however, are the detailed molecular mechanisms of SAC activation and silencing coming into sharp focus, thanks to a recent surge in structural and biochemical studies of the APC/C, its interactions with the MCC, and the mechanisms of MCC assembly and disassembly. This review covers several aspects of SAC function that have recently seen significant advances, beginning with the structure and function of the APC/C itself, and the mechanism of its inhibition by the MCC. I then move to the sites of MCC assembly—kinetochores—and outline the mechanisms of chromosome-microtubule attachment sensing and MCC assembly at unattached kinetochores. Finally, I address how the SAC is silenced after kinetochore-microtubule attachment, paying particular attention to a newly discovered pathway for direct MCC disassembly. Throughout, I attempt to place recent structural and biochemical work into the larger framework of SAC function that has been refined, through the work of many, over the 25 years since the discovery of this pathway.

2 The APC/C: Target of the Spindle Assembly Checkpoint

As the master regulator of anaphase onset, the mechanisms of the APC/C, particularly how it is regulated through the cell cycle and how it recognizes substrates, are of considerable interest. Because of its immense size and complexity, these

questions were unanswerable until recent advances in cryo-electron microscopy (cryo-EM) began to provide high-resolution pictures of the APC/C in a variety of functional states (Chang et al. 2014, 2015; Brown et al. 2015, 2016; Zhang et al. 2016c; Yamaguchi et al. 2016; Alfieri et al. 2016). This structural work, coupled with in vitro and in vivo functional analysis, has significantly improved our understanding of APC/C substrate recognition, the role of "coactivator" proteins such as Cdc20 in that recognition, and how the APC/C is inhibited by the MCC. In particular, the previously enigmatic roles of Cdc20, a key APC/C coactivator that also acts as an inhibitor when incorporated into the MCC, have been significantly clarified.

2.1 Overall APC/C Architecture

The APC/C is a 19-subunit complex (20 when counting a bound coactivator; see below) with a total molecular weight of ~1.2 MDa (Fig. 1a) (Sudakin et al. 1995; King et al. 1995; Chang et al. 2014). It contains two E3 ubiquitin ligase subunits: Apc2 is related to the Cullin subunits of SCF ubiquitin ligases, while Apc11 contains a RING-type E3 ligase domain. These subunits bind several different E2 activating enzymes to mediate substrate ubiquitination, with different E2s responsible for ubiquitin chain initiation and elongation. The bulk of the APC/C forms two large structures, the so-called TPR lobe (or "arc lamp") named for the tetratricopeptide (TPR) repeats found in this lobe's subunits, and the platform. Together, the TPR lobe and platform define a large central cavity and serve to juxtapose functional modules responsible for substrate recognition with those responsible for ubiquitination (Fig. 1a). For a detailed discussion of APC/C architecture, the reader is referred to recent reviews on the subject (Primorac and Musacchio 2013; Chang and Barford 2014; Barford 2015).

2.2 APC/C Substrate Recognition Is Mediated by Coactivator Proteins

In order to recognize its substrates, the APC/C requires one of a family of "coactivator" proteins, which bind the APC/C in a cell cycle-regulated manner and dictate substrate specificity by binding directly to conserved "degron" motifs in those substrates. All APC/C coactivators are structurally related, with a central WD40 β-propeller domain responsible for degron recognition, and conserved motifs at the N- and C-terminus that mediate docking between the APC/C's TPR lobe and platform (Figs. 1a and 2b) (Zhang and Lees 2001; Schwab et al. 2001; Vodermaier et al. 2003; Thornton et al. 2006; Matyskiela and Morgan 2009; Izawa and Pines 2012; Chang et al. 2015; Zhang et al. 2016c). While detailed discussion is

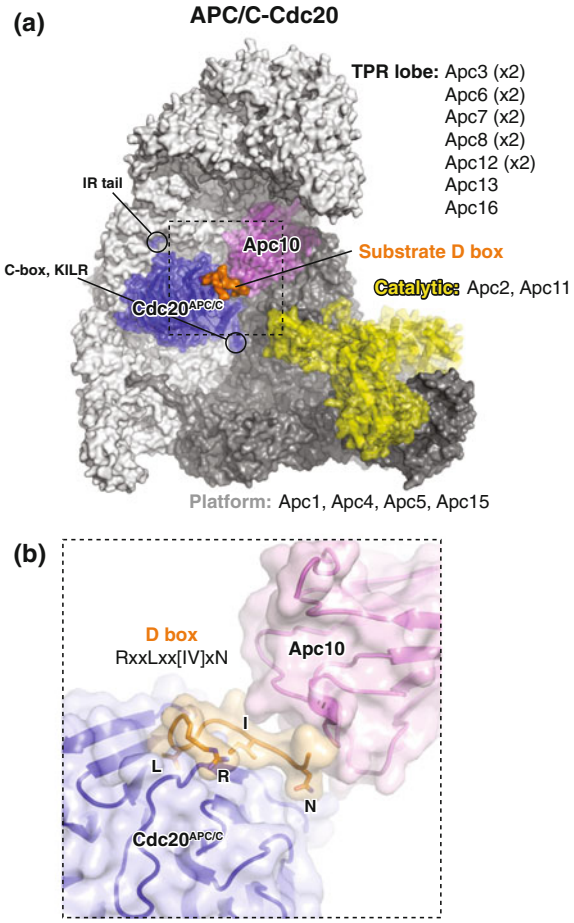


Fig. 1 Architecture of the APC/C and interactions with Cdc20, substrate degron motifs, and the MCC. **a** Structure of the human APC/C-bound to coactivator Cdc20 and high-affinity substrate Hsl1 (Zhang et al. 2016c) (PDB 5G04). The TPR lobe and platform of the APC/C are colored *light* and *dark gray*, respectively, with the catalytic module *yellow* and the substrate-recognition module *pink* (Apc10) and *blue* (Cdc20). Cdc20 binding to the APC/C is mediated by its N-terminal C box and KILR motifs, and the C-terminal IR tail (see Fig. 2b for Cdc20 domain structure). The D-box of Hsl1, *orange*, is sandwiched between Cdc20 and Apc10. **b** Close-up of Hsl1 D-box recognition by Cdc20 (*blue*) and Apc10 (*pink*). View is equivalent to (**a**)

outside the scope of this review, binding of the different coactivator proteins to the APC/C is regulated through phosphorylation of both the coactivators themselves (Zachariae et al. 1998; Jaspersen et al. 1999; Lukas et al. 1999; Kramer et al. 2000; Labit et al. 2012; Chang et al. 2015) and subunits of the APC/C (Lahav-Baratz et al. 1995; Shteinberg et al. 1999; Kramer et al. 2000; Golan et al. 2002; Kraft et al. 2003; Zhang et al. 2016c; Qiao et al. 2016). The end result of this regulation is that the bound coactivator, and therefore the APC/C's substrate specificity, depends on

