Current Biology 22, R966–R980, November 20, 2012 ©2012 Elsevier Ltd All rights reserved http://dx.doi.org/10.1016/j.cub.2012.10.006

The Spindle Assembly Checkpoint

Review

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During mitosis and meiosis, the spindle assembly checkpoint acts to maintain genome stability by delaying cell division until accurate chromosome segregation can be guaranteed. Accuracy requires that chromosomes become correctly attached to the microtubule spindle apparatus via their kinetochores. When not correctly attached to the spindle, kinetochores activate the spindle assembly checkpoint network, which in turn blocks cell cycle progression. Once all kinetochores become stably attached to the spindle, the checkpoint is inactivated, which alleviates the cell cycle block and thus allows chromosome segregation and cell division to proceed. Here we review recent progress in our understanding of how the checkpoint signal is generated, how it blocks cell cycle progression and how it is extinguished.

Introduction — Challenges Faced by Eukaryotes

The survival of all organisms requires the production of genetically identical daughter cells through a process known as cell division. To ensure genetic identity, the genome must be replicated and segregated prior to the actual division process. In archaea and bacteria, organisms whose genomes are typically contained in a single circular chromosome, replication and segregation are coupled; once DNA replication has been initiated at the single origin, the two genomes start migrating to opposite ends of the cell while the remainder of the chromosome is duplicated [1]. One advantage of this strategy is that DNA replication can be re-initiated before cell division is complete; thus, daughters inherit a chromosome that has already initiated a new round of DNA replication (Figure 1A). Consequently, under ideal conditions, *Escherichia coli* can grow with a doubling time of about 20 minutes, considerably faster than the ~33 minutes it takes to complete one round of chromosome replication.

The situation in eukaryotes is rather different. In contrast to asexual reproduction exhibited by prokaryotes, the sexual reproduction strategy employed by eukaryotes means that the latter typically have at least two chromosomes, one from each parent. Moreover, the genome is normally segmented into sets of linear chromosomes (Figure 1B), with humans, for example, having two sets of 23. In addition, the architecture of the eukaryotic cell cycle is fundamentally different; genome replication and segregation are uncoupled, defining separate S and M phases (DNA synthesis and mitosis, respectively), and these are often separated by growth or gap phases (Figure 1B). Consequently, eukaryotes face a unique challenge in the effort to ensure accurate genome transmission. Specifically, during M phase, the array of chromosomes must be segregated so that each daughter inherits a complete complement: mistakes at this point in the cell cycle can result in daughters with deviations from the normal karyotype, which in turn can result in a loss of fitness or, in the case of multicellular organisms, various diseases [2].

The solution to this challenge is sister chromatid cohesion, mediated by cohesin, a multimeric protein ring structure that encircles the replicated sister chromatids [3]. Following DNA replication in S phase, original and new chromatids are held together by cohesin, thus maintaining their identity as sisters. Cohesion is maintained throughout the rest of S phase, G2 and into early mitosis where the chromosomes align at the centre of the cell on the microtubule spindle apparatus. At anaphase onset, the cohesin ring is opened, chromatid cohesion is lost and the sisters separate, allowing spindle forces to pull them to opposite sides of the cell (Figure 2). The solution provided by cohesion, however, presents a new challenge: to ensure accurate chromosome segregation, sister chromatid cohesion must be maintained until all the chromosomes are correctly aligned on the spindle.

This challenge is solved by the spindle assembly checkpoint (SAC, sometimes referred to as the 'mitotic checkpoint' or 'M-phase checkpoint'), a quality control mechanism that prevents anaphase until all the chromosomes are stably attached to the spindle [4–6]. Note that while the term 'spindle assembly checkpoint' is firmly entrenched, it is a misnomer; the SAC does not monitor spindle assembly *per se* but rather the status of kinetochore–microtubule attachment. In the presence of unattached kinetochores the SAC is 'on' and anaphase is inhibited. The SAC only becomes satisfied when all kinetochores are stably bound to microtubules; inhibition of anaphase is then alleviated and the cell cycle can continue. This review will focus on three questions. Firstly, how do unattached kinetochores generate the on signal? Secondly, how does the SAC inhibit anaphase onset? And, thirdly, how does kinetochore–microtubule attachment satisfy the SAC and extinguish the on signal?

Discovery of the SAC Network

Following pioneering work leading to the concept of cell cycle checkpoints [7], the SAC was discovered by genetic screens in the budding yeast *Saccharomyces cerevisiae* [8,9] which identified most of the key components, namely Mad1, Mad2, Mad3, Bub1 and Bub3. Subsequently, other SAC components such as Mps1 [10] were identified. What the SAC was monitoring was initially unclear, but elegant experiments in higher eukaryotic cells focused attention on kinetochores (Figure 3). In particular, micromanipulation and laser ablation experiments led to the notion that unattached kinetochores generated an inhibitory signal that delays anaphase onset [11,12]. Indeed, discovery of vertebrate SAC components showed that they all localised to unattached kinetochores, and it is now widely accepted that the SAC on signal is generated exclusively by events at kinetochores.

The downstream target of the SAC is the anaphase promoting complex, or cyclosome (APC/C), an E3 ubiquitin ligase that targets several proteins for proteolytic degradation, including mitotic cyclins [13]. The APC/C, which ubiquitylates proteins containing D-box and/or KEN-box degrons, requires activation by one of two co-factors, Cdc20 or Cdh1. The discovery that Mad2 binds Cdc20 linked unattached

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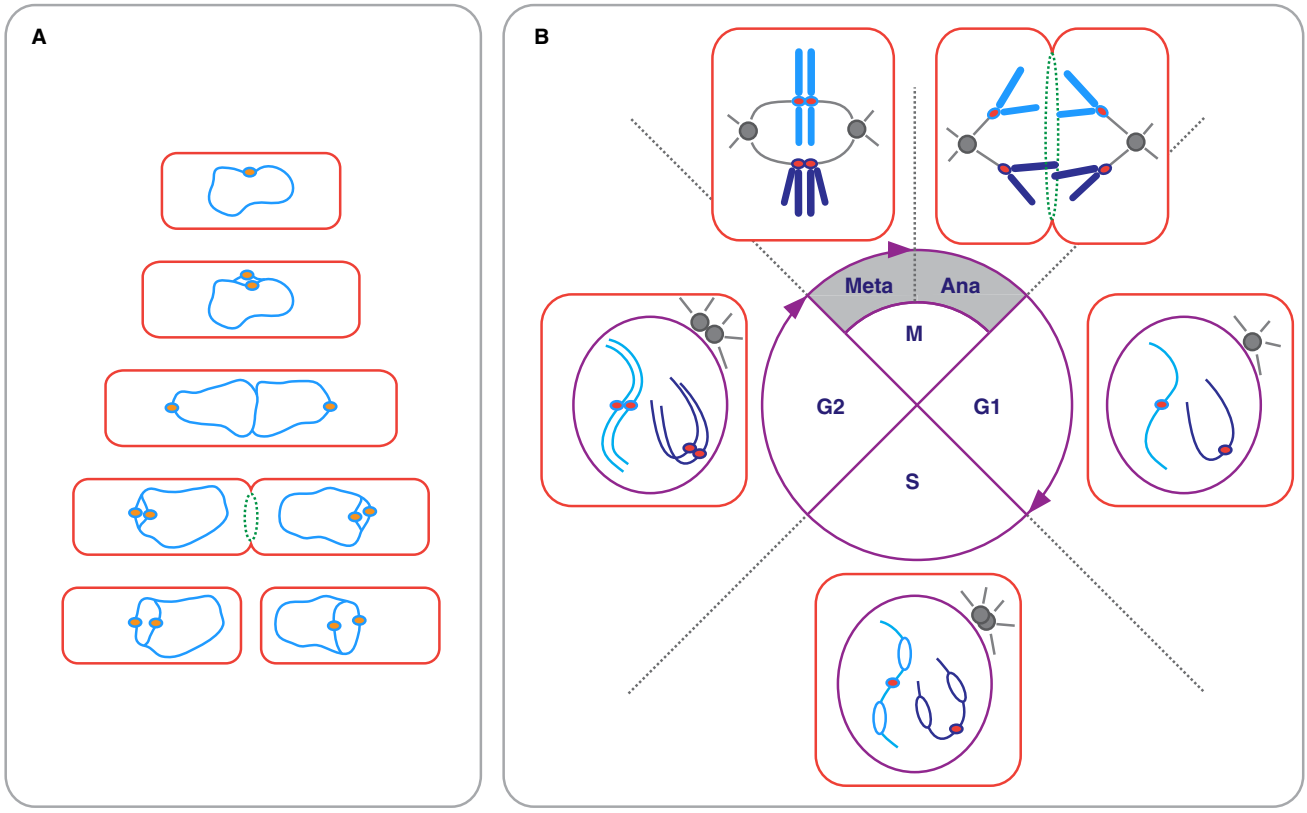

Figure 1. Fundamentals of chromosome segregation.

(A) Archaea and bacteria typically have a single circular chromosome (light blue) whose replication and segregation are coupled; once DNA replication initiates at the single origin (orange circle), the two genomes migrate to opposite ends of the cell while the remainder of the chromosome is duplicated. When DNA replication is complete, the cytokinetic ring (green) divides the cell. Under optimal growth conditions, the next round of DNA replication can re-initiate before the completion of cell division. (B) Eukaryotes have segmented genomes represented by sets of linear chromosomes (light and dark blue). Somatic cell cycles are subject to several quality control mechanisms, known as cell cycle checkpoints (purple arrow heads). In G1, each chromosome is represented by a single chromatid. DNA replication in S phase initiates at multiple origins and must be completed before progression into mitosis. The microtubule organising centre (grey circle) also duplicates during S phase. Throughout S phase and G2, cohesion keeps the replicated sister chromatids together. In mitosis, the chromosomes condense, the nuclear envelope (purple) breaks down and microtubules (grey) assemble a bipolar spindle apparatus. Chromosomes attach to microtubules via their kinetochores (red circles), with sister kinetochores attaching to opposite spindle poles. Metaphase is defined as the point when all the chromosomes are correctly aligned at the spindle equator. Loss of cohesion, which marks the onset of anaphase, allows the chromatids to be pulled to opposite sides of the spindle. Cytokinesis, mediated by a contractile ring (green) divides the cytoplasm. (In contrast to the situation in prokaryotes, eukaryotic genomes are physically separated from the cell's cytoplasm by a nuclear membrane, which only breaks down during mitosis to facilitate chromosome segregation. Note, however, that fungi — including the model organisms *S. cerevisiae* and *S. pombe* — adopt a closed mitosis whereby the nuclear envelope remains intact for the entire cell cycle. Note also that many eukaryotes, including certain algae and fungi, live predominantly as haploids.

kinetochores with cell cycle regulation [14,15]. The notion that Mad2 is an important APC/C inhibitor was then confirmed by several observations. Firstly, it was demonstrated that Mad2 can inhibit APC/C^{Cdc20} *in vitro* [16,17]. Secondly, purification of an APC/C inhibitor from HeLa cells identified the mitotic checkpoint complex (MCC) comprising Mad2, BubR1, Bub3 and Cdc20 [18], with BubR1 later shown to potentiate Mad2's ability to inhibit APC/C activity [19,20]. (Note: BubR1 is the vertebrate homolog of Mad3 but has a carboxy-terminal kinase domain not present in fungi.) The MCC is currently regarded as the predominant APC/C inhibitor generated by the SAC.

Identification of securin as an APC/C substrate and elucidation of the securin–separase–cohesin pathway [3] then provided a framework describing how the SAC regulated anaphase onset and mitotic exit (Figure 2). Since then, the key questions outlined above have focused in on how the SAC module is assembled at kinetochores, how this module generates the MCC and how the MCC inhibits the APC/C. And, from the standpoint of SAC inactivation, key remaining problems include how microtubule binding inhibits further MCC production at kinetochores and how existing APC/C inhibitory complexes are inactivated.

Other proteins have been implicated in SAC function, including Rae1, Nup98, Prp4, Tao1 and PICH, RASSF1A and RED. However, it is now clear that Tao1 and PICH are not SAC components [21,22], and a recent genome-wide RNAi library screen that identified all the established SAC proteins did not reveal Rae1, Nup98, Prp4, RASSF1A or RED (Topham and Taylor, unpublished), so to maintain focus we will not consider these further. Several SAC proteins are also required for chromosome alignment, but again, to

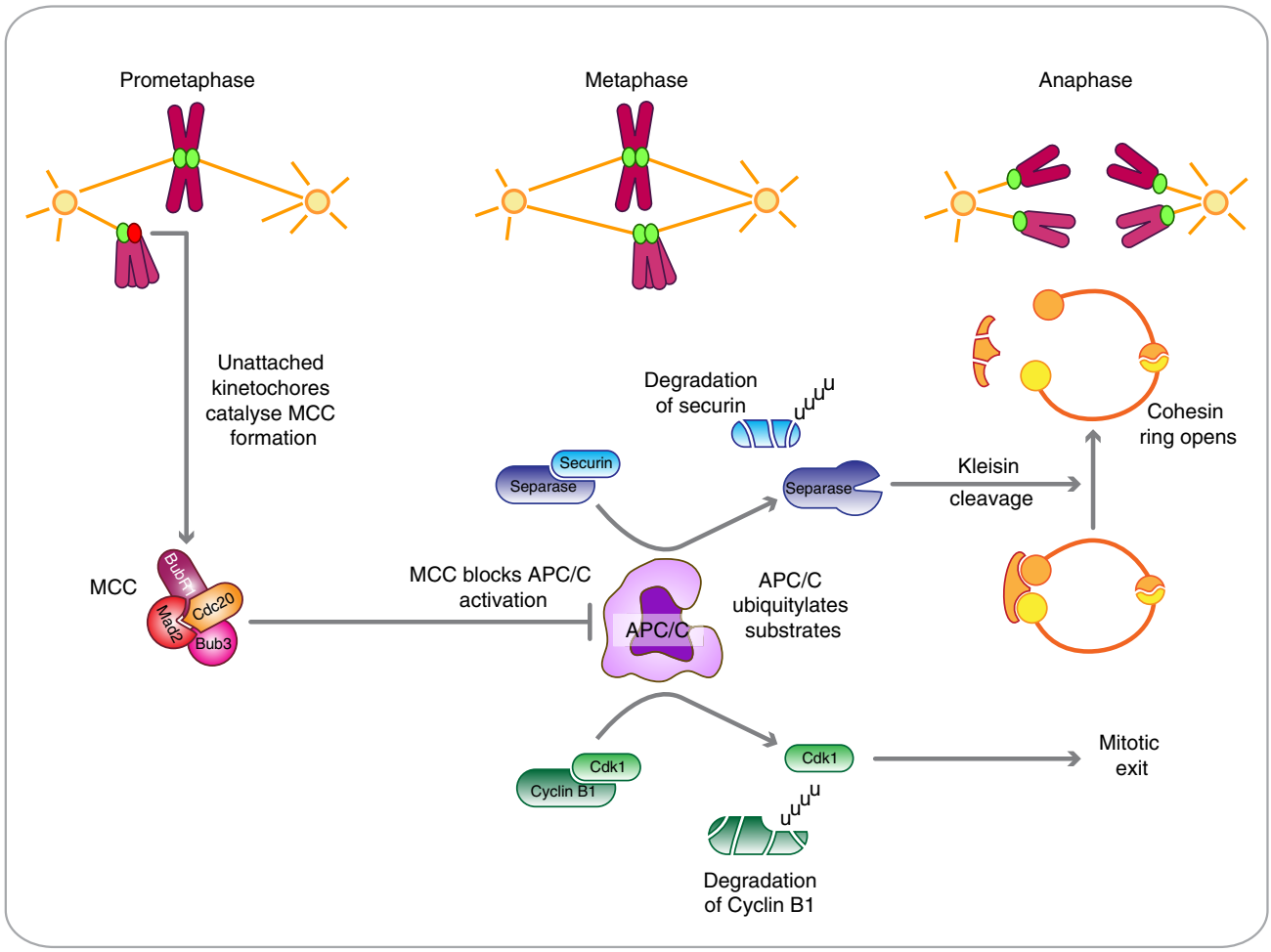

Figure 2. SAC principles.

During the early stages of mitosis (prometaphase), unattached kinetochores catalyse the formation of the mitotic checkpoint complex (MCC) composed of BubR1, Bub3, Mad2 and Cdc20, leading to inhibition of the APC/C. Once all the chromosomes are aligned with their kinetochores attached to the spindle (metaphase), generation of the MCC ceases, allowing Cdc20 to activate the APC/C, leading to the ubiquitylation and degradation of securin and cyclin B1. Degradation of securin liberates separase which in turn cleaves the Scc1 kleisin subunit of the cohesin ring structure; this opens the ring, allowing sister chromatids to separate (anaphase). Meanwhile, degradation of cyclin B1 inactivates Cdk1, leading to mitotic exit.

maintain focus, here we will only discuss mechanisms regulating anaphase.

How Do Unattached Kinetochores Generate the 'On' Signal?

Kinetochores are complex structures comprising at least 80 different proteins assembled at the centromere of each sister chromatid [23,24] (Figure 4A,B). Centromeric chromatin is specified by nucleosomes with the histone H3 variant CENP-A [25], which recruits the constitutive centromere-associated network (CCAN), a complex of at least 16 different proteins [23,24]. As cells enter mitosis, the CCAN assembles the KMN network, consisting of the KNL1, Mis12 and Ndc80 sub-complexes (Figure 4C), thus forming the outer kinetochore [23,24]. Importantly, the Ndc80 complex, comprising Ndc80/Hec1, Nuf2, Spc24 and Spc25, is essential for load-bearing attachments to microtubules (reviewed in [26]). Other kinetochore proteins regulating microtubule attachment include the RZZ complex comprising Zw10, Rod and Zwilch, which is recruited by Ndc80 and in turn recruits the dynein/dynactin minus-end motor complex [27]. As detailed below, RZZ is essential for recruitment of Mad1 and Mad2 [27] (Figure 4C), and thus RZZ is often classified as a SAC component. An important regulator of kinetochore assembly is Aurora B, the protein kinase component of the chromosome passenger complex (CPC) that also includes INCENP, Borealin and Survivin. Aurora B and the CPC have been extensively reviewed [28,29] so they will not be discussed here.

Exactly how the SAC module is assembled is unclear. SAC proteins are recruited to kinetochores in a step-wise fashion, with Bub1, an essential SAC kinase [9,30,31], binding in early prophase followed by the others [32–35]. Moreover, Bub1 is required to recruit the majority of downstream SAC components, including BubR1, Bub3, Mad1 and Mad2 [30,31,33–37]. This suggests that Bub1 links the SAC module to the outer kinetochore, so understanding exactly how Bub1 binds the kinetochore is an important question.

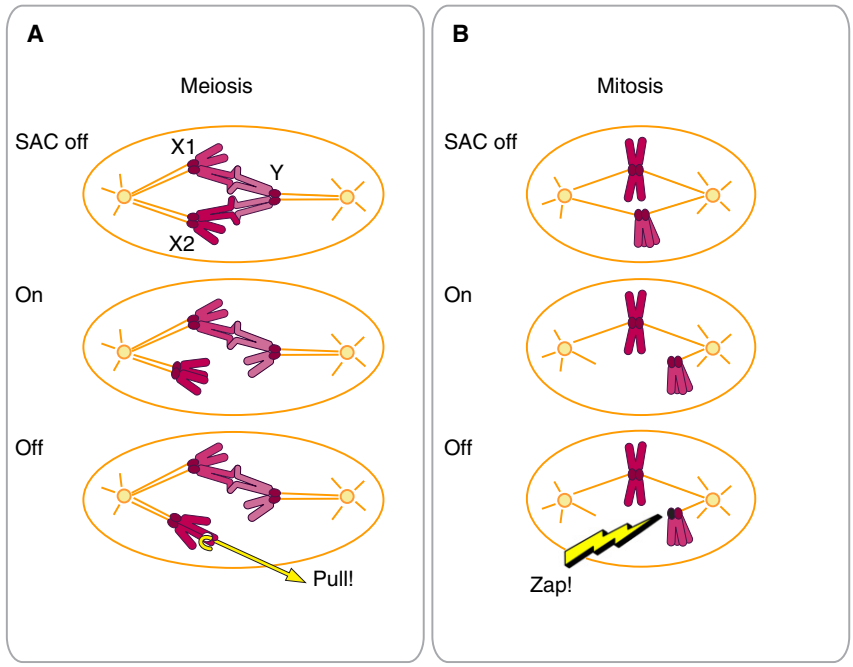

Figure 3. Kinetochores regulate anaphase onset.

Summary of experiments showing that in both meiosis [12] and mitosis [11], unattached kinetochores delay anaphase. (A) Praying mantis spermatocytes contain three sex chromosomes, X_1, X_2 and a Y, which in meiosis I are normally paired to form a trivalent. This ensures that following anaphase, the daughter cells inherit either both X chromosomes or the Y. Occasionally, cohesion between one of the X chromosomes and the Y is lost prematurely. Because the X could now segregate with the Y, this represents a potential genetic disaster. To avoid this, the presence of an unpaired X delays anaphase for many hours, presumably due to persistent SAC activation, and eventually the spermatocyte undergoes apoptosis. However, when an unpaired X was artificially manipulated using a micromanipulation needle such that the attached kinetochores came under tension, the spermatocyte underwent anaphase [12]. The current interpretation of this experiment is that placing the kinetochores on the unpaired X under tension stabilised microtubule binding and therefore satisfied the SAC. (B) Similarly, in mitotic PtK1 cells, derived from rat kangaroo, the presence of a single unattached kinetochore can delay anaphase for many hours [156]. However, when the last unattached kinetochore was destroyed using a laser microbeam, anaphase initiated on time [11]. The interpretation of this experiment is that the lack of microtubule attachment allows kinetochores to generate the SAC 'on' signal.

Bub1 and the Role of KNL1

Kinetochore localisation of Bub1 is mediated by a region in its amino-terminal half [38]. Bub3 also binds this region and early analysis showed that Bub1's kinetochore localisation domain and Bub3-binding site are synonymous, residing downstream of a conserved domain containing tetratricopeptide repeats (TPRs) [39] (Figure 4D). Because Bub1 is also required for kinetochore recruitment of Bub3 [30,33,36,39,40], these two proteins may bind the kinetochore as a complex. The Bub3-binding site was also shown to be essential for BubR1's ability to bind kinetochores [39]. However, fluorescence recovery after photo-bleaching (FRAP) studies showed that while Bub3 and BubR1 cycle rapidly at kinetochores, Bub1 is a more stable component [34,41]. This raises the possibility that other mechanisms might contribute to kinetochore localisation of Bub1.

Recently, the KMN network protein KNL1 (also known as Blinkin, AF15q14, CASC5, Spc7 and Spc105) was suggested to be one such mechanism, as depletion of KNL1 abolishes kinetochore localisation of both Bub1 and BubR1 [42–46]. Moreover, KNL1's amino-terminal "KI" motifs were shown to directly interact with the TPR domains of both Bub1 and BubR1 [45,47]. In addition, mutating the TPR domains not only prevented KNL1 binding but also rendered Bub1 and BubR1 defective in terms of their abilities to support chromosome alignment and SAC function, respectively [45]. These observations led to the notion that kinetochore localisation of Bub1 and BubR1 is mediated via direct interactions between their TPR domains and KNL1 [45,48] (Figure 4D).

More recently, crystal structures of the Bub1–KNL1 and BubR1–KNL1 complexes have been solved, identifying residues that participate in the interactions [49,50]. Crucially, mutating amino acids in Bub1 or BubR1 that abolish KNL1 binding did not significantly affect their kinetochore localisation [49,50]. By contrast, mutating the Bub3 binding site had a dramatic effect [50]. Thus, while KNL1 is required for kinetochore targeting of Bub1 and BubR1, this function is not mediated via the direct interaction between their TPR domains and KNL1. This distinction is important; for example, when a Bub1 mutant lacking the TPR domain was shown to restore SAC function following Bub1 RNAi, it was concluded that Bub1's role was kinetochore-independent [51]. However, the Bub3-binding site remained intact, so this mutant is fully expected to bind kinetochores, therefore negating the 'kinetochore-independent' conclusion. The role of the KNL1–BubR1 interaction is also less clear now as the integrity of the TPR domain is required for MCC assembly, independently of KNL1 binding [52,53].

Despite these complexities, it is possible that KNL1 recruits Bub1 to kinetochores through a different mechanism. For instance, in yeast and *Caenorhabditis elegans*, KNL1 is required for Bub1 recruitment, despite the fact that in these organisms the KI motifs of KNL1 are not conserved [42–44,46,47]. Moreover, recent studies showed that Bub1 recruitment in yeast depends on phosphorylation of KNL1's 'MELT' motifs by Mps1 [42–44] (Figure 4D). However, the universality of this model is unclear as there are conflicting reports regarding whether Mps1 is required for Bub1 recruitment in human cells [54–58]. Interestingly, *Schizosaccharomyces pombe* Bub1 only binds phosphorylated KNL1 *in vitro* in the presence of Bub3 [42], consistent with the notion that Bub1 and Bub3 bind the kinetochore as a complex [39]. Clearly, further dissecting the relationship between Bub1–Bub3, KNL1 and Mps1 is important, as Bub1 mutants incapable of kinetochore binding cannot promote SAC function [51,59].

The Mad2 Template Model

Our understanding of how unattached kinetochores generate the MCC has been helped by formulation of the 'Mad2 template model' (Figure 5). Mad2 was the first SAC

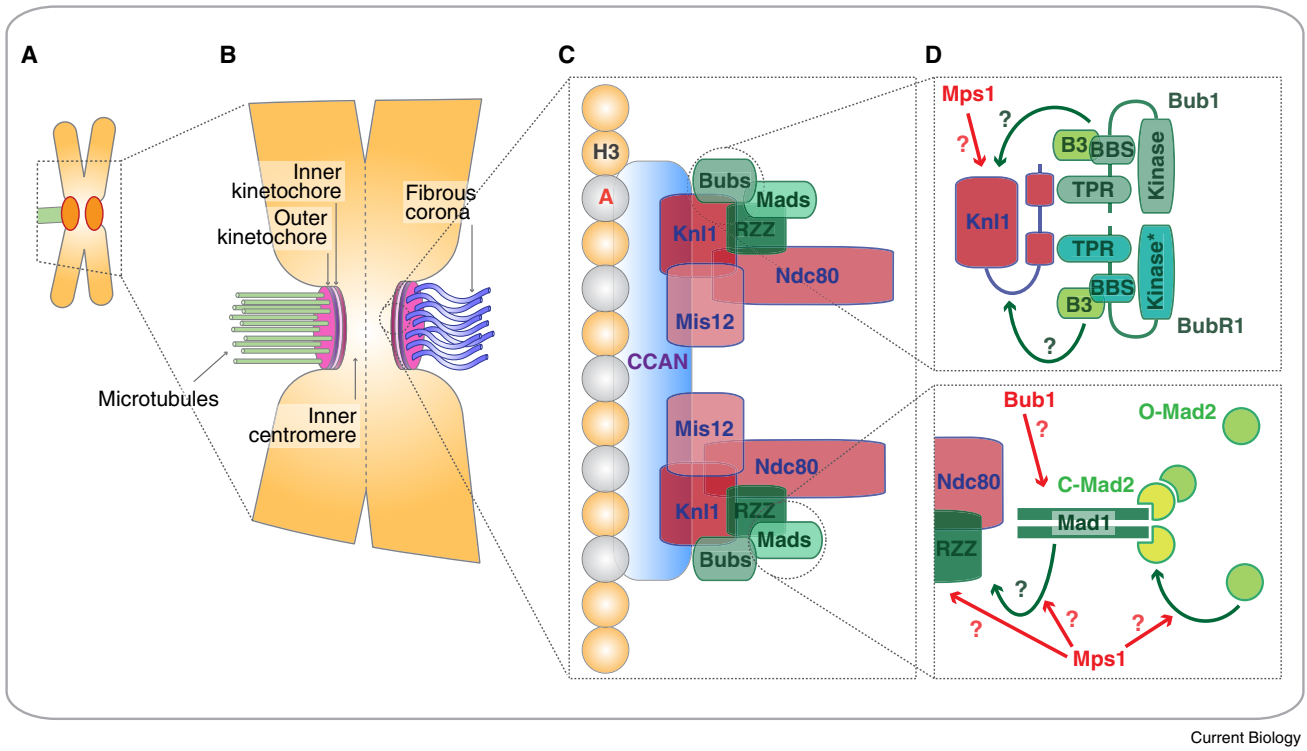

Figure 4. Kinetochore structure.

(A) Schematic of a single chromosome with one attached and one unattached kinetochore. (B) Schematic of kinetochore ultrastructure showing sister kinetochores, composed of inner and outer regions, assembled on the inner centromere. The unattached kinetochore has a fibrous corona. (C) Simplified schematic of the vertebrate kinetochore's molecular architecture, showing an array of histone H3 and Cenp-A containing nucleosomes, which recruits the constitutive centromere-associated network (CCAN). The CCAN directs assembly of the KMN network comprising the KNL1, Mis12 and Ndc80 subcomplexes. The KNL1 subcomplex in turn recruits SAC components, either directly as in the case of Bub1/Bub3 or via RZZ/Ndc80 in the case of the Mad1/Mad2. Note that vertebrate kinetochores are modular in nature and for simplicity only two units are shown here. (D) Models of Bub and Mad kinetochore recruitment. Bub1 and BubR1 have similar domain structures, comprising an amino-terminal TPR domain, a Bub3-binding site (BBS) and a carboxy-terminal kinase domain. *Note that BubR1's kinase domain is not catalytically active. While the TPR domains bind directly to KNL1, kinetochore targeting of Bub1 and BubR1 depends on their interactions with Bub3. Kinetochore localisation of the Mad1–C-Mad2 core complex is via the RZZ and Ndc80 complexes and it is promoted by Mps1 and Bub1. O-Mad2 is then recruited to the kinetochore via dimerisation with C-Mad2 and Mps1 also participates here. The exact mechanism of Bub1 and BubR1 recruitment and the role of Mps1 remain to be determined.

component shown to be conserved in vertebrate cells [60,61] and has thus received considerable attention, with structural studies and FRAP experiments being particularly informative [5,62,63]. In brief, Mad2 adopts two distinct conformations; when unbound, it adopts an open conformation (O-Mad2) but upon binding to Mad1 (or Cdc20), two β-sheets move across the face of the protein to create the closed conformation (C-Mad2), with Mad1 now trapped within this fold [63–67]. Upon mitotic entry, the Mad1–C-Mad2 core complex is recruited to kinetochores. Because Mad2 can dimerise [63,66,68], O-Mad2 from the cytosol can then be recruited to kinetochore-bound Mad1–C-Mad2 [41,69]. Indeed, FRAP studies revealed that kinetochore-bound Mad2 exists in two populations; one that is relatively stable, corresponding to the Mad1–C-Mad2 core complex, and a mobile fraction that arises due to the transient recruitment of O-Mad2 to Mad1–C-Mad2 [34,41,69]. Crucially, O-Mad2 bound to Mad1–C-Mad2 somehow captures Cdc20, thus creating a C-Mad2–Cdc20 complex, the first step in MCC assembly [63]. In this manner, C-Mad2 within the Mad1–C-Mad2 core complex acts as a prion-like template, catalysing the conversion of additional O-Mad2 proteins to the closed conformation and in doing so binding Cdc20.

Importantly, when kinetochores bind microtubules, the Mad1–C-Mad2 core is ejected via a mechanism known as 'stripping' and O-Mad2 is no longer recruited [60,61]. If Mad1 is artificially tethered to kinetochores so that it is not ejected upon microtubule capture, Mad2 is still recruited and anaphase onset is delayed in a Mad2-dependent manner [70]. Thus, the activity of the Mad1–C-Mad2 core at kinetochores may be the critical and only step in the SAC mechanism to indicate that a kinetochore is unattached. Consequently, while the template model is well supported, understanding how Mad1–C-Mad2 is recruited to kinetochores, how the template reaction is restricted to (or activated at) kinetochores, and how Mad1–C-Mad2 is ejected following microtubule capture are all important questions.

In principle, C-Mad2–Cdc20 complexes could recruit O-Mad2 and catalyse the formation of additional Mad2–Cdc20 complexes in the cytosol, thereby amplifying the SAC signal downstream of kinetochores [5,62,63]. However, when either p31^{comet}, a negative regulator of the SAC, or BubR1/Mad3 binds to C-Mad2–Cdc20, the dimerisation interface of Mad2 is blocked [52,71]. Indeed, a recent study in budding yeast shows that Mad2 dimerisation does not amplify the SAC signal downstream of kinetochores [72].

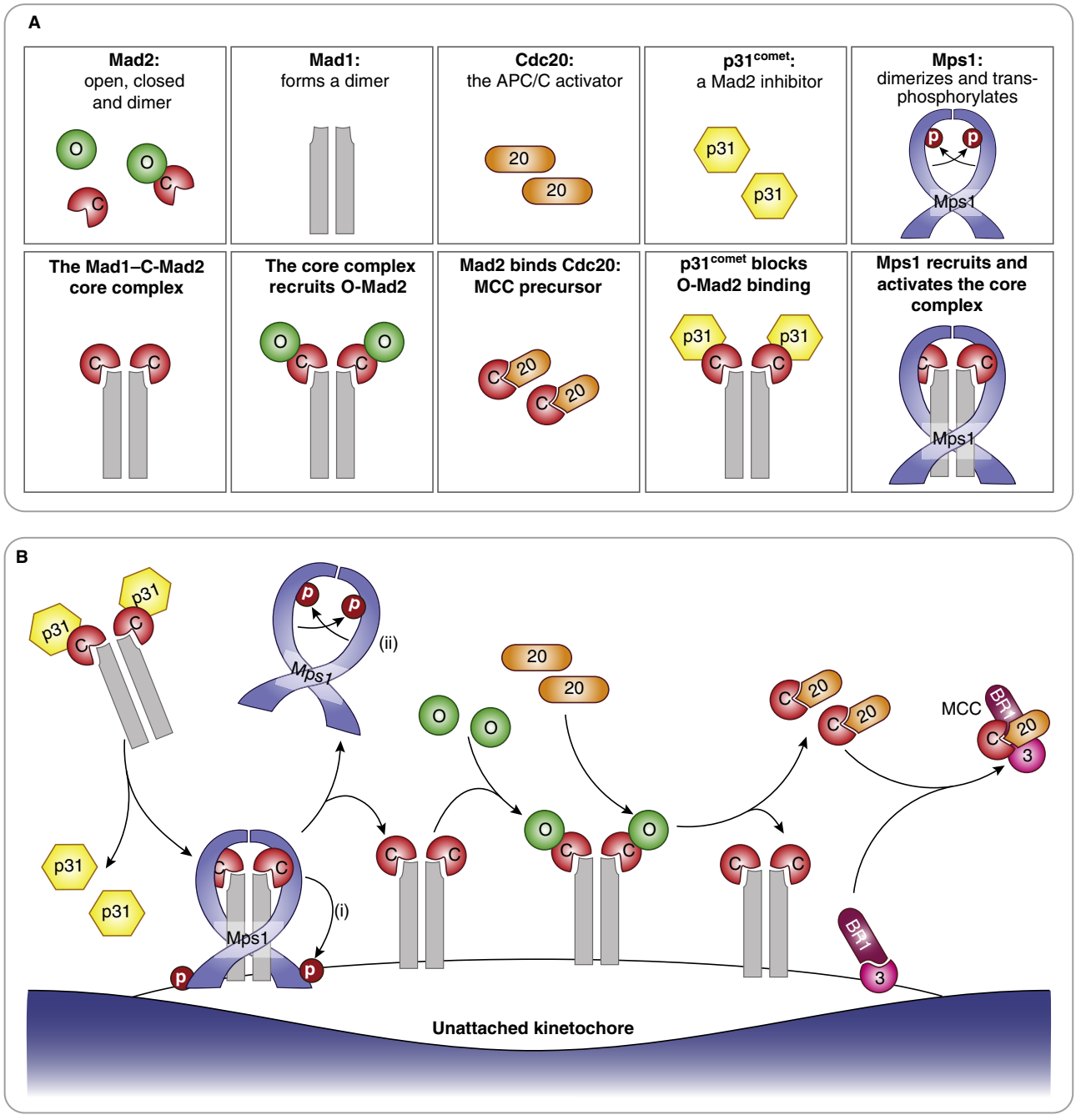

Figure 5. Regulation of the Mad2 template mechanism.

(A) The key components, including Mad2 in the open (O) and closed (C) conformations, which can dimerise; Mad1, the kinetochore receptor for Mad2, is a ligand for C-Mad2; the APC/C activator Cdc20 is also a ligand for C-Mad2; p31^{comet} which binds C-Mad2 at the dimerisation interface; and Mps1, a protein kinase which can dimerise and transphosphorylate. (B) Mad2 binds Mad1 to form the Mad1–C-Mad2 core complex. In interphase, and when not bound to kinetochores, this core complex is 'capped' by p31^{comet}. Upon kinetochore binding, p31^{comet} is somehow released, allowing recruitment of O-Mad2 via Mad2 dimerisation. O-Mad2 is then 'handed over' to Cdc20, and in doing so closes to form a C-Mad2-Cdc20 subcomplex, which then binds BubR1-Bub3, thereby forming the MCC. Mps1 plays a two-step role: (i) upon mitotic entry, Mps1 activity is required to recruit Mad1–C-Mad2 to the kinetochore; (ii) Mps1 activity is also required continuously during mitosis to promote its own release from kinetochores and in doing so allow recruitment of O-Mad2 to Mad1–C-Mad2 (see text for details). Adapted from [5].

Therefore, it appears that unattached kinetochores are the primary source of C-Mad2–Cdc20 [63–65,73]. Of note, in interphase Mad1 and Mad2 are present at nuclear pores, most likely as a Mad1–C-Mad2 complex [74], yet this does not recruit O-Mad2 [58]. Moreover, when Mad1 is artificially tethered to chromosome arms, the SAC is not activated, despite the fact that Mad2 is recruited — presumably in the closed conformation only [70]. Thus, the Mad2 template

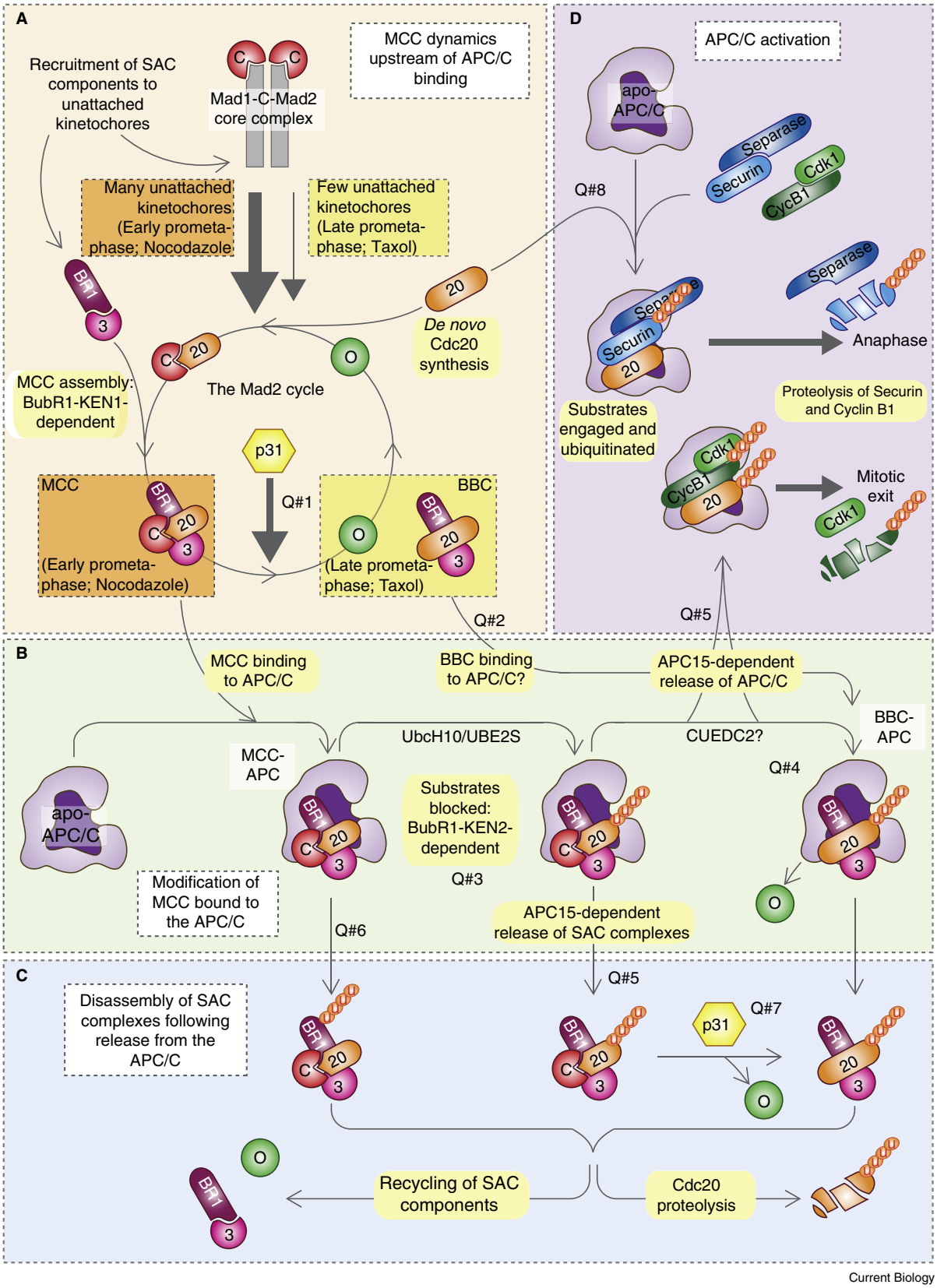

Figure 6. MCC assembly and disassembly.

(A) MCC dynamics upstream of APC/C binding. (B) Modification of the MCC bound to the APC/C. (C) Disassembly of SAC complexes following release from the APC/C. (D) APC/C activation. (Note that different APC/C^{Cdc20} complexes do not necessarily discriminate between securin and

reaction appears to be inhibited away from kinetochores, most likely via a mechanism that blocks the recruitment of O-Mad2 to the core complex [5,63,69].

The Role of Mps1

The Mps1 kinase has emerged as an important regulator of the Mad2 template mechanism. Advances in our understanding of Mps1 function have been facilitated by the discovery of several small molecule inhibitors, including AZ3146, Reