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# The Molecular Biology of Spindle Assembly Checkpoint Signaling Dynamics

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**The spindle assembly checkpoint is a safeguard mechanism that coordinates cell-cycle progression during mitosis with the state of chromosome attachment to the mitotic spindle. The checkpoint prevents mitotic cells from exiting mitosis in the presence of unattached or improperly attached chromosomes, thus avoiding whole-chromosome gains or losses and their detrimental effects on cell physiology. Here, I review a considerable body of recent progress in the elucidation of the molecular mechanisms underlying checkpoint signaling, and identify a number of unresolved questions.**

## Introduction

The spindle assembly checkpoint (SAC, also known as mitotic or metaphase checkpoint) is a feedback-control system that operates during cell division in eukaryotic cells [1–3]. The SAC monitors chromosome bi-orientation on the mitotic spindle, and as long as improperly attached chromosomes remain, it halts cells in mitosis and precludes passage into the final phases of cell division (Figure 1). The ultimate function of the SAC is therefore to prevent loss of sister chromatid cohesion (the initiation of anaphase) and premature chromosome segregation in the presence of unattached or incorrectly attached chromosomes. This function preserves the genome from alterations in chromosome copy number and protects cells from the dire consequences that follow them [4].

Assaying for SAC function is simple. Spindle poisons that depolymerize or hyperstabilize microtubules cause long-term SAC arrests (~20 hours or more in human cell lines and several hours in the budding yeast *Saccharomyces cerevisiae*). Measuring the duration of this SAC response after depletion of any given protein reveals the possible involvement of the latter in the SAC response (see [5] for an example). More sophisticated SAC assays based on live-cell sensors have also been described [6,7]. The components of the SAC pathway are nearly ubiquitous in eukaryotes [3], but their genetic ablation may have from mild to dramatic consequences for viability in different organisms — probably a reflection of differences in the robustness of kinetochore–microtubule attachment pathways.

Like other signaling pathways, the SAC consists of a sensory apparatus that monitors the state of chromosome attachment to the mitotic (or meiotic) spindle, and an effector system that targets the basic cell-cycle machinery. In between these two end points of the pathway are proteins believed to act as catalysts for the accumulation of the SAC effector (Figure 2A). Similar to other pathways, reversible protein phosphorylation is a crucial regulator of the SAC signaling and its downstream effects [8–10]. Table 1 lists the main features of SAC proteins.

The SAC effector is named the mitotic checkpoint complex (MCC). It targets the anaphase-promoting complex or cyclosome (APC/C; Figure 1). This ubiquitin ligase triggers mitotic exit by polyubiquitination of two crucial substrates, Cyclin B and Securin, in turn promoting their rapid destruction by the proteasome [11,12]. By inhibiting the APC/C, the MCC stabilizes these substrates, effectively preventing mitotic exit.

## Kinetochores

Kinetochores mediate chromosome attachment to microtubules. They are complex multi-subunit structures with an 'inner' layer interfacing with the unique centromeric chromatin present on each chromosome, and an 'outer' layer involved in microtubule binding and SAC control (Figure 2) [13,14]. Within the outer layer, the Knl1 complex–Mis12 complex–Ndc80 complex (abbreviated as KMN network) promotes microtubule binding through a calponin-homology (CH) domain on the Ndc80/Hec1 subunit of the Ndc80 complex (Figure 2A) [15–18].

Despite some organism-to-organism variability, during prometaphase (i.e. during the early phases of kinetochore–microtubule attachment), all SAC proteins become recruited to kinetochores (see for instance [19]). Most notably for Mad1, Mad2, and Mps1, and to a lesser degree also for the other SAC proteins, the kinetochore levels then decline with the progression of attachment (Figure 2A–C) [19–24]. When active, kinetochores are believed to act as 'catalytic platforms' that determine the collective rate of production of MCC as a function of microtubule attachment status (Figure 2C). Physical tethering of certain SAC proteins, such as Mad1 and Mps1, to KMN subunits is sufficient to cause a permanent metaphase arrest in some systems [25–31]. Whether the MCC is created partly or entirely at kinetochores or in the cytosol, however, is still unclear. On the other hand, it seems likely that the MCC can diffuse freely within the cell to seize control over the APC/C.

In their classic experiment, Rieder and colleagues demonstrated that laser ablation of the last unattached kinetochore accelerates mitotic exit [32], indicating that unattached kinetochores extend the duration of the SAC response, and that the latter has an otherwise limited half-life. Although the molecular details of this dynamic regulation remain obscure, some general concepts have started to emerge. First, the checkpoint has different 'strengths' depending on the severity of the conditions that trigger it [33], which is more elegantly recapitulated by saying that it "acts as a rheostat rather than a toggle switch" [6]. Likely,

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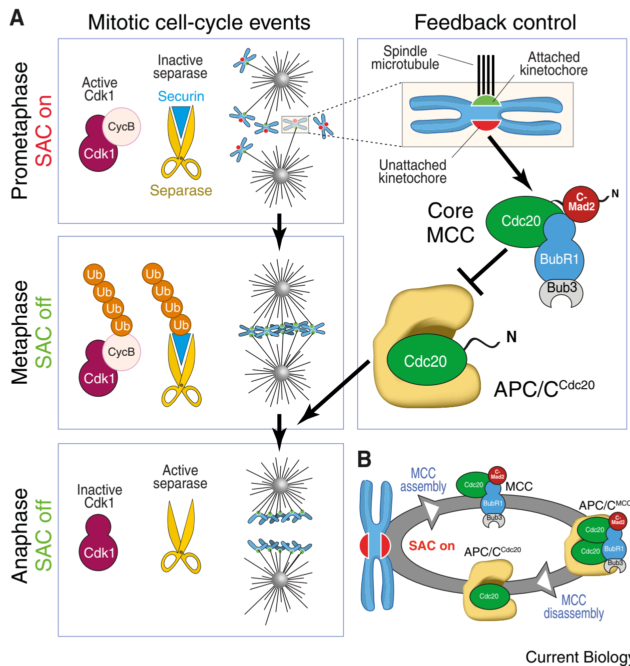


**Figure 1. The SAC mechanism.**
(A) Mitosis is instated by activation of the Cdk1:Cyclin B complex. The SAC is active during prometaphase, when chromosomes attach to the mitotic spindle. Attachment involves kinetochores. Properly attached kinetochores (green) 'satisfy' the SAC, which stops signaling. Unattached or improperly attached kinetochores (red) emit the SAC signal. MCC, the SAC effector, is shown to originate at red kinetochores. It binds and inhibits APC/C$^{Cdc20}$, which is required for the metaphase–anaphase transition, thus preventing entry into anaphase. When the SAC is satisfied on all kinetochores (at metaphase), activation of APC/C$^{Cdc20}$ promotes Cyclin B and Securin ubiquitination and proteolysis. Their destruction starts mitotic exit and sister chromatid separation, the latter through activation of the cohesin-protease separase. (B) Even during SAC activation, pathways of MCC production and MCC inactivation coexist in the cell. This may be required for a responsive SAC response: the continued presence of 'red' kinetochores is required to support a sufficiently high rate of MCC production at any given time to counteract MCC disassembly.

the difference in strength reflects the number of unattached or improperly attached kinetochores, which in turn determines the overall rate of MCC assembly [7]. Thus, the more severe is the condition that generates the signal, the more robust is the SAC response. Second, the MCC is continuously disassembled and re-assembled during checkpoint activation (reviewed in [34]). This homeostatic control likely reflects a strategy to impart responsiveness to the SAC network, essentially making the presence of improperly attached kinetochores imperative for the continuation of the SAC response (Figure 1B). If the SAC responds to even a single unattached kinetochore, as it does [7,32], these conditions imply that the rate at which even a single kinetochore generates the MCC must be sufficient to allow steady-state accumulation of the MCC to levels that are sufficient to maintain the mitotic arrest [7]. Third, recruitment of SAC proteins to kinetochores does not only have stimulatory effects on the SAC response. SAC silencing, which is emerging as an active, energy consuming process, may also require kinetochore function.

In summary, the KMN network is the fulcrum of the sensory mechanism of the SAC (Figure 2) [10,14]. It is required for kinetochore recruitment of probably all SAC proteins, and it somehow generates a dynamic control system that determines the duration of the SAC in response to microtubule binding. How the 'sensory' apparatus of the SAC embeds itself into the microtubule-binding machinery of the kinetochore, however, is incompletely understood and partly controversial. Aurora B appears to be crucial for operation (as it is required for kinetochore recruitment of the SAC-promoting kinase Mps1), while it also counteracts the recruitment of the SAC-silencing phosphatase PP1 [10,35].