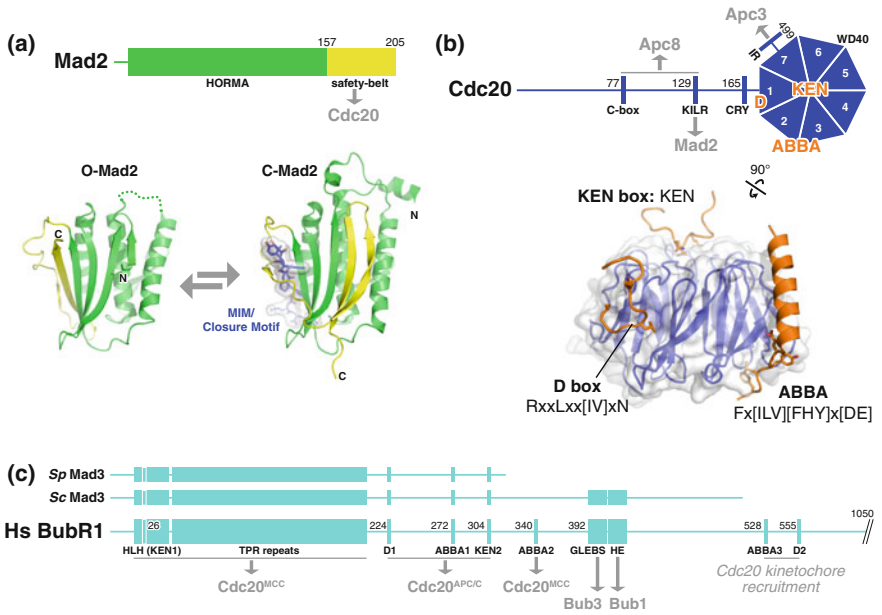
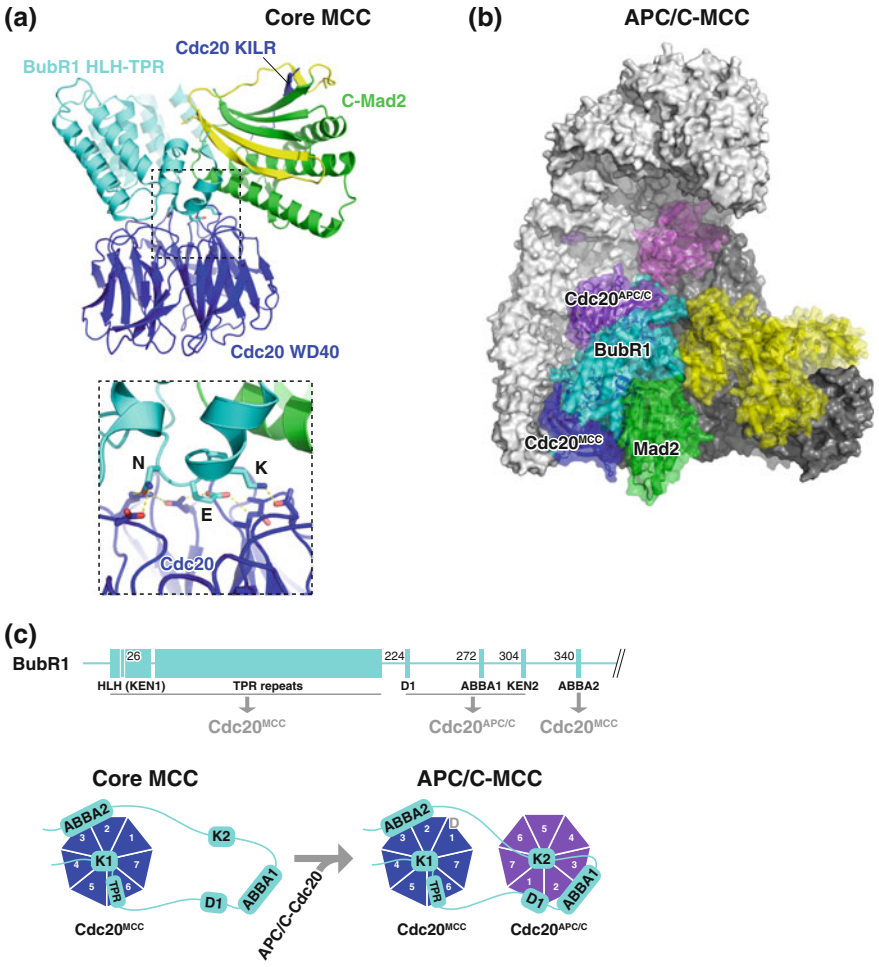


Fig. 2 Domain structure of core MCC subunits. **a** *Top* Domain structure of Mad2, with the HORMA domain core in green and the C-terminal safety belt *yellow*. *Bottom* Structures of Mad2 in the open (O-Mad2) and closed (C-Mad2) conformations. In O-Mad2, the safety belt occupies the binding site for Mad2-interacting motifs (MIMs). In C-Mad2, the safety belt wraps entirely around a bound MIM/closure motif (*blue sticks*), locking it in place. **b** *Top* Domain structure of Cdc20. The KILR motif (residues 129-132 in human Cdc20) can bind Mad2 as a MIM (in $Cdc20^{MCC}$) or contribute to APC/C binding (in $Cdc20^{APC/C}$). The CRY box (residues 165–167) of $Cdc20^{MCC}$ is recognized by $Cdc20^{APC/C}$ in the APC/C-MCC complex (Alfieri et al. 2016) and contributes to $Cdc20^{MCC}$ ubiquitination and degradation (Reis et al. 2006). *Bottom* Structure of *S. cerevisiae* Cdh1 (*blue*) bound to an inhibitor, Acm1 (*orange*), that contains all known degrons: D box, KEN box, and ABBA motif (He et al. 2013) (PDB 4BH6). Consensus residues in each degron motif are shown as sticks. **c** Domain structure and interactions of human BubR1 and its orthologs (called Mad3) in *S. pombe* and *S. cerevisiae*

cell-cycle stage: Cdh1 is bound during interphase, and Cdc20 is bound in mitosis. Here, I focus entirely on the APC/C-Cdc20 complex, which controls anaphase onset and constitutes the target of the SAC.

When bound to the APC/C as a coactivator, Cdc20 recognizes several different degron motifs via distinct surfaces on its central WD40 β-propeller domain (Figs. 1b, 2b and 3a) (reviewed in Davey and Morgan 2016). Recognition of one such motif, the destruction box (D-box) (Glotzer et al. 1991), is bipartite: this motif becomes sandwiched between Cdc20 and an adjacent APC/C subunit, Apc10 (Fig. 1b) (Buschhorn et al. 2011; da Fonseca et al. 2011; Chang et al. 2014). Recognition of the two other known degron motifs—the KEN box (named for its sequence: lysine-glutamate-asparagine) (Pfleger and Kirschner 2000) and ABBA motif (also termed A-motif, Phe-box, or IC20BD) (Burton et al. 2011; He et al.



2013; Lischetti et al. 2014; Diaz-Martinez et al. 2015; Di Fiore et al. 2015)—is mediated solely by Cdc20 (Fig. 3a). Together, binding of one or more degrons by the APC/C-Cdc20 complex positions a substrate for ubiquitination by the catalytic module (Chang et al. 2014; Brown et al. 2015).

3 The Mitotic Checkpoint Complex Inhibits APC/C-Cdc20

The key element of SAC signaling is the four-protein MCC, which is generated at unattached kinetochores and directly binds and inhibits the APC/C-Cdc20 complex. The conserved "core" MCC comprises Mad2, Cdc20, and BubR1 (Mad3 in fungi),

◂**Fig. 3** MCC architecture and APC/C inhibition. **a** Structure of the *S. pombe* core MCC, containing the BubR1 (Mad3) N-terminal region (HLH and TPR; *cyan*), Cdc20 (*blue*), and Mad2 (*green*) (Chao et al. 2012) (PDB ID 4AEZ). *Inset* Close-up view of the BubR1 KEN1 motif interacting with Cdc20. **b** Structure of the human APC/C-Cdc20 bound to the MCC (Alfieri et al. 2016). Core MCC subunits are colored as in (**a**), and the APC/C is colored as in Fig. 1a except for $Cdc20^{APC/C}$ (*purple*). The C-terminal region of BubR1, as well as Bub3, are conformationally flexible and were not included in the model. MCC binding rotates $Cdc20^{APC/C}$ away from Apc10 and occupies the D-box binding site. BubR1 also occupies the space where E2 enzymes bind the catalytic module. **c** Interactions of BubR1 with $Cdc20^{MCC}$ and $Cdc20^{APC/C}$. In the core MCC, BubR1 interacts with $Cdc20^{MCC}$ mainly through its KEN1/TPR motif (Chao et al. 2012). In the APC-C-MCC structure, electron density in the ABBA-motif binding site of $Cdc20^{MCC}$ (Alfieri et al. 2016) was originally assigned to ABBA3, but recent data suggest that in fact ABBA2 occupies this site (Di Fiore et al. 2016). Core MCC assembly leaves the BubR1 D1, ABBA1, and KEN2 motifs available. Upon MCC binding to APC/C-Cdc20, these motifs bind $Cdc20^{APC/C}$ to mediate APC/C inhibition. While the structure of APC/C-MCC showed density in the $Cdc20^{MCC}$ D-box binding site (*gray* in schematic), this density cannot be confidently assigned to a specific region of BubR1 (Figure adapted from Chao et al. 2012 and Di Fiore et al. 2016)

with BubR1 forming a constitutive dimer with Bub3 in a subset of organisms including humans. Cdc20's role as an APC/C coactivator is outlined above; for many years, how Cdc20 also functions as an APC/C inhibitor was unknown. Recent structural work on both the isolated MCC and its complex with the APC/C have clarified this question, resulting in a simple, yet elegant, model for APC/C inhibition by the MCC and for the dual roles of Cdc20.

3.1 Mitotic Checkpoint Complex Architecture

Mad2 was the first protein demonstrated to bind and inhibit the APC/C (Li et al. 1997). Mad2 contains a HORMA domain (Aravind and Koonin 1998) that can adopt two different conformations: an inactive "open" conformation (O-Mad2), and a "closed" conformation (C-Mad2) that binds short peptide motifs called Mad2-interacting motifs (MIMs) or, more generally, closure motifs (Fig. 2a) (reviewed in Mapelli and Musacchio 2007; Luo and Yu 2008). These two conformations differ in the structure of the C-terminal region of the protein, termed the "safety belt": in C-Mad2, this segment wraps entirely around a bound closure motif to form a topologically linked complex (Luo et al. 2002). In O-Mad2, the safety belt is docked against the closure motif binding site (Luo et al. 2000), and the protein is therefore unable to bind a closure motif. The bulk of soluble Mad2 in the cell is in the O-Mad2 state (Luo et al. 2004); the rate-limiting step of MCC assembly is the recruitment of O-Mad2 to unattached kinetochores, where it is converted to C-Mad2 and associates with a closure motif in Cdc20, termed the KILR motif (Fig. 2b) (Hwang et al. 1998; Kim et al. 1998; Fang et al. 1998; Kallio et al. 1998; Luo et al. 2002).

BubR1 is the third member of the so-called "core" MCC (Hardwick et al. 2000; Tang et al. 2001; Sudakin et al. 2001; Fang 2002), and directly interacts with both

Mad2 and Cdc20, significantly stabilizing the overall complex (Figs. 2c and 3a) (Sczaniecka et al. 2008; Tipton et al. 2011; Chao et al. 2012; Faesen et al. 2017). BubR1, which arose along with its paralog Bub1 from a gene duplication event (Suijkerbuijk et al. 2012a; Vleugel et al. 2012; Di Fiore et al. 2016), has a complex domain structure featuring at least seven degron-like motifs: the N-terminal TPR-repeat domain contains a KEN box, and this domain is followed by a second KEN box, two D-boxes, and three ABBA motifs (Fig. 2c). In the core MCC, BubR1 binds Cdc20 through its N-terminal KEN box (KEN1) and the adjacent TPR-repeat domain (Sczaniecka et al. 2008; Chao et al. 2012), and also through one of its ABBA motifs (most likely ABBA2; Di Fiore et al. 2016). The TPR domain also binds MAD2, completing the cooperative assembly of the highly stable core MCC (Fig. 3a).

3.2 APC/C-Cdc20 Binding and Inhibition by MCC

The fully assembled MCC contains a copy of BubR1 with a series of degron motifs —D1, ABBA1, and KEN2—unoccupied (Fig. 3c). The presence of these degrons, and their importance for APC/C-Cdc20 inhibition by the MCC, led to a proposal that BubR1 could bind two copies of Cdc20, one as part of the MCC (termed $Cdc20^{MCC}$) and a second bound to the APC/C as a coactivator (termed $Cdc20^{APC/C}$) (Primorac and Musacchio 2013). An important biochemical and cryo-EM analysis of APC/C-Cdc20 and APC/C-MCC complexes purified from HeLa cells provided early evidence that this might be the case, as the stoichiometry of Cdc20 was doubled in APC/C-MCC versus APC/C-Cdc20 (Herzog et al. 2009). The relatively low resolution (by today's standards) of that study's EM analysis, however, prevented a clear visualization of the two copies of Cdc20 in APC/C-MCC. More recently, it was shown biochemically that the fully assembled MCC could bind a second copy of Cdc20 that was already bound to the APC/C, and that this binding was disrupted by mutating BubR1's D1 degron motif (Izawa and Pines 2014). More recent high-resolution structures of the APC/C-MCC complex have clearly shown the positions of two copies of Cdc20 in this complex, confirming the above findings (Fig. 3b) (Yamaguchi et al. 2016; Alfieri et al. 2016). These structures, plus detailed biochemical and genetic analysis with BubR1 mutants, also finally reveal the roles of BubR1's many degron-like motifs: in the APC/C-MCC complex, BubR1 winds between $Cdc20^{MCC}$ and $Cdc20^{APC/C}$, occupying all degron-binding sites of both copies (Fig. 3c) (Alfieri et al. 2016; Di Fiore et al. 2016). BubR1 binds $Cdc20^{MCC}$ through its KEN1 motif and TPR domain as described above, and also through its ABBA2 motif (Figs. 2c and 3c) (Diaz-Martinez et al. 2015; Alfieri et al. 2016; Di Fiore et al. 2016). Between these motifs, BubR1 wraps around the WD40 domain of $Cdc20^{APC/C}$, binding through its D1, ABBA1, and KEN2 motifs (Fig. 3c) and also causing a significant rotation of $Cdc20^{APC/C}$ that disrupts the bipartite D-box recognition site (Alfieri et al. 2016). APC/C-bound MCC also sterically occludes the binding of E2 enzymes to the APC/C catalytic module, further inhibiting

activity (Yamaguchi et al. 2016; Alfieri et al. 2016). Thus, MCC targets already-assembled APC/C-Cdc20 for inhibition, binding through a series of degron motifs in BubR1. Because $Cdc20^{APC/C}$ remains bound to the APC/C in this complex, reactivation of the APC/C upon SAC silencing requires only removal or disassembly of the bound MCC (see Sect. 5 and Fig. 4).

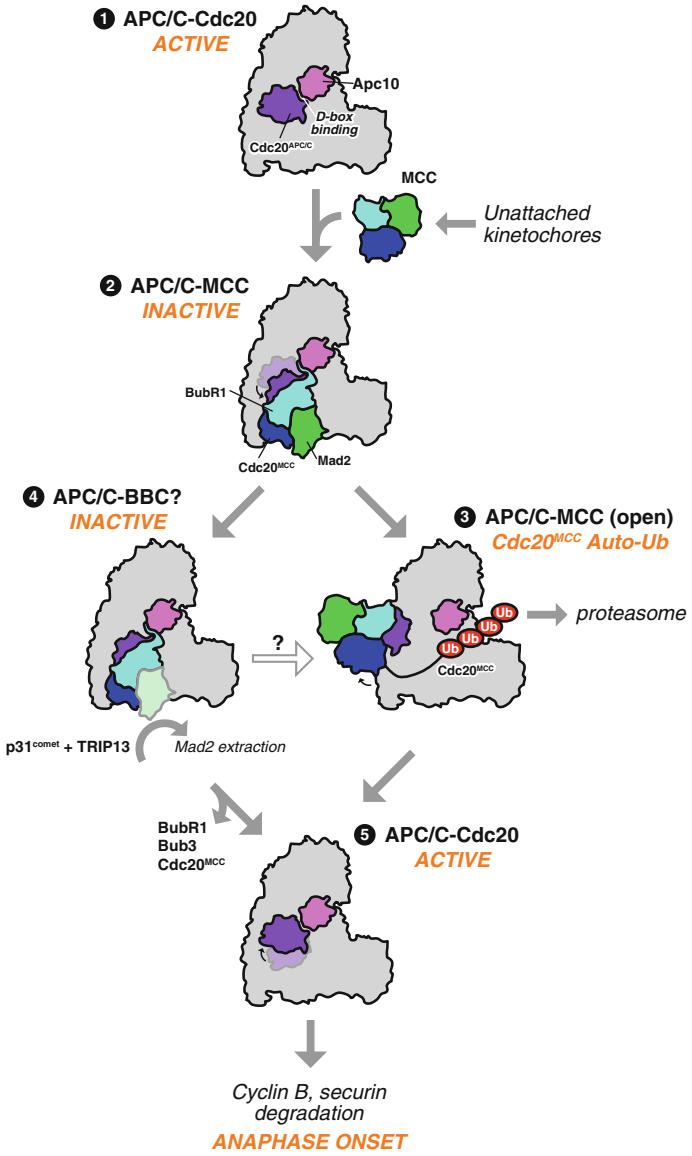
4 Assembly of the Mitotic Checkpoint Complex at Unattached Kinetochores

The key molecular event monitored by the SAC is kinetochore-microtubule attachment. Kinetochores are complex multi-megadalton structures that assemble on each chromosome's centromere, where they both mediate chromosome-microtubule attachment and serve as signaling hubs for the checkpoints monitoring attachment status. The architecture and function of kinetochores are covered in recent excellent reviews (Pesenti et al. 2016; Nagpal and Fukagawa 2016); here I focus mainly on a conserved outer kinetochore complex, the KMN network, that serves as the major sensor of microtubule attachment and a scaffold for MCC assembly.

4.1 The KMN Network: A Scaffold for SAC Signaling and MCC Assembly

The KMN network is a highly conserved outer kinetochore complex that serves as both the main microtubule-binding component of the kinetochore, and a platform for MCC assembly when microtubules are not bound (Fig. 5a) (Cheeseman et al. 2006; Varma and Salmon 2012). The KMN network contains three subcomplexes with distinct roles: the Mis12 complex anchors the network to the inner kinetochore, the Ndc80 complex binds microtubules, and the Knl1 complex is responsible for recruiting SAC proteins.

Knl1 (Spc105 in *Saccharomyces cerevisiae*, Spc7 in *Schizosaccharomyces pombe*, Knl1/CASC5/Blinkin in humans) contains a large disordered N-terminal region with multiple conserved motifs. Nearest the N-terminus is a phosphatase-binding site, termed SILK/RVSF (Hendrickx et al. 2009; Liu et al. 2010). When kinetochores are not attached to microtubules, phosphatase binding is inhibited through phosphorylation of this site by the Aurora B kinase (Liu et al. 2010). Following the SILK/RVSF motif in Knl1 are multiple short motifs, termed MELT repeats (Desai et al. 2003; Nekrasov et al. 2003; Cheeseman et al. 2004; Vleugel et al. 2015b), that are phosphorylated by the Mps1 kinase when kinetochores are not attached to microtubules (London et al. 2012; Shepperd et al. 2012; Yamagishi et al. 2012). Phosphorylated MELT repeats (P-MELT) recruit the SAC



protein Bub3 along with its binding partners, Bub1 and BubR1 (Yamagishi et al. 2012; Primorac et al. 2013; Vleugel et al. 2013, 2015b; Krenn et al. 2014; Zhang et al. 2014; Overlack et al. 2015). As mentioned above, Bub1 and BubR1 are paralogs with similar overall structures, but each has evolved to fulfill distinct roles in the checkpoint: Bub1 serves as the major hub for MCC assembly by recruiting SAC proteins, and BubR1 is a subunit of the MCC (Bub1 and BubR1's evolution

◂**Fig. 4** Life cycle of the APC/C in mitosis. (*1*) After CDK phosphorylation (not shown) and binding of $Cdc20^{APC/C}$ (*purple*), the APC/C is active. (*2*) Unattached kinetochores trigger the assembly of the MCC (see Fig. 5), which binds and inhibits APC/C-Cdc20 by occupying all degron-recognition sites and rotating $Cdc20^{APC/C}$ away from Apc10 (Alfieri et al. 2016). (*3*) Upon SAC silencing, two pathways lead to APC/C reactivation. First, rotation of the bound MCC to the "open" position (stabilized by Apc15) allows auto-ubiquitination of the $Cdc20^{MCC}$ N-terminal tail, triggering proteasome-mediated destruction. (*4*) Second, $p31^{comet}$ and TRIP13 extract Mad2 from the MCC, potentially resulting in a partially bound state (BBC: BubR1-Bub3-Cdc20) in which the remaining MCC subunits are less-stably bound and prone to dissociation (potentially also involving $Cdc20^{MCC}$ ubiquitination; *outline arrow*). (*5*) After APC/C-Cdc20 reactivation, it ubiquitinates B-type cyclins and securin to promote anaphase onset and mitotic exit

from a bifunctional ancestor is discussed more fully in Suijkerbuijk et al. 2012a; Di Fiore et al. 2016). Both Bub1 and BubR1 bind Bub3 through their so-called GLEBS motifs (Taylor et al. 1998; Wang et al. 2001; Larsen et al. 2007), and the resulting complex is competent to bind Knl1 P-MELT repeats (Fig. 5b). Interestingly, Bub1:Bub3 binds much more strongly to P-MELT repeats than does BubR1:Bub3 (Primorac et al. 2013; Overlack et al. 2015), and the bulk of BubR1:Bub3 is recruited to kinetochores indirectly, through a pseudo-symmetric Bub1-BubR1 dimer interaction (Overlack et al. 2015; Zhang et al. 2015). Some BubR1:Bub3 is recruited directly to Knl1 P-MELT repeats, however, and preliminary evidence suggests that this pool may be the major source of BubR1:Bub3 that is incorporated into the MCC (see Sect. 4.3) (Zhang et al. 2016a). The requirement for BubR1 localization to kinetochores varies between organisms, however, as some BubR1 orthologs—such as *S. pombe* Mad3—lack both Bub3 and Bub1 binding motifs (Fig. 2c).

Once recruited to Knl1 P-MELT motifs, Bub1 and BubR1 recruit the remaining SAC components necessary for MCC assembly: Cdc20 and a complex of Mad1 bound to C-Mad2. Cdc20 is recruited by both Bub1 and BubR1, through homologous degron-like motifs C-terminal to these proteins' GLEBS motifs (BubR1 ABBA3 (Lischetti et al. 2014; Di Fiore et al. 2015), Bub1 KEN-ABBA (Vleugel et al. 2015a)). Mad1:Mad2 is also probably recruited by Bub1, with direct interactions between Mad1 and Bub1 having been reported in multiple organisms including fungi, nematodes, and humans (London and Biggins 2014; Moyle et al. 2014; Ji et al. 2017). Humans and other complex eukaryotes also possess a separate complex, known as RZZ (Rod-Zwilch-ZW10) that binds Bub1 and recruits Mad1:Mad2 (Wang et al. 2004; Kops et al. 2005; Buffin et al. 2005; Karess 2005; Barisic and Geley 2011; Zhang et al. 2015; Caldas et al. 2015; Silió et al. 2015). Regardless of its recruitment pathway, kinetochore-localized Mad1:Mad2 is necessary to recruit soluble O-Mad2 and mediate its conversion to C-Mad2, binding to the Cdc20 KILR motif, and assembly into the MCC (see Sect. 4.3).

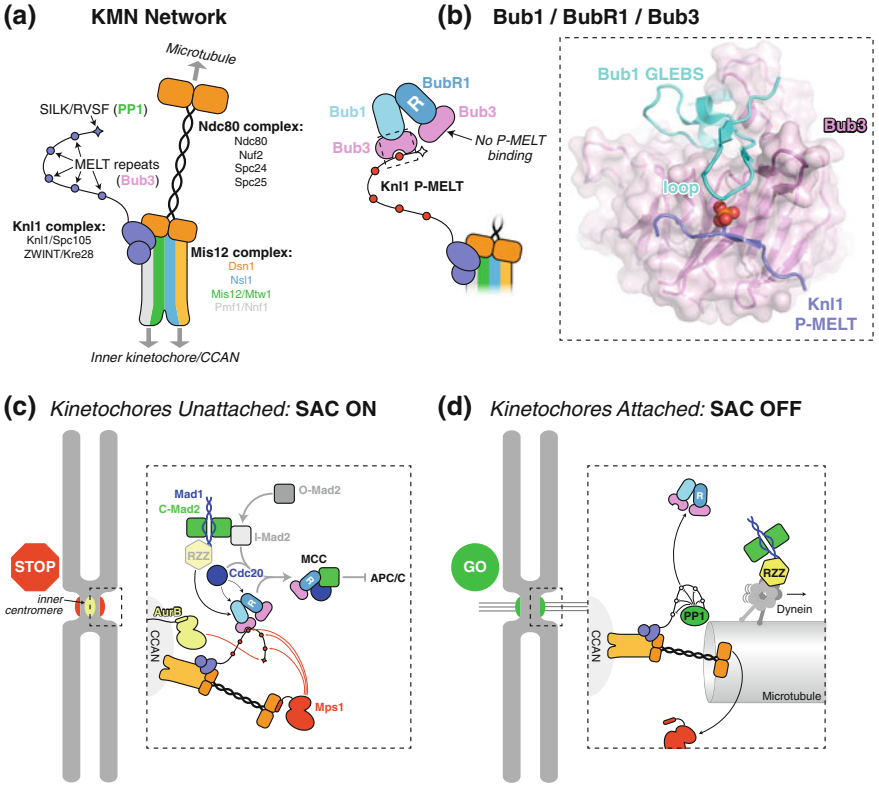


Fig. 5 Assembly of the MCC at unattached kinetochores. **a** Overall architecture of the outer kinetochore KMN network, consisting of the Knl1, Mis12, and Ndc80 subcomplexes. The Mis12 complex binds inner kinetochore protein complexes, also termed the "constitutive centromere-associated network" or CCAN. The Ndc80 complex contains the main microtubule-binding activity in the kinetochore, while Knl1 contains conserved SILK/RVSF and MELT motifs. **b** Interactions of Bub1, BubR1, and Bub3. Bub3 associates with P-MELT repeats when bound to the Bub1 GLEBS motif, but not the BubR1 GLEBS motif (detail view, right from PDB ID 4BL0 Primorac et al. 2013). BubR1 and Bub1 form a pseudo-symmetric dimer interaction involving their "helical extension" segments ("HE" in Fig. 2c) C-terminal to the GLEBS motif (Overlack et al. 2015). **c** MCC assembly at unattached kinetochores. Mps1 is recruited to Ndc80 in the absence of microtubules, and phosphorylates Knl1 MELT repeats to mediate recruitment of Bub1-Bub3 (*thin red arrows*). Aurora B (tethered at the inner centromere; *light yellow*) phosphorylates the Knl1 SILK/RVSF motif to inhibit PP1 binding (*thin red arrow*). Bub1 recruits Mad1:C-Mad2 (in some organisms, through the RZZ complex), and both Bub1 and BubR1 recruit Cdc20 (*thin black arrows*). Mad1-bound C-Mad2 converts soluble O-Mad2 to C-Mad2 concomitant with Cdc20 binding, followed by binding of BubR1 to complete MCC assembly (*thick gray arrows*). **d** After kinetochore-microtubule attachment, multiple mechanisms inactivate MCC assembly. In the absence of Mps1, PP1-mediated dephosphorylation of Knl1 MELT repeats causes loss of Bub1/Bub3, and in those organisms with RZZ, Mad1:C-Mad2 is actively "stripped" from kinetochores through coupling to dynein motors

4.2 The Mps1 Kinase Coordinates Attachment Sensing with MCC Assembly

As noted above, the kinase Mps1 phosphorylates Knl1 MELT repeats to initiate recruitment of SAC components to unattached kinetochores (the diverse roles of Mps1 are reviewed in Lan and Cleveland 2010; Liu and Winey 2012). Mps1 recruitment, therefore, must be responsive to kinetochore-microtubule attachment status. It is not surprising, therefore, that the key determinant of Mps1 recruitment is the Ndc80 complex, the major kinetochore complex responsible for microtubule binding (Martin-Lluesma et al. 2002; Nijenhuis et al. 2013; Zhu et al. 2013; Hiruma et al. 2015; Ji et al. 2015; Aravamudhan et al. 2015; Dou et al. 2015). Exactly how Mps1 kinase activity is coordinated with Ndc80-microtubule binding is not yet firmly established. One mechanistic model involves a direct competition between Mps1 and microtubules for Ndc80 binding. Supporting this idea, two groups recently showed that Mps1 binds directly to the Ndc80 CH domain, which is also responsible for microtubule binding (Wei et al. 2007; Ciferri et al. 2008; Hiruma et al. 2015; Ji et al. 2015). These studies showed that Mps1-Ndc80 binding is suppressed in vitro by microtubules, suggesting that Mps1 and microtubules compete directly for Ndc80 binding (Hiruma et al. 2015; Ji et al. 2015). Another possible mechanism is that Mps1 remains associated with Ndc80 even after microtubule attachment, but its ability to phosphorylate Knl1 is inhibited once attachment occurs (Aravamudhan et al. 2015). In either case, active Mps1 promotes MCC assembly at unattached kinetochores in several ways. First and most importantly, it phosphorylates the MELT repeats in the N-terminal region of Knl1 (London et al. 2012; Shepperd et al. 2012; Yamagishi et al. 2012), which in turn recruit Bub1:Bub3 as described above. Mps1 also phosphorylates Bub1 directly, and this phosphorylation was recently shown to be required for Bub1's ability to recruit Mad1:Mad2 to kinetochores (London and Biggins 2014; Ji et al. 2017). Finally, Mps1 phosphorylates Mad1 in its poorly characterized C-terminal RWD domain, promoting a direct Mad1-Cdc20 interaction that contributes to MCC assembly and SAC signaling (Hardwick et al. 1996; Faesen et al. 2017; Ji et al. 2017).

4.3 Assembling the MCC

Once all SAC components are recruited to unattached kinetochores, they participate in a complex structural dance, still incompletely understood, that ultimately results in fully assembled MCC. The first, and rate-limiting, step of MCC assembly is the association of Mad2 with Cdc20 (Simonetta et al. 2009; Faesen et al. 2017). This occurs when kinetochore-localized Mad1:Mad2 recruits soluble O-Mad2 to kinetochores through a pseudo-symmetric Mad2 homodimer interaction (Fig. 6) (Luo et al. 2004; Howell et al. 2004; Shah et al. 2004; de Antoni et al. 2005; Vink et al.

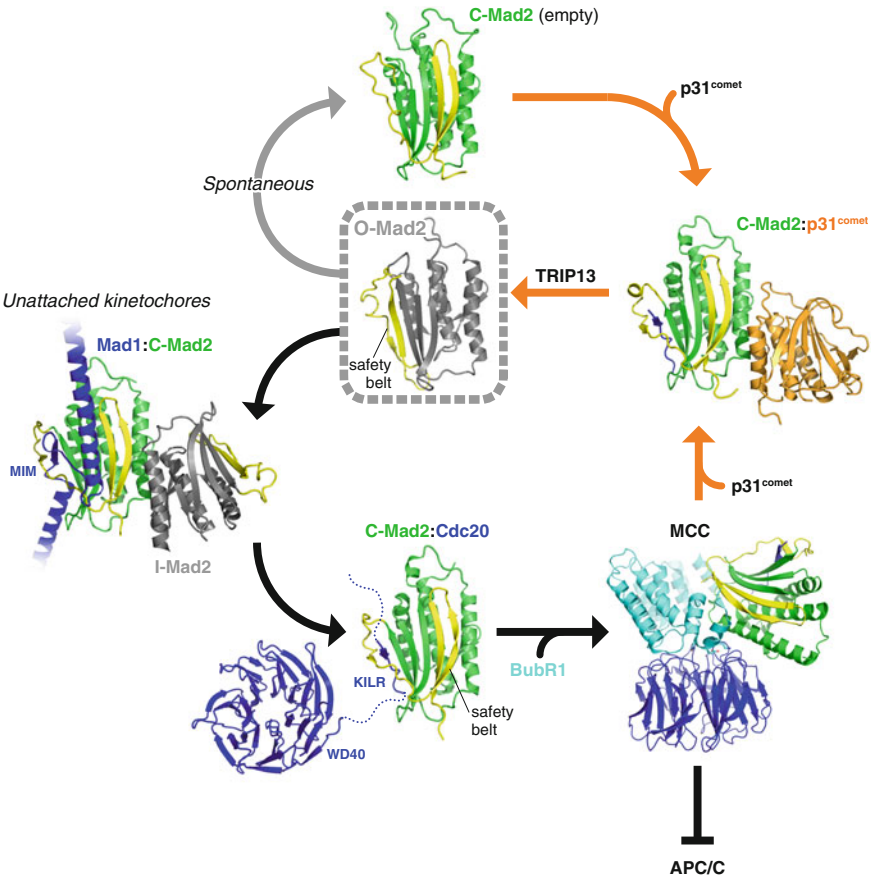


Fig. 6 The Mad2 conformational cycle. The majority of soluble cellular Mad2 is in the open (O-Mad2) conformation, with its C-terminal safety belt region (*yellow*) occluding the MIM/closure motif binding site (Luo et al. 2004) (structure from Luo et al. 2000); PDB 1DUJ). **SAC pathway** (*black arrows*): Upon SAC activation, unattached kinetochores recruit a complex of Mad1 bound to closed Mad2 (C-Mad2), which in turn dimerizes with soluble O-Mad2 to generate "intermediate" Mad2 (I-Mad2), primed for conversion to C-Mad2 and binding to Cdc20 [(Hara et al. 2015); structure shown is a composite of PDBs 1GO4 (Mad1:Mad2) (Sironi et al. 2002) and 2V64 (C-Mad2:I-Mad2) (Mapelli et al. 2007)]. After Mad2:Cdc20 binding, BubR1 (with associated Bub3, not shown) binds to complete the assembly of the MCC, which is competent for APC/C inhibition (Chao et al. 2012; PDB 4AEZ). **Recycling pathway** (*orange arrows*): $p31^{comet}$ binds C-Mad2 in the MCC in a manner akin to Mad2 homodimerization (Yang et al. 2007; PDB 2QYF). C-Mad2:$p31^{comet}$ is recognized and disassembled by TRIP13, reforming O-Mad2 (Ye et al. 2015). **Spontaneous pathway** (*gray arrow*): O-Mad2 spontaneously converts to ligand-free C-Mad2 with a lifetime ($1/k$) of $\sim$10 h (Luo et al. 2004; PDB 1S2H). $p31^{comet}$ and TRIP13 recycle this C-Mad2 to maintain a soluble pool of O-Mad2 for SAC activation (Ma and Poon 2016)

2006; Nezi et al. 2006). The resulting C-Mad2:O-Mad2 dimer has been visualized in two different x-ray crystal structures (Mapelli et al. 2007; Hara et al. 2015), and in both cases the O-Mad2 protomer adopts a subtly altered conformation compared to its structure in solution. This conformational shift is believed to promote dissociation of the C-terminal safety belt motif from its position occluding the closure motif binding site, resulting in a transient partially unfolded state (Mapelli and Musacchio 2007; Hara et al. 2015). Partially unfolded Mad2 is competent to associate with the Cdc20 KILR motif and refold into the closed state. The unique Mad1:Mad2-mediated conversion of soluble O-Mad2 to C-Mad2 has been termed the "Mad2 template model" (de Antoni et al. 2005; Musacchio and Salmon 2007; Mapelli and Musacchio 2007). After Mad2-Cdc20 binding, MCC assembly is completed when BubR1 binds both proteins as described above (Chao et al. 2012).

It has been understood for some time that in solution, O-Mad2 is less stable than C-Mad2 and will spontaneously convert to C-Mad2 with a half-time of several hours (Luo et al. 2004). Given that conversion of O-Mad2 to C-Mad2 is the rate-limiting step of MCC assembly, why does spontaneous conversion in solution not result in Cdc20 binding and MCC assembly? The key difference is likely the presence of Cdc20: binding of Mad2 to a closure motif can probably only occur in the transient partially unfolded state between O-Mad2 and C-Mad2, when the safety belt is disengaged from the HORMA domain core. Thus, the presence of Cdc20 at the time and place of Mad2 conformational conversion is likely key for complex formation. Further control over this assembly could be mediated by Mad1's functionally mysterious C-terminal RWD domain, which is phosphorylated by Mps1 at unattached kinetochores, interacts directly with Cdc20, and may be required for initial Cdc20-Mad2 association (Faesen et al. 2017; Ji et al. 2017). Away from kinetochores, spontaneous O-Mad2 to C-Mad2 conversion probably results in "empty" C-Mad2 that not only does not nucleate MCC assembly (Fig. 6), but is actively harmful in that it cannot be recruited to kinetochores when needed (as Mad1:Mad2 specifically recruits O-Mad2). For this reason, spontaneous O-Mad2 to C-Mad2 conversion must be continually counteracted in the cell to maintain a functional SAC (see Sect. 5.2 and Fig. 6) (Ma and Poon 2016).

5 Silencing the SAC

5.1 Kinetochore Transformations

After all kinetochores become attached to microtubules, the SAC must be silenced to allow anaphase onset. To accomplish SAC silencing, kinetochores undergo a number of structural and compositional changes. First, Ndc80 binding to microtubules suppresses Mps1 activity, either by removing it from kinetochores or spatially segregating it from its substrates. At the same time, the activity of the Aurora B kinase, which phosphorylates a number of outer kinetochore components

when kinetochores are not attached, including the Knl1 SILK/RVSF motif, is suppressed (regulation of Aurora B is discussed in detail in Lampson and Cheeseman 2011; Carmena et al. 2012; van der Horst and Lens 2014; Krenn and Musacchio 2015). The loss of these two kinase activities alters the balance of kinase/phosphatase activity at the outer kinetochore, first enabling Protein Phosphatase 2A (PP2A), recruited by BubR1, to dephosphorylate the Knl1 SILK/RVSF motif (Espert et al. 2014; Nijenhuis et al. 2014). The SILK/RVSF motif then binds protein phosphatase 1 (PP1) (Liu et al. 2010; Rosenberg et al. 2011; Meadows et al. 2011; London et al. 2012), which in turn dephosphorylates the Knl1 MELT repeats, resulting in loss of Bub1:Bub3 and all associated SAC components. The delicate balance of kinase and phosphatase activities at kinetochores, and how this balance is affected by microtubule attachment and other events, is outside the scope of this review but is covered in detail elsewhere (Suijkerbuijk et al. 2012b; Foley and Kapoor 2013; Espert et al. 2014; Nijenhuis et al. 2014; Etemad and Kops 2016). Finally, in organisms that possess the RZZ complex, RZZ and an associated protein called Spindly mediate the active "stripping" of Mad1:Mad2 from kinetochores upon microtubule attachment by linking Mad1:Mad2 to the microtubule minus-end directed motor dynein (Starr et al. 1998; Howell et al. 2001; Gassmann et al. 2008, 2010; Yamamoto et al. 2008; Chan et al. 2009; Barisic et al. 2010). Thus, microtubule attachment sets in motion a series of events that result in the dissociation of all SAC components from kinetochores, thereby halting MCC assembly.

5.2 MCC Disassembly and Degradation

In addition to halting assembly of new MCC, SAC silencing requires that existing MCC, both soluble and APC/C-Cdc20 bound, be disassembled and/or degraded. Two separate pathways have been identified that contribute to MCC turnover, one involving ubiquitination and degradation of $Cdc20^{MCC}$, and the other involving direct disassembly of the MCC complex through the extraction of Mad2.

The first pathway for reactivation of inhibited APC/C-MCC complex involves the ubiquitination and subsequent degradation of $Cdc20^{MCC}$ (Pan and Chen 2004; King et al. 2007; Reddy et al. 2007; Ge et al. 2009; Foe et al. 2011). As noted above, the MCC not only occupies the degron-binding sites of the APC/C, it also sterically occludes binding of E2 enzymes to the APC/C catalytic module. Recent cryo-EM analysis of APC/C-MCC identified a minor conformational state (termed APC/C-MCC-open) in which the bound MCC is rotated away from the catalytic module, allowing binding of an E2 enzyme (Fig. 4) (Alfieri et al. 2016). A structure of the APC/C-MCC-open state with a bound E2, UbcH10, revealed how $Cdc20^{MCC}$ can be ubiquitinated while still bound to the APC/C (Alfieri et al. 2016). This work also revealed why the small APC/C subunit Apc15 is required for $Cdc20^{MCC}$ ubiquitination (Mansfeld et al. 2011; Foster and Morgan 2012; Uzunova et al.

2012): in the absence of Apc15, the APC/C-MCC-open state is not accessed, meaning that E2 binding and $Cdc20^{MCC}$ ubiquitination cannot occur (Alfieri et al. 2016). After ubiquitination of $Cdc20^{MCC}$, this protein is presumably targeted to the proteasome for degradation, resulting in the reactivation of APC/C-Cdc20.

A second pathway for MCC turnover involves the direct disassembly of MCC by two proteins, $p31^{comet}$ and TRIP13 (Pch2 in yeast). $p31^{comet}$ is a HORMA domain protein distantly related to Mad2, that was first identified as a Mad2-binding protein (Habu et al. 2002; Xia et al. 2004). TRIP13 is a AAA^+ family ATPase, which was first identified as a regulator of the Mad2-related HORMAD proteins in meiotic prophase (San-Segundo and Roeder 1999; Borner et al. 2008; Wojtasz et al. 2009; Vader 2015). Recently, TRIP13 was found to cooperate with $p31^{comet}$ in MCC disassembly and SAC inactivation (Teichner et al. 2011; Tipton et al. 2012; Eytan et al. 2014; Wang et al. 2014; Miniowitz-Shemtov et al. 2015; Ma and Poon 2016). Together, $p31^{comet}$ and TRIP13 specifically recognize C-Mad2 and convert it to the unbound O-Mad2 conformation (Ye et al. 2015). This enzymatic activity has multiple important functions, depending on context. First, $p31^{comet}$ and TRIP13 can directly disassemble soluble MCC (Mansfeld et al. 2011; Eytan et al. 2014). $p31^{comet}$ can also bind to APC/C-MCC, and some evidence suggests that Mad2 can be extracted from within APC/C-bound MCC, albeit less efficiently than from soluble MCC (Mansfeld et al. 2011; Westhorpe et al. 2011). Mad2 extraction from either soluble MCC or APC/C-MCC is a possible source of the BBC complex (BubR1, Bub3, Cdc20), which has been found to bind and inhibit the APC/C under certain conditions (Nilsson et al. 2008; Kulukian et al. 2009; Westhorpe et al. 2011; Han et al. 2013). It is likely that after Mad2 extraction, the remaining subunits of the MCC are still able to inhibit APC/C-Cdc20 to some extent. Ultimately, however, Mad2 extraction by $p31^{comet}$ and TRIP13 would destabilize the MCC, promoting dissociation and APC/C-Cdc20 reactivation.

$p31^{comet}$ and TRIP13 are conserved in plants, animals, and insects, but a clear fungal homolog of $p31^{comet}$ is missing, raising doubts about the conservation of the $p31^{comet}$/TRIP13-mediated MCC disassembly pathway. Recently, however, a radically shortened $p31^{comet}$ relative, termed Tiny yeast comet 1 (Tyc1), has been identified in *S. cerevisiae* (Schuyler S.C., personal communication). The structural mechanisms of this protein, and how it relates to more canonical $p31^{comet}$ proteins, will be exciting to explore in the future.

Another question that remains largely unexplored is whether the two known pathways for MCC turnover, $Cdc20^{MCC}$ ubiquitination and $p31^{comet}$/TRIP13-mediated Mad2 extraction, are functionally linked. Addition of $p31^{comet}$ to cell extracts arrested in metaphase by nocodazole treatment has been shown to promote Cdc20 ubiquitination (Reddy et al. 2007), suggesting that Mad2 extraction might promote formation of the APC/C-MCC-open state and thereby promote $Cdc20^{MCC}$ ubiquitination. The two pathways are not perfectly intertwined, however, as RNAi depletion of $p31^{comet}$ and the priming E2 enzyme UbcH10 (necessary for Cdc20 ubiquitination) has an additive effect on SAC inactivation

(Reddy et al. 2007). Overall, the functional relationship between $Cdc20^{MCC}$ ubiquitination and MCC disassembly by $p31^{comet}$ and TRIP13 remains to be fully explored.

As noted above, O-Mad2 is unstable and spontaneously converts to C-Mad2 in vitro with a half-time of several hours (Luo et al. 2004). Based on the idea that this spontaneous conversion likely also occurs in the cell, we proposed that $p31^{comet}$ and TRIP13 might be involved in "recycling" this C-Mad2 by converting it back to O-Mad2 (Fig. 6) (Ye et al. 2015). This was recently shown to be the case: knockout of TRIP13 in human cells causes a profound defect in SAC activation, rendering these cells unresponsive to microtubule poisons such as nocodazole (Ma and Poon 2016). Biochemical examination shows that, indeed, Mad2 overwhelmingly adopts the closed conformation in these cells, and there is no detectable Mad2 binding to other MCC subunits (Ma and Poon 2016). Addition of exogenous TRIP13 to extracts from TRIP13-knockout cells re-establishes the predominance of O-Mad2 in solution (Ma and Poon 2016). In overall agreement with these results, work in *Caenorhabditis elegans* has also shown that $p31^{comet}$ and TRIP13 homologs (CMT-1 and PCH-2, respectively) are important for Mad2 recruitment to unattached kinetochores, and that loss of these factors causes defects in SAC activation (Nelson et al. 2015). Thus, $p31^{comet}$ and TRIP13 contribute to both SAC activation and inactivation by catalyzing the closed-to-open conformational change in Mad2.

6 Conclusion

Recent years have seen tremendous advances in our understanding of the molecular structures and protein–protein interactions underlying the SAC: the structure and mechanisms of the APC/C, its mode of inhibition by the MCC, and the mechanisms of MCC assembly and disassembly. We know much less about what occurs at kinetochores, including how they promote MCC assembly, and how their structure and composition changes in response to microtubule attachment/detachment and other signals. Finally, our understanding of SAC dynamics, particularly how it is able to respond quickly to changes in kinetochore-microtubule attachment status, is in its infancy. Thus, while recent advances in molecular understanding of SAC mechanisms represent an important step forward, a true holistic understanding of this fascinatingly complex pathway still awaits.

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