## Aurora B and Mps1

A detailed account of the complex structural organization of Aurora B and of its multiple functions goes beyond the scope of this review, and the reader is referred to excellent recent reviews of the field [36,37]. Here, suffice it to say that Aurora B is a serine/threonine (S/T) protein kinase, that it is a subunit of a targeting and activating complex named the chromosome passenger complex (CPC), and that during mitosis it is greatly enriched in the region between kinetochores, from which it can phosphorylate kinetochore substrates, including centromeric protein A (CENP-A) in the inner kinetochore and the subunits of the KMN network in the outer kinetochore [36,37]. Aurora B-dependent phosphorylation of kinetochore substrates is strictly linked to the state of kinetochore–microtubule attachment, and declines when bi-orientation ensues (Figure 2) [38–42]. This decline in phosphorylation probably results from a decrease in the ability of Aurora B to reach its substrates rather than from a reduction of its intrinsic catalytic activity. A few hypotheses for how the specific topology of the centromere–kinetochore interface may support this change in activity await further validation or disproval [35,43,44]. Importantly, the activity of Aurora B not only controls the SAC, but is also required to promote correct kinetochore–microtubule attachment [36,37]. Given the very restricted spatial localization of Aurora B between kinetochores during prometaphase, the hypothesis that the same pool of active Aurora B controls both pathways at the same time, albeit on at least partly distinct substrates, appears simpler than alternative functional hypotheses [45].

Recruitment of Mps1 to kinetochores is considered one of the crucial contributions of Aurora B to SAC signaling [46,47], but an exact molecular description of this contribution is lacking (Figure 2B). Be that as it may, once at kinetochores, Mps1 phosphorylates the phosphodomain of Knl1 at several Met-Glu-Leu-Thr (MELT) motifs [48–50], thus creating docking sites for the hierarchical recruitment of additional SAC proteins, including Bub3, Bub1, BubR1 (known as Mad3 in yeast), Mad1, Mad2, and Cdc20, which play a crucial role in the assembly of MCC, either as MCC subunits, or by supporting MCC assembly (Figure 2C) [51–61].

The 4-subunit Ndc80 complex within the KMN network, regarded as the kinetochore's main microtubule receptor, binds directly to Mps1 to promote its recruitment to kinetochores [62–68]. Recent studies identified a direct interaction of Mps1 with the Ndc80/Hec1 CH domain [62,63,65,66,68,69]. This finding stimulated the interesting hypothesis that microtubule binding may displace Mps1 from kinetochores, either by a mechanism of direct competition [68], or by direct competition alleviated by a 'partly noncompetitive mechanism' [62]. If correct,

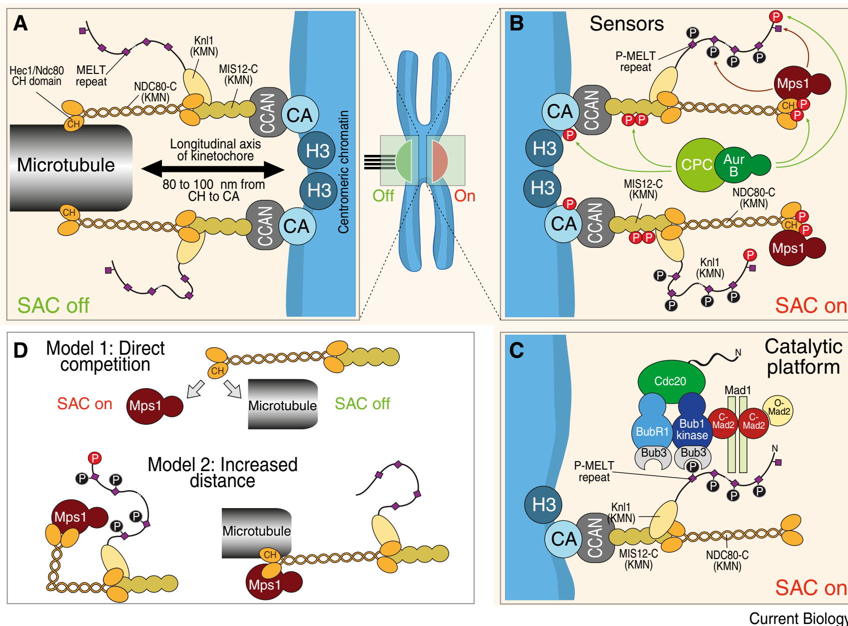


**Figure 2. Kinetochores and the SAC.**
(A) The proteins or protein complexes indicated as MIS12-C, NDC80-C and Knl1 are sub-complexes of the outer kinetochore KMN network, which binds microtubules with the CH domain of Ndc80/Hec1 and coordinates the SAC mechanism. The KMN links through the constitutive centromere associated network (CCAN) to specialized centromeric chromatin containing the histone H3 variant CENP-A (CA). (B) Aurora B (Aur B), the catalytic subunit of the chromosome passenger complex (CPC), phosphorylates (green arrows) various substrates of unattached kinetochores and promotes recruitment of the SAC kinase Mps1. Mps1 phosphorylates (red arrows) so-called MELT repeats in Knl1 (reviewed in [10]). (C) The SAC protein Bub3 recognizes the phosphorylated MELT sequences (P-MELT). The Bub3:Bub1 complex recruits BubR1:Bub3. Bub1 also contributes to recruiting Cdc20, and the Mad1:C-Mad2 complex, which in turn recruits O-Mad2. BubR1:Bub3, Cdc20, and Mad2 interact in the MCC. Incorporation of Mad2 in MCC requires its transformation from O-Mad2 to C-Mad2, a reaction accelerated by Mad1:C-Mad2. (D) Two models (discussed in the main text) for reducing Mps1 activity in the kinetochore. Model 1: Direct competition of microtubules and Mps1 for the same site on Ndc80. Model 2: Increased distance between Mps1 and Knl1 (MELT repeats) along the longitudinal axis of the kinetochore. There is at present no clear molecular understanding of how increased distance is generated.

these models would argue that there is direct coupling between microtubule binding to kinetochores and SAC silencing (Figure 2D). The models will now have to be reconciled with previous evidence demonstrating recruitment of Mps1 on kinetochores holding to robust microtubule fibers [70] and to new evidence showing Mps1 recruitment to attached kinetochores in *S. cerevisiae* [63]. Furthermore, Mps1 can maintain the SAC arrest caused by tethering Mad1 to kinetochores even after it has been removed from kinetochores, suggesting that its removal from kinetochores is not sufficient for SAC silencing [25]. Previous studies had suggested that removing Mps1 from kinetochores may be necessary for SAC satisfaction and mitotic exit [28,29], but it needs to be ascertained beyond reasonable doubt that tethering Mps1 to kinetochores does not disturb kinetochore–microtubule attachment, activating the checkpoint rather than preventing its silencing.

An alternative hypothesis is that microtubule binding does not directly compete for Mps1 binding to the Ndc80 complex, but rather increases the distance along the longitudinal axis of the kinetochore between Mps1 and its crucial substrate for SAC signaling, the phosphodomain of Knl1 (Spc105 in *S. cerevisiae*) [63]. This exciting model is strongly supported by evidence that any artificial perturbation of this distance, either by tethering Mps1 at more interior sites along the longitudinal axis of the kinetochore, or by tethering the phosphodomain of Knl1/Spc105 at more exterior sites, re-activates SAC signaling [63]. This model offers a mechanistic basis to previous studies identifying intra-kinetochore tension as a crucial parameter in SAC signaling and silencing [71,72]. Because the ability of Aurora B kinase to reach substrates in the kinetochore also decreases with microtubule attachment (see above), this model might also explain why Mps1, which requires Aurora B for kinetochore recruitment, becomes released from kinetochores as attachment proceeds.

## A Structural Perspective on the MCC Subunits

MCC assembles from the interaction of the three SAC proteins Mad2, BubR1/Mad3, and Bub3 with Cdc20 [73–77]. I now discuss some of the essential structural features of these proteins. With 1050 residues in humans, BubR1 is the largest of the MCC subunits. BubR1 is stuffed with functional motifs and structural domains (Figure 3A). These include an amino-terminal helix-loop-helix motif (HLH, a motif characterized by two $\alpha$-helices connected by a loop), which embeds KEN1 and is implicated in Cdc20 binding; a contiguous tetratricopeptide repeat (TPR) region; a D-box followed by KEN2, both implicated in binding the second Cdc20 subunit of MCC [11,78]; a binding site for Bub3, known as GLEBS or Bub3-binding domain, and embedding a short 'loop' motif [51,56,79–81]; a helical extension to the GLEBS that is not required for Bub3 binding but promotes dimerization with Bub1 and is required for kinetochore localization of BubR1 [51]; an 'internal Cdc20-binding site' (IC20BD, not shown in Figure 3A) of uncertain functional significance, recently shown to embed a conserved motif variably identified as A-motif (the original formulation), ABBA-motif, or Phe-box, closely followed by another D-box [57,59,60,82–87]; a kinetochore attachment regulatory domain (KARD) motif, mediating a phosphorylation-dependent interaction with the PP2A phosphatase [88–90]; and a carboxy-terminal pseudo-kinase domain [91] (Figure 3A). The complex interplay of these motifs and domains is an active area of investigation.

Bub3 (328 residues in humans) consists entirely of a seven-bladed WD40 $\beta$-propeller. It forms tight, probably constitutive complexes with the Bub3-binding motifs (GLEBS) of BubR1 and Bub1 [80]. Bub1 is a BubR1 paralog that does not become embedded in MCC-like particles. When bound to Bub1, Bub3 acts as a signaling adaptor — it binds to the phosphorylated MELT (P-MELT) repeats on the KMN subunit Knl1

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**Table 1. List of SAC proteins and their essential features (adapted from [174])**

| Protein name | Essential features | Main role in SAC | Main binding partners |
|---|---|---|---|
| Aurora B | S/T protein kinase | Recruitment of Mps1, inhibition of recruitment of PP1, SKA complex, Astrin:SKAP | Other CPC subunits |
| Bub1 | S/T protein kinase, domain- and motif-rich | Kinetochore recruitment of BubR1:Bub3 and Cdc20 | Bub3, Cdc20, P-MELT sequences |
| BubR1 | Pseudokinase, domain- and motif-rich | Component of MCC | Bub3, Mad2, Cdc20, Bub1:Bub3 complex |
| Bub3 | β-propeller, phosphoaminoacid adaptor | Component of MCC | BubR1, Bub3, P-MELT sequences |
| Cdc20 | β-propeller, adaptor for degrons | APC/C co-activator, component of MCC | APC/C, BubR1, Mad2, Bub1, several substrates including Cyclin B and Securin |
| Mad1 | Coiled-coil rich | Component of Mad1:C-Mad2 template complex | Mad2 |
| Mad2 | HORMA domain | Component of Mad1:C-Mad2 template complex and component of MCC | Mad1 and Cdc20 |
| Mps1 | S/T protein kinase | Phosphorylation of MELT repeats of Knl1 | Ndc80 for kinetochore recruitment |
| p31$^{comet}$ | HORMA domain | Dissociation of MCC by binding to C-Mad2, Capping of Mad1:C-Mad2 template | C-Mad2, Trip13 |
| Rod | α-solenoid | Subunit of RZZ complex that contributes to recruitment of Mad1:C-Mad2 to kinetochores | Other RZZ subunits, Spindly, Mad1:Mad2 |
| Zwilch | Mixed α- and β-structure | Subunit of RZZ complex that contributes to recruitment of Mad1:C-Mad2 to kinetochores | Other RZZ subunits, Spindly, Mad1:Mad2 |
| ZW10 | α-solenoid | Subunit of RZZ complex that contributes to recruitment of Mad1:C-Mad2 to kinetochores | Other RZZ subunits, Spindly, Mad1:Mad2 |
| Trip13 | AAA (triple A) ATPase | Conversion of C-Mad2 to O-Mad2 in silencing | p31$^{comet}$ |
| PP1 | S/T phosphatase | SAC silencing, counteracting Mps1 and Aur B | Knl1 |
| PP2A | S/T phosphatase | SAC silencing, counteracting Mps1 and Aur B | BubR1 |

(Figure 3B) [56]. The contribution of Bub1 to this interaction is small but crucial. The short 'loop' region within the Bub3-binding motif of Bub1 is required for Bub3 to promote Mps1-dependent recruitment of the Bub1:Bub3 complex to P-MELT sequences at kinetochores [51]. The equivalent loop region in BubR1 does not perform the same function, thus preventing direct kinetochore recruitment of the BubR1:Bub3 complex through P-MELT motifs (Figure 3C) [51]. Rather, kinetochore recruitment of BubR1 requires a direct, 'pseudo-symmetric' interaction with its paralog Bub1 already docked on Knl1's P-MELT, and Bub3 is required for this interaction (Figure 3C) [51,92]. Thus, Bub3 is involved in kinetochore recruitment of both Bub1 and BubR1, but the molecular mechanisms are distinct, for reasons and with functional consequences that need further clarification and that are discussed more thoroughly below [51]. Bub3 may also contribute directly to the MCC by promoting binding of BubR1 to Cdc20 and APC/C inhibition [93].

Cdc20 (499 residues in humans) also folds primarily as a WD40 β-propeller, but has amino- and carboxy-terminal extensions (Figure 4A). Cdc20 has a double-life. As a pre-anaphase co-activator of the APC/C, it performs a crucial function as an anaphase activator. As part of the MCC, it is a *bona fide* SAC protein and an anaphase inhibitor. Specific mutations in the Mad2-interaction motif (MIM) of Cdc20 can separate functions and cause SAC override (Figure 4B) [77]. Thus, Cdc20 is at the intersection where the SAC and the cell cycle meet (reviewed in [9,10]). As an APC/C co-activator, it presents substrates to the APC/C for ligation to ubiquitin [11]. Cdc20 interacts with its substrates through short linear degron motifs (i.e. as destruction motifs that mediate interactions with the ubiquitin ligase system), the best characterized of which are the destruction box (D-box) and the lysine-glutamate-asparagine (KEN) box [11]. Binding sites for these degrons, as well as for the A-box, map to distinct regions of the Cdc20 β-propeller (Figure 4C). Additional regulatory sequences fall in the amino-terminal and carboxy-terminal extensions of Cdc20, and include the C-box, important for Cdc20's co-activator function [11], the MIM [77,94,95], and the IR tail, which mediates Cdc20 binding to the APC/C. The MIM partly overlaps with a region of Cdc20, distinct from the C-box, that is also required for Cdc20's co-activator function [96]. While point mutations in this region can separate the functions of Cdc20 as an anaphase activator or inhibitor, as discussed earlier in this paragraph, more extensive mutations affect the co-activator function of Cdc20 as well [96]. Cdh1, which is closely related to Cdc20 and also acts as a co-activator of the APC/C, is not a target of the SAC. Its function as an APC/C co-activator is inhibited by phosphorylation during mitosis and only resumes upon mitotic exit [11].

Mad2 (205 residues in humans) is a HORMA (Hop1, Rev7, Mad2) domain protein with the remarkable property of adopting two distinct protein topologies, named open and closed Mad2 (O-Mad2 and C-Mad2, respectively) (Figure 4D–E) [97,98]. The C-Mad2 conformation is adopted in complex with Cdc20 or with Mad1 (the latter being the Mad2 receptor at kinetochores, as explained below). O-Mad2 is the conformation adopted by Mad2 when not bound to these partners. Topological switching

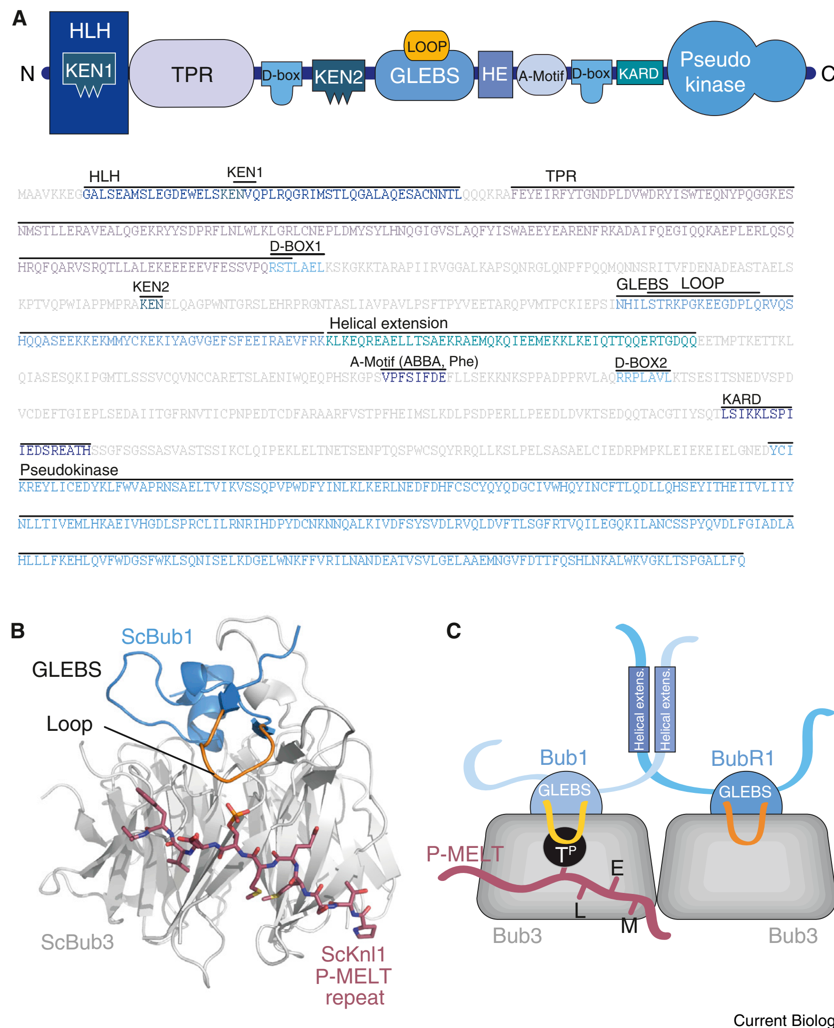


**Figure 3. Domain organization and interactions of BubR1 and Bub1.**
(A) Schematic representation of domains and motifs of BubR1, and their identification in the BubR1 sequence. HLH, helix-loop-helix; TPR, tetratricopeptide repeat; HE, helical extension. (B) Cartoon model of the Bub3:Bub1$^{GLEBS}$:P-MELT complex of *S. cerevisiae* (PDB ID 4BL0) [56]. The Bub1 loop region is shown in orange. (C) The mechanism of kinetochore recruitment of BubR1:Bub3 is based on a pseudo-symmetric interaction with Bub1:Bub3 involving analogous structural elements in the two structures, including a helical extension [51]. The loop of BubR1 does not support binding to P-MELT.

entails the reorganization of a mobile element encompassing the carboxy-terminal region of the Mad2 structure and referred to as the ‘safety belt [94,95,99,100]. The safety belt wraps around short linear MIMs of Cdc20 or Mad1, recently aptly defined as ‘closure motifs’ to emphasize that they cause Mad2 (and possibly, more generally, other HORMA domain proteins) to transition from the open to the closed conformation (Figure 4D,E) [101,102].

## A Structural Perspective on the MCC

It was originally proposed that MCC isolated from mitotic HeLa cells contains a single copy of Mad2, BubR1, Bub3, and Cdc20 [75], but it is not quite so simple. A ‘core’ MCC containing a single copy of each subunit (core MCC or MCC$^{1Cdc20}$) may bind a second molecule of Cdc20 to form MCC$^{2Cdc20}$ (Figure 5A) [78]. The existence of MCC$^{2Cdc20}$ had been previously advocated to resolve a number of puzzling observations [11]. For instance, it has been shown that BubR1 contains two KEN boxes. KEN1 and KEN2 (with the latter being adjacent to a recently identified D-box [78]) allow BubR1 to interact with Cdc20, qualifying BubR1 as a pseudo-substrate inhibitor of Cdc20 [103]. Because both KEN boxes of BubR1 are required for effective inhibition of the APC/C [103–106], and because Cdc20 hosts a single KEN-box binding site, binding of two Cdc20 subunits to BubR1 in MCC was to be expected [11]. Furthermore, the presence of two molecules of Cdc20 allowed a more straightforward interpretation of the mechanism of MCC inactivation, as discussed more thoroughly below [11,34,107].

The determination of the crystal structure of the Mad2:Cdc20:Mad3/BubR1 complex from *Schizosaccharomyces pombe* (PDB ID 4AEZ) was a milestone in MCC studies [108]. In this ternary assembly, the amino-terminal HLH motif of Mad3/BubR1, which contains KEN1, wedges between the β-propeller of Cdc20 and the αC helix of Mad2 (Figure 5B). The contiguous TPR superhelix of Mad3/BubR1 makes additional contacts with both Cdc20 and Mad2 [108]. The Mad2 safety belt traps the MIM closure motif embedded in the flexible amino-terminal region of Cdc20. Collectively, these interactions predict a cooperative binding mechanism in which each subunit contributes to the reinforcement of the binding affinity of the other two. Indeed, Mad2 is required for BubR1/Mad3 to bind Cdc20 [74,84,103,109,110], and Mad2 and BubR1 synergize to inhibit Cdc20-mediated activation of APC/C [83,109]. The assembly revealed by the structure of the Mad2:Cdc20:Mad3/BubR1 complex likely coincides with the very stable core MCC (MCC$^{1Cdc20}$). The presence of a single Cdc20 subunit in this assembly is unsurprising in view of the fact that KEN2, which is required for the binding of the second Cdc20 subunit of MCC [11,78,105], was omitted from the Mad3/BubR1 constructs used for crystallization [108]. Importantly, the stability of the core MCC is insensitive to inactivation of KEN2 [59,105].

Not only does the second Cdc20 subunit bind to a different segment of BubR1/Mad3, but its MIM is dispensable for the interaction with preformed MCC$^{1Cdc20}$ (core MCC) [78]. Thus, the two Cdc20 subunits are incorporated in MCC through different interactions. It has been proposed that MCC$^{1Cdc20}$ encounters and inhibits the second Cdc20 subunit when the latter is already bound to the APC/C (APC/C$^{Cdc20}$, i.e., active APC/C)

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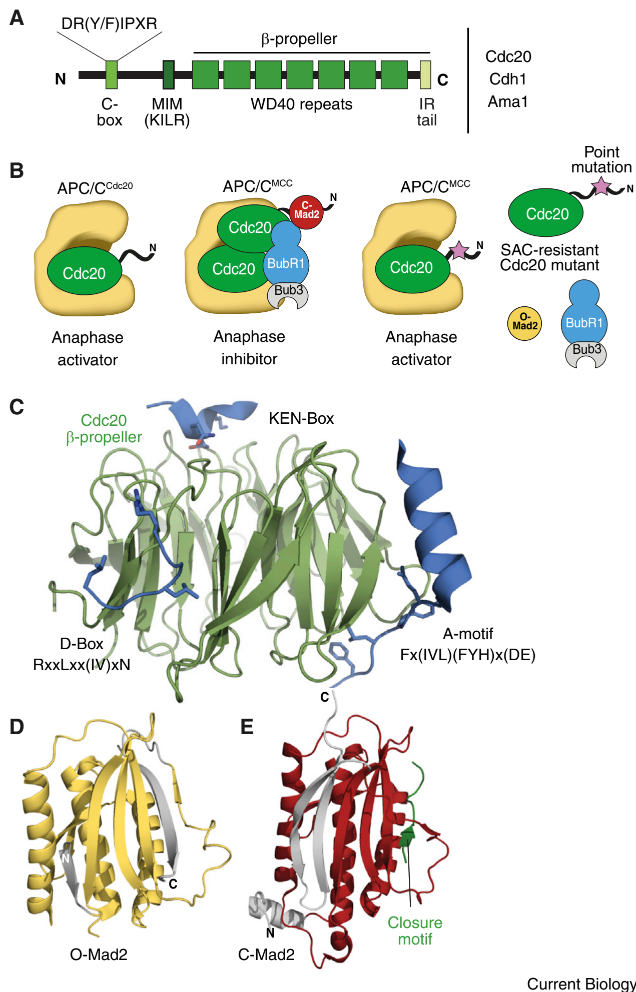


**Figure 4. Cdc20 and Mad2.**
(A) Domain organization of Cdc20. (B) The double life of Cdc20 and effects of a separation-of-function mutant [76]. (C) Cartoon model of the Cdc20 β-propeller with bound KEN- and D-boxes and A-motif. The cartoon is a composite obtained by superposition of SpCdc20 onto ScCdh1 bound to Acm1 (PDB ID 4BH6). (D) Cartoon model of O-Mad2 (PDB ID 2V64). The invariant part of the structure is shown in yellow, while the variable elements near the N- and C-termini are in grey. (E) Cartoon model of C-Mad2 (PDB ID 2V64), with the invariant structure shown in red and the restructured elements in grey. The closure motif has been embraced by the 'safety belt' encompassing the carboxy-terminal region of Mad2, which has repositioned away from its position in O-Mad2, replacing the amino-terminal strand of the latter [94,95,100,173].

(Figure 5A) [78]. This model, which is consistent with previous observations in reconstituted systems [105,111,112], is attractive because it suggests a way in which kinetochores, by promoting rapid assembly of MCC$^{1Cdc20}$, may exercise dominant control over active APC/C$^{Cdc20}$. *In vitro*, the first Cdc20 subunit enters a complex with Mad2 and BubR1 with higher affinity than the second [78]. This is consistent with the idea that MCC$^{1Cdc20}$ may be the primary product of SAC activation, but formal proof of this is currently missing. In summary, the current data support the idea that BubR1 interacts with Cdc20 in core MCC through KEN1, and with a second Cdc20 subunit through a combination of a D-box and KEN2 [11,78]. The order of subunit assembly in MCC, and the exact composition of MCC, are crucial for a molecular understanding of the APC/C inhibitory process, and require further investigations.

The function of the A-motif of BubR1 remains unclear. Its mutational inactivation, or deletions of the entire internal Cdc20 binding site of BubR1 in which the A-motif is embedded, has only mild negative effects on SAC robustness in cells [57,59,60,86]. *In vitro*, the A-motif of BubR1 is required for efficient inhibition of APC/C ubiquitylation activity when Mad2 is excluded from the assays, but not in its presence [57,60]. The A-motif of BubR1 has also been implicated in kinetochore recruitment of Cdc20 and in SAC silencing [57,60,93], but at least the role in Cdc20 recruitment is controversial, because a different study showed BubR1 to be dispensable for kinetochore recruitment of Cdc20 [59]. Other studies have identified Bub1 as the Cdc20 kinetochore receptor, and pointed to BubR1 as an ancillary factor in the Cdc20 recruitment mechanism [60,87]. Like BubR1, also Bub1 contains an A-motif, and the latter was shown to be necessary for Cdc20 recruitment to kinetochores [87]. Furthermore, the A-motif of Bub1 is also required for the (quite poorly understood) SAC functions of Bub1 [60,87].

## The MCC–APC/C Interaction

Recent developments in single-particle electron microscopy (EM) of the APC/C [113] raise hopes for high-resolution analyses that may clarify which segments of BubR1 and/or Cdc20 promote binding of MCC to the APC/C. The available EM reconstruction of APC/C$^{MCC}$ was carried out on a sample that, in view of the specific details of biochemical isolation, was likely to contain MCC$^{2Cdc20}$ [114]. Due to limited resolution, however, docking into this EM reconstruction of individual MCC subunits is merely tentative [108,114]. This uncertainty notwithstanding, comparison of the APC/C$^{MCC}$ reconstruction with reconstructions of APC/C:co-activator complexes [113,115] suggests that Cdc20 in MCC has to be dislodged from the position it normally occupies as an APC/C co-activator [108,114]. This result goes together with the observation that two Cdc20 motifs that mediate binding to APC/C in the catalytic cycle, the C-box and the IR tail, may be dispensable for binding of MCC to APC/C [96] (although it must be formally ascertained if this condition applies to both Cdc20 subunit in the APC/C$^{MCC}$ complex). Thus, if the core MCC bound to APC/C$^{Cdc20}$ to create APC/C$^{MCC-2Cdc20}$, it would be expected to force (at least partial) dissociation of the Cdc20 co-activator from APC/C$^{Cdc20}$ to allow its repositioning in the inhibited complex. Because the dissociation halftime of co-activator from APC/C is significantly slower than the time it takes the checkpoint to instate mitotic arrest [7,116], MCC may be able to accelerate co-activator dissociation and repositioning.

The amino-terminal region of Cdc20 has been implicated in allosteric catalytic activation of the APC/C [115,117–119]. By binding to the MIM in the amino-terminal region of Cdc20, Mad2 competes directly against the co-activator function of Cdc20 [96]. Artificially tightening the interaction of Mad2 with Cdc20 in *S. cerevisiae* sequesters Cdc20 away from APC/C and is sufficient for robust mitotic arrest even in the absence of Mad3. If free Cdc20 is present, however, the arrest is readily overridden, suggesting that MCC binding to the APC/C is ultimately crucial for checkpoint function [120]. Promoting the docking of MCC onto the APC/C may be one of three crucial functions of BubR1/Mad3, together with the cooperative stabilization of

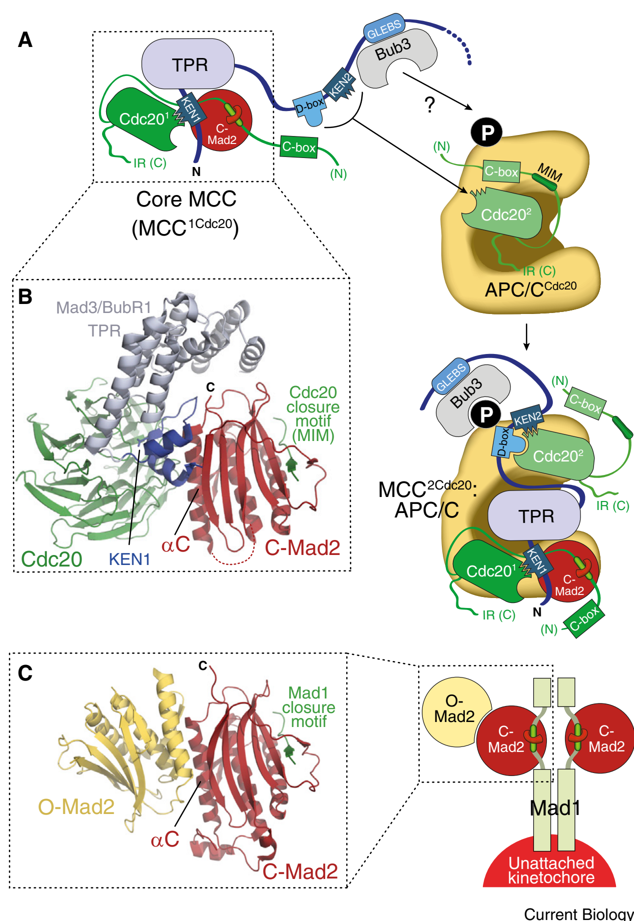


**Figure 5. The MCC.**

(A) Schematic representation of the MCC with one Cdc20 bound (called 'core MCC' or "MCC$^{1Cdc20}$"). BubR1 (blue tones) uses its N-terminal KEN1 box and the TPR (tetratricopeptide repeat) to interact with Cdc20 and C-Mad2 [108]. The latter uses its 'safety belt' to bind a region that is required for the interaction of Cdc20 with the APC/C. The interaction of this core MCC is expected to be cooperative because each of the three participating proteins makes contacts with the other two (see panel B). Carboxy-terminal to the TPR of BubR1 are a D-box and KEN2, both of which are required for binding a second molecule of Cdc20 [78]. The GLEBS sequence binds Bub3 [11]. Bub3 increases the binding affinity of the MCC for the APC/C [93]. In the figure, Bub3 is shown to bind a phosphorylated residue on the APC/C, but this is purely speculative. Additional functional sequences of BubR1 have been omitted for clarity, with the exception of the pseudo-kinase domain. If core MCC binds APC/C$^{Cdc20}$, two molecules of Cdc20 may bind on MCC-inhibited APC/C ("MCC$^{2Cdc20}$") [78]. A speculative view of this arrangement is shown. (B) Crystal structure of fission yeast MCC$^{core}$ (PDB ID 4AEZ) [108]. (C) Schematic view of the Mad1:C-Mad2:O-Mad2 complex (right) and crystal structure of the human O-Mad2:C-Mad2 conformational dimer (PDB ID 2V64). C-Mad2 in B and C is shown in the same orientation. Binding of C-Mad2 to O-Mad2 or BubR1:Cdc20 is mutually exclusive.

the interaction of Mad2 with Cdc20 in the core MCC through the HLL and TPR repeats, and the direct inhibition of Cdc20 as a pseudo-substrate through both KEN-boxes and other motifs. Furthermore, as already discussed, Bub3 may further increase the binding affinity of MCC for APC/C [11,51,93]. The mechanism for this is unknown, but in view of a role of Bub3 as a binding adaptor for phosphorylated motifs, it may be speculated that it involves phosphorylation of the APC/C.

## What Is the Real Identity of the SAC Effector?

As a summary, MCC appears to be a pseudo-substrate inhibitor of the APC/C (through BubR1) *and* an agent sequestering a segment of Cdc20 away from a direct stimulatory interaction with the APC/C (through C-Mad2), combined in a cooperative assembly of Cdc20, C-Mad2, and BubR1:Bub3 that binds directly to the APC/C (Figure 5). While these features of MCC are compatible with the possibility that MCC is the ultimate APC/C inhibitor, analysis of the stoichiometry of MCC subunits bound to the APC/C during SAC arrest or after reconstitution *in vitro* has provided an alternative interpretation. Specifically, analysis of APC/C demonstrated that Mad2 may be sub-stoichiometric with respect to Cdc20 and BubR1:Bub3 (which together form the BBC sub-complex of MCC) [110,112,121]. This observation has led to the suggestion that C-Mad2, in spite of its being required for BubR1:Cdc20 binding, may be dispensable for APC/C inhibition as an MCC subunit [86,110,112]. In this scheme of events, binding of C-Mad2 to Cdc20 was pictured as a catalytic step required for rapid assembly of the BBC (Figure 6A, left) [86].

When considering this model, it is important to remember that MCC forms spontaneously *in vitro* at low reactant concentrations, implying that it has the properties expected for a thermodynamically stable complex (to the point of crystallizing) [108]. If the only function of C-Mad2 was the catalytic generation of a Cdc20 intermediate in the pathway to forming BBC, this binding might be expected to be of relatively low affinity. Instead, Mad2 binds spontaneously to Cdc20 *in vitro* with a dissociation constant (~100 nM) that approximates the cellular concentrations of Mad2, one of the most abundant SAC proteins [75,95,122,123]. These are not the properties expected of a neutral catalytic agent but rather of a binding partner.

Furthermore, if C-Mad2 were only a catalytic activator for BBC formation, a crucial prediction is that the equilibrium concentrations of BBC in the presence or absence of Mad2 would be identical (Figure 6A, right). The model proposed by Han and colleagues [86] built on the observation that the BBC does not accumulate in the absence of Mad2. A crucial detail in the interpretation of this result is whether the measurements were carried out at equilibrium (when there is no net change of reaction product over time) or not. If the measurements were carried out at equilibrium (point T2 in Figure 6A), the model would be falsified, because the concentrations of BBC should be the same with or without the catalyst C-Mad2 at equilibrium. If instead the measurements were not carried out at equilibrium but rather at an arbitrary intermediate time point (T1 in Figure 6A), then the observation that less BBC accumulates in the absence of C-Mad2 is in principle compatible with a model picturing C-Mad2 as a catalyst, but in the absence of a measurement at equilibrium no meaningful conclusions can be drawn.

## The BBC

In conclusion, MCC is likely to be significantly more stable than its sub-complexes, Cdc20:C-Mad2 and the BBC (Figure 6B), and its stability might be further accrued after binding to APC/C (Figure 6C). This does not question the relevance of BBC, which is clearly found associated with the APC/C as a *bona fide* inhibitor, although probably of reduced potency in comparison to MCC [6,86,105,110], but raises the question of

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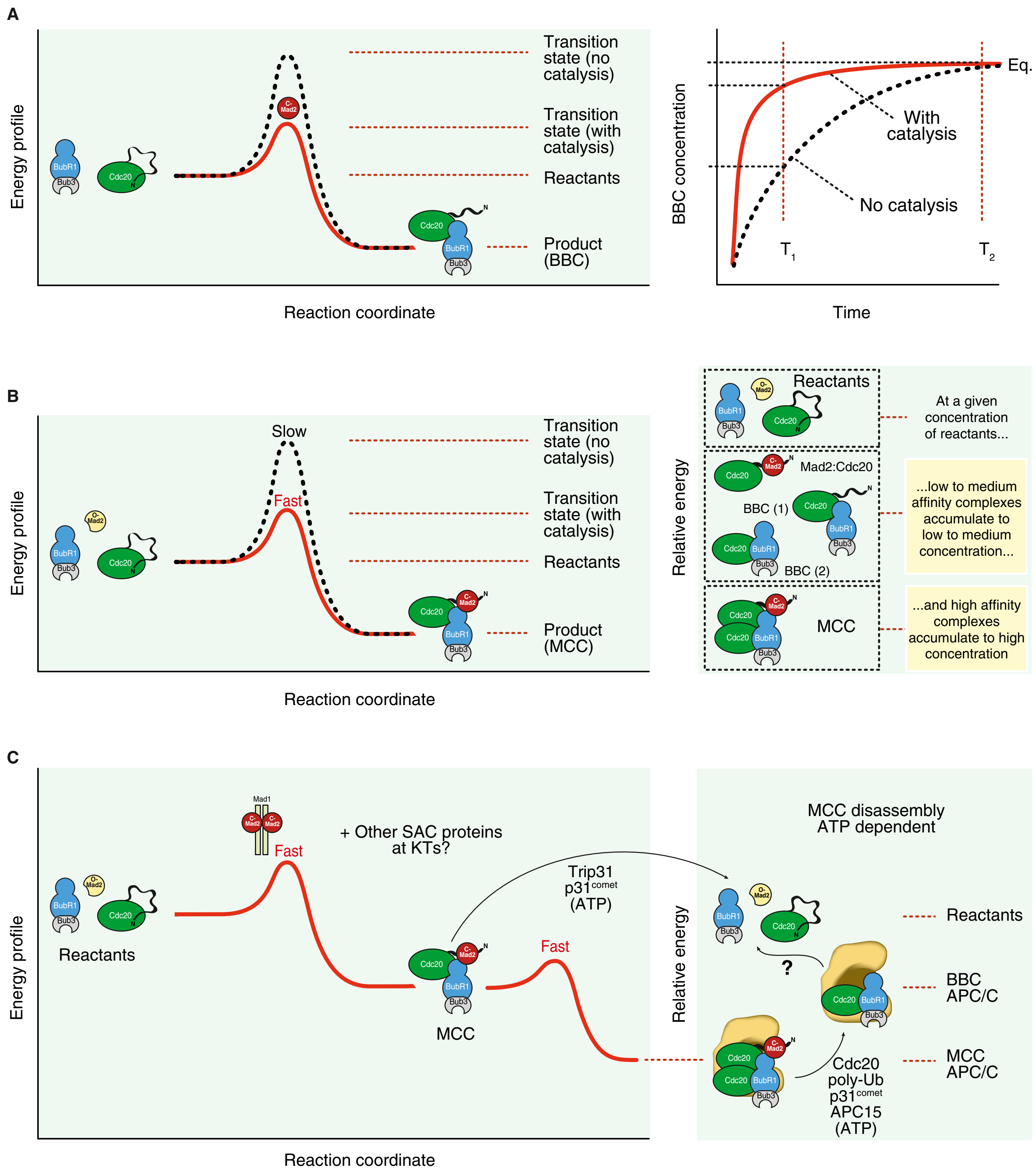


**Figure 6. Binding energy and binding kinetics.**
(A) It has been proposed that C-Mad2 catalytically promotes the interaction of Cdc20 with BubR1 [86], i.e., that it accelerates the accumulation of the Cdc20:BubR1 complex. The model predicts that the equilibrium concentration of Cdc20:BubR1 does not change, only the rate at which the equilibrium concentration is reached. At time T1, more Cdc20:BubR1 has formed with C-Mad2, but at time T2 both reactions have reached equilibrium and the concentrations of Cdc20:BubR1 are identical. Measuring at T1 is not sufficient to demonstrate that Mad2 is a catalyst, because a difference in the concentration of Cdc20:BubR1 when Mad2 is present or absent may be also due to Mad2 binding and stabilization of Cdc20:BubR1, not only to catalytic acceleration of the reaction. For a meaningful conclusion, a measurement at equilibrium is required. The model is falsified if less Cdc20:BubR1 is observed at equilibrium in the absence of Mad2.

*(legend continued on next page)*

the origin of BBC. The answer is that APC/C$^{BBC}$ likely originates from APC/C$^{MCC}$ after extraction of C-Mad2 and (probably of) the specific Cdc20 subunit that is bound to it (Cdc20$^{1}$ in Figure 5A).

At least two mechanisms appear to work on the dissociation of MCC (reviewed in [34], shown in Figure 6C). In one mechanism, the APC/C subunit APC15 and a protein named p31$^{comet}$ promote the APC/C-dependent ubiquitination and destruction of Cdc20 and the concomitant release of C-Mad2 from an APC/C$^{MCC}$ precursor [107,121,124,125]. Depletion of these proteins or addition of a proteasome inhibitor leads to accumulation of the APC/C$^{MCC}$ precursor and prevents the conversion of APC/C$^{MCC}$ to APC/C$^{BBC}$ [34]. This course of events, in which Cdc20 needs to be degraded for the rapid transformation of MCC into BBC, led to the hypothesis that there may be two molecules of Cdc20 in APC/C$^{MCC}$ [11,107]. Because during the transformation of APC/C$^{MCC}$ to APC/C$^{BBC}$, the degradation of Cdc20 occurs concomitantly with the release of C-Mad2, it is reasonable to assume that Cdc20$^{1}$ (bound to C-Mad2 in the core MCC) is the target of ubiquitin-dependent proteolysis. What remains on the APC/C, the BBC, may consist of BubR1: Bub3 bound to Cdc20$^{2}$ (Figure 6C). The reaction consumes ATP, which is not surprising when considering that if MCC is thermodynamically stable at the cellular concentrations of its constituents, it would accumulate on the APC/C unless it were actively forced to dissociate from it to keep its concentration away from equilibrium. Importantly, the transformation of MCC into BBC may facilitate checkpoint silencing, because BBC is likely to be significantly less stable than MCC and may dissociate more readily from the APC/C [121], but whether dissociation of BBC is spontaneous or rather requires an active process is currently unclear. The steady-state levels of APC/C$^{MCC}$ may be higher in cells with unattached kinetochores, while APC/C$^{BBC}$ may predominate in the final phases of attachment [121]. Interference with Cdc20 degradation by the APC/C in mitosis delays mitotic exit, and so does the artificial stabilization of the Cdc20:C-Mad2 interaction, in line with the notion that Mad2 contributes directly to APC/C inhibition and that the dissociation of Cdc20:C-Mad2 is required for APC/C re-activation [78].

In another, probably related mechanism, the triple A (AAA) ATPase Trip13, in conjunction with p31$^{comet}$, also contributes to MCC dissociation, possibly by focusing on the pool of MCC not bound to APC/C [102,108,126,127]. Interestingly, recent studies point to Trip13 as a HORMA-domain specific folding factor [128]. *In vitro*, Trip13 catalyzes the conversion of C-Mad2 to O-Mad2 [102,126]. Substrates for this mechanism may be the MCC itself, or its C-Mad2:Cdc20 sub-complex. Both mechanisms contribute to maintaining the steady-state condition shown in Figure 1B.

## The Mad2 Template Model and the Role of Catalysis in MCC Assembly

It might be useful at this point to propose an operational criterion to name a SAC protein 'catalyst' for assembly of MCC (or parts thereof). The SAC protein should, at sub-stoichiometric concentrations, influence the *rate constant* of MCC assembly but not the *equilibrium concentration* of the MCC, both of which ought to be measured. A catalytic function of any given SAC protein may, of course, co-exist with additional SAC-promoting functions, such as the addition of modifications that alter the equilibrium concentration of MCC by changing the affinity of the interaction of its individual subunits (e.g. phosphorylation sites mediating binding of interacting subunits), or that increase the rate of MCC formation by changing their local concentration.

As already clarified, a role of C-Mad2 as a catalytic activator of Cdc20 for BBC assembly may require further investigation. At this point in time, the only SAC protein formally passing the test as catalyst for MCC assembly is the Mad1:C-Mad2 complex [112,122]. The Mad1:C-Mad2 complex at kinetochores promotes the recruitment of O-Mad2 through dimerization with C-Mad2. This preludes the further 'processing' of O-Mad2 that leads to its conversion to C-Mad2 and to Cdc20 binding and incorporation in the MCC. The 'Mad2-template model' posits that the transient 'conformational' dimerization of O-Mad2 with a C-Mad2 'template' in the Mad1:C-Mad2 complex facilitates the conversion of O-Mad2 to a C-Mad2 'copy' bound to Cdc20 (Figure 7A) [99,129]. The Mad1:C-Mad2 is predicted to be a crucial determinant of the overall rate of MCC production during SAC activation, for the reason that the topological change of Mad2 entails large activation energies and is therefore extremely slow, to the point of being rate-limiting for the otherwise energetically downhill accumulation of MCC (Figure 6B,C) [122]. Removal of the Mad1:C-Mad2 template from kinetochores upon microtubule attachment, a function performed by a complex of the cytoplasmic motor dynein with the Rod–Zwilch–ZW10 (RZZ) complex and Spindly, is required for SAC silencing (Figure 7B) [130–135]. Forced retention of Mad1:C-Mad2 at kinetochores prevents SAC silencing even in cells that have an otherwise apparently normal metaphase plate, that is, cells that are not expected to engage the SAC [25–27].

## Elusive Catalysts

*In vitro*, Mad1:C-Mad2 accelerates binding of O-Mad2 to Cdc20 approximately by a factor of 10 and without influencing the equilibrium concentration of the Cdc20:C-Mad2 complex [112,122], thus satisfying the criterion discussed at the beginning of the previous section. This degree of acceleration of the rate of formation of Cdc20:C-Mad2, however, is deemed largely insufficient for the establishment of a rapid SAC response [122], suggesting that additional contributions to catalysis must exist. Identifying additional factors contributing to catalysis in MCC formation remains one of the crucial unresolved questions in the SAC field.

Recent studies in different model systems have focused on the significance for SAC activation of a direct physical interaction of Bub1:Bub3 with Mad1:C-Mad2, which is clearly discernible in *S. cerevisiae* and *Caenorhabditis elegans* but less so in *S. pombe*

(B) Likely MCC is a thermodynamically stable complex, more stable than its sub-complexes, but formal proof of this is required. BBC1 and BBC2 are two possible complexes of Cdc20 with BubR1:Bub3 engaging KEN1 or KEN2, respectively. (C) Mad1:C-Mad2 has been shown to accelerate a crucial conversion for the accumulation of MCC [112,122], the conversion of O-Mad2 to C-Mad2 for binding to Cdc20. Binding of MCC to APC/C may generate a very stable complex, whose disruption requires energy. The latter may be provided in the form of ATP-dependent reactions, such as ubiquitination and the subsequent destruction of one of the two Cdc20 subunits (probably the one bound to Mad2) [11], or directly by ATP-dependent disassembly through the Trip13 ATPase [102,108,126,127].

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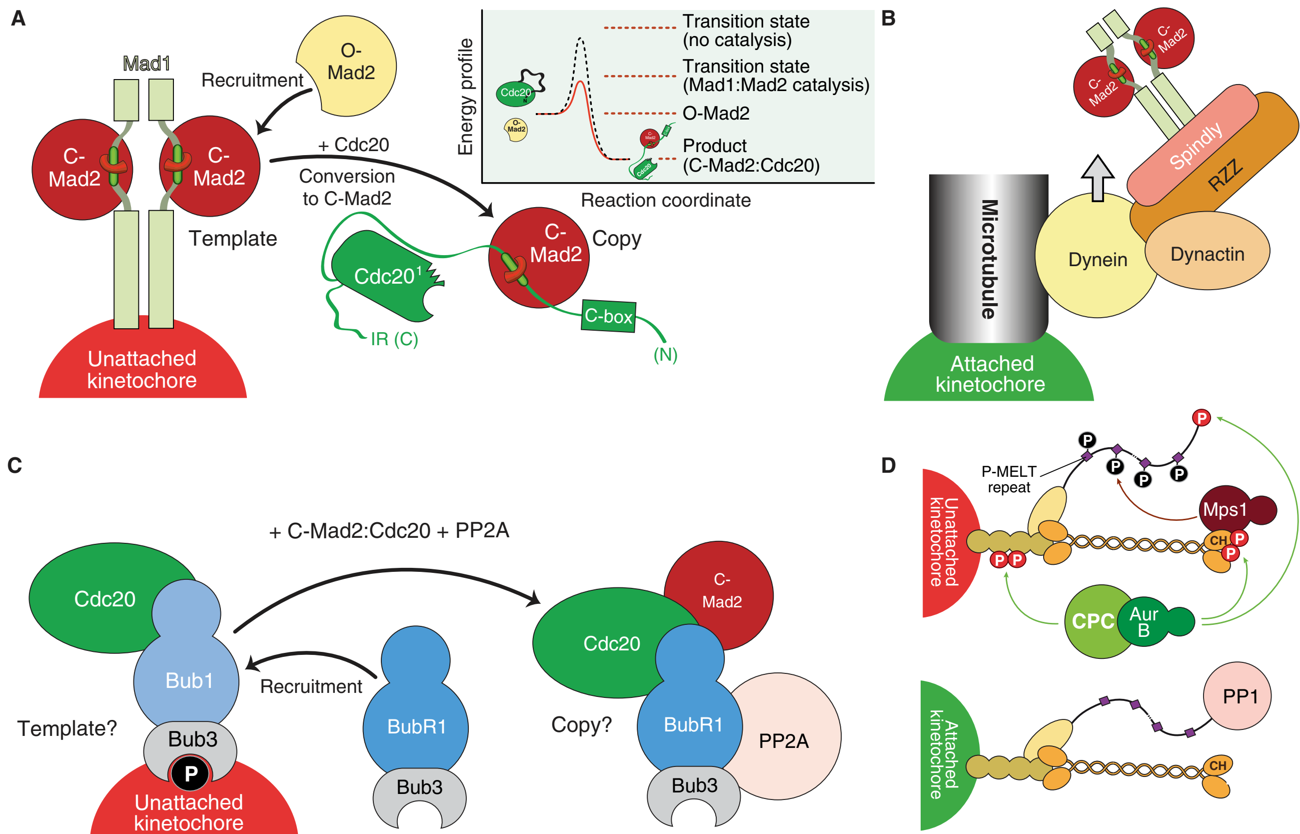


**Figure 7. Templates and silencing mechanisms.**

(A) Schematic representation of the Mad2 template model with its kinetic implications in the conversion of O-Mad2 to C-Mad2. (B) Dynein promotes tracking of RZZ–Spindly–Mad1:Mad2 complexes on microtubules away from kinetochores, silencing the SAC. (C) BubR1:Bub3 recruitment onto Bub1:Bub3 at kinetochores may be important for loading PP2A onto BubR1:Bub3, and possibly onto the entire MCC, to promote SAC silencing (in ways that require further analysis). (D) Aurora B suppresses the recruitment of PP1 to kinetochores by phosphorylating a PP1 docking site at the amino terminus of the Knl1 subunit of the KMN. Dephosphorylation of the site promotes recruitment of PP1 to facilitate SAC silencing. See main text for details.

or in human cells [30,31,52,55,61,136]. Bub1:Bub3 is a good candidate for a catalytic function in the SAC, because, as already clarified, it is required for kinetochore recruitment of BubR1: Bub3, at least in human cells [51,92,137]. It has been pointed out that there is a parallel linking the physical interactions of Bub1:Bub3 with BubR1:Bub3 and between Mad1:C-Mad2 with O-Mad2 [51]. In both cases, the former member of the pair acts as a kinetochore receptor for the latter, which ultimately enters MCC (Figure 7C). It is remarkable that in both cases this is achieved through pseudo-symmetric interactions — between paralogs for the Bub1:Bub3/BubR1:Bub3 pair, and between structural conformers for the Mad1:C-Mad2/O-Mad2 pair.

Despite these interesting considerations, pinpointing the exact role of Bub1 in the SAC response has proven experimentally difficult, not least because of an apparently crucial role of the penetrance of Bub1 depletion on the outcome of the experiments, with less than complete Bub1 depletions being compatible with SAC function (e.g. [21,138]), and more efficient Bub1 depletions abrogating SAC function [139–141]. At least in part, the role of Bub1 in the SAC may have to do with recruiting Cdc20 through the Bub1 A-motif [60,87], as already discussed.

## The Ambiguous Role of Bub3 between SAC Activation and Anaphase Onset

Conversely, it seems well established that kinetochore recruitment of BubR1:Bub3, one of the functions of Bub1:Bub3, is not strictly required for a robust SAC response. In at least two organisms, *C. elegans* and *S. cerevisiae*, Mad3 may not localize to kinetochores at all [142,143]. In human cells, the interaction of BubR1 with Bub3 is required for kinetochore localization and SAC function [51,85,93,105], but this does not imply that the BubR1 kinetochore-targeting function of Bub3 is necessary for the SAC, because alternative mutants of the BubR1:Bub3 complex impaired in Bub1 binding and kinetochore localization are checkpoint-proficient [51]. These observations may support the conclusion that kinetochore recruitment of BubR1:Bub3 is not important for SAC signaling. This conclusion, however, deserves a second thought in light of recent evidence suggesting that Bub3, Bub1 and BubR1 play unexpected functions in checkpoint silencing and anaphase onset (the latter independently of checkpoint signaling).

Specifically, Bub3 depletion in *S. pombe* was shown to cause a SAC silencing defect [144,145], and to restore the SAC defect

caused by mutation of all MELT repeats [50]. The discovery of this role of Bub3 as a SAC-silencing factor was facilitated by the fact that Bub3 is not part of MCC in *S. pombe* and is dispensable for SAC activity [145]. In other organisms, a SAC defect caused by inactivation of Bub3 would have probably partly obscured its function in SAC silencing (because with a defective SAC response, there is no SAC to silence), but there is evidence of an ongoing role of Bub3 in SAC silencing also in species where Bub3 is part of MCC. A mutant BubR1:Bub3 complex impaired in Bub1:Bub3 binding and kinetochore localization fails to interact with PP2A [51], a phosphatase required for efficient SAC silencing (as well as for kinetochore–microtubule attachment) [88–90,146–148]. Whether Bub1 contributes to loading PP2A on BubR1:Bub3 beyond its role in kinetochore recruitment of BubR1:Bub3 is currently unclear (Figure 7C). Recent studies also showed that Bub1 or Bub3 deletions in *C. elegans* or *S. cerevisiae* delay anaphase onset in a way that depends on kinetochores (but not on Bub1 kinase activity) and that cannot be bypassed by deletion of other SAC proteins, and therefore defined as 'SAC-independent' [149,150]. These interesting observations hint at a role of Bub3 and Bub1 in APC/C activation required for anaphase onset, possibly through a stabilization of Cdc20.

Thus, kinetochore recruitment of (at least a subset of) SAC proteins might affect not only SAC activation, but also SAC silencing or the timing of anaphase onset. In principle, the latter two effects have the potential to mask detrimental effects on the SAC from impaired kinetochore recruitment. As already discussed, a dual role of kinetochores in SAC activation, SAC silencing, and timing of anaphase onset may be crucial for maintaining responsiveness to the state of kinetochore–microtubule attachment and to promote rapid mitotic exit once proper attachments to microtubules have been established (Figure 1B). In addition to the role of PP2A at kinetochores, recent studies have also focused on PP1, which becomes recruited to a docking site in the amino-terminal region of Knl1 in a manner that is directly opposed by Aurora B kinase (Figure 7D) [151–158]. The combination of PP1 and PP2A at the kinetochore of bi-oriented chromosomes might be expected to wipe out existing phosphorylation, effectively terminating SAC signaling [159].

## Template Model: Further Proof Required

Initial proof of the Mad2 template model was built on separation of function mutants of Mad2 impaired in dimerization, conformational switching, or both [99,160,161]. Mutants locked as O-Mad2 are recruited normally to kinetochores even if they cannot interact with Mad1 or Cdc20. Conversely, dimerization mutants of Mad2 cannot be recruited to kinetochores [99]. These interactions were later reproduced *in vitro* with purified components [162].

Genetic evidence in support of the template model is that the dimerization mutants of Mad2 fail to support checkpoint function in human cells and in yeast [99,161]. The crystal structure of the MCC core [108], however, suggested that this genetic evidence requires re-examination. The reason is illustrated in Figure 5B,C. The C-Mad2 interface with O-Mad2 partially overlaps with the binding site for Mad3/BubR1 in core MCC. It follows that Mad2 dimerization and its incorporation into the MCC are mutually exclusive [108,163]. Thus, mutations originally believed to affect exclusively conformational dimerization of Mad2 may also undermine MCC stability, providing an alternative plausible explanation for their deleterious effects on the checkpoint.

The overlap in the interaction surfaces of Mad2 involved in dimerization and BubR1 binding is large but not complete, and at least some of the original mutants may pass the test as separation-of-function mutants that prevent Mad2 dimerization while allowing BubR1/Mad3 binding to C-Mad2 (Figure 5) [164]. Indeed, the structure of core MCC was obtained with a Mad2 mutant (Arg133 to Ala) originally identified for its ability to disrupt Mad2 dimerization and to cause a checkpoint defect [165]. This mutant may therefore be considered a separation of function mutant, but formal proof that it does not perturb the stability of core MCC is needed.

A second important implication of the competitive nature of the interactions of BubR1/Mad3 and O-Mad2 with C-Mad2 is that the conformational dimerization of Mad2 that occurs on the template (the Mad1:C-Mad2 complex) is suppressed on the copy (the Cdc20:C-Mad2 sub-complex of the MCC). This predicts that the template-based conversion of O-Mad2 to C-Mad2 occurring at kinetochores is unlikely to be amplified in the cytosol by the Cdc20:C-Mad2 complex, at least as long as it is part of MCC [164]

## The MCC in Interphase Generates a Timer for Early Mitosis

It was proposed over a decade ago that some of the SAC gene products may act not only as components of the checkpoint signaling pathways, but also as part of a kinetochore-independent mitotic timer determining the peak time of anaphase entry in the absence of kinetochore–microtubule attachment problems [5]. More recent observations suggest that the role of SAC components in determining the peak time of anaphase entry reflects events that take place in interphase, prior to mitotic entry [106,166]. The crucial observation is that the inactivation or depletion of checkpoint proteins results in severely reduced Cyclin B and Securin levels already in G2 [106,166,167]. APC/C undergoes Cdk1-dependent activation in mitosis [11], but the observed reduction of APC/C substrates in G2 suggests that in the absence of a functional checkpoint, APC/C may become partially active already in late G2, although evidently not to a level that would compromise mitotic entry. Indeed, that MCC begins to form already in interphase had been noted concomitantly with its discovery [75], and was later confirmed in several other studies [106,166,167]. The interphase levels of MCC, however, may be significantly lower than those present in mitosis [111].

Precocious APC/C activation before mitotic entry may provide a simple explanation for why the peak timing of anaphase exit is different in cells with a functional or dysfunctional checkpoint pathway. Cyclin B degradation takes place for the entire duration of prometaphase, even during SAC activation [6,168], albeit at a low rate in comparison to the rates post SAC satisfaction. Cells entering mitosis with substantially reduced levels of Cyclin B are likely to leave mitosis ahead of time in comparison to cells that enter mitosis with normal levels of Cyclin B, because the threshold levels of Cyclin B required to begin anaphase will be reached faster in the former than in the latter. Even when the checkpoint is completely inactive, mitotic exit does not occur

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before 10–14 minutes after entry into mitosis (iconized by nuclear envelope breakdown, NEBD). This provides a minimal estimate for the time required to degrade Cyclin B until the threshold required for mitotic exit is reached [5,6,166]. In cells with an active checkpoint, this time would be more than sufficient for a single unattached kinetochore to instate robust checkpoint arrest after NEBD [7]. Thus, a role of the SAC in protecting Cyclin B from being degraded prior to NEBD may be especially important for cells that are endowed with very robust and errorless kinetochore–microtubule attachment mechanisms, and which are therefore expected to activate the SAC only weakly, if at all, in early mitosis [166].

Kinetochores are unlikely to be involved in the production of MCC in interphase, as they are partly disassembled and do not host SAC proteins at this time. It has been proposed recently that nuclear pores play a crucial role in the production of MCC in interphase by localizing there the Mad1:C-Mad2 complex [166]. Mad1:C-Mad2 at nuclear pores is capped by p31$^{comet}$ and may not be able to interact freely with O-Mad2 [169]. Evidently, inhibition by p31$^{comet}$ must be incomplete and Mad1:C-Mad2 can deliver at least part of its activity as a catalyst for MCC production. Depletions of BubR1 and Mps1 also have negative effects on the levels of interphase MCC [106,167]. How interphase MCC is produced is clearly a matter of interest for future studies.

## A Small Calculation and Final Considerations

The analysis of interaction kinetics and thermodynamics of SAC protein is crucial to understand the SAC and to identify missing links. The analysis of Mad2 dynamics, for instance, has been particularly instructive. Mad2 turns over at kinetochores with halftimes ($t_{1/2}$) of between 6 and 20 seconds [19,170]. As there are $\sim$1000 Mad2 binding sites at kinetochores of a somatic vertebrate cell [171], $\sim$3000 Mad2 molecules may be expected to turn over on a single kinetochore every minute (assuming $t_{1/2}$ = 10 seconds). It takes approximately 3–4 minutes to instate the checkpoint after Mad2 has been recruited onto an unattached kinetochore [7], and given the estimated flux at kinetochores, we can expect up to 9000–12000 Mad2 molecules to be incorporated into MCC at each kinetochore during 3–4 minutes if the conversion efficiency is 100% (a highly optimistic assumption). Assuming a cell volume of 2 pL, the concentration of 9000 molecules of MCC would be $\sim$7.5 nM, approximately one order of magnitude lower than the estimated concentration of APC/C [75,83]. In line with a published prediction [172], these 'back-of-the-envelope' calculations may be taken as an indication that details are overwhelmingly important for a quantitative description of checkpoint function. Do kinetochores increase the rate of MCC formation beyond what is provided by the Mad1:C-Mad2 template? And if so, how? Are there catalytic steps acting in the cytosol, in addition to those taking place at kinetochores? The concentration of the SAC proteins at kinetochores, the at least partial suppression of pathways that silence the checkpoint, and direct catalytic steps, including steps harnessing energy, are all likely to be important to some extent, but we still need to build a comprehensive understanding of the relative weights of these processes. Excellent progress towards a quantitative description of these processes has been recently made, and future strides will finally bring to a quantitative description of the SAC network.

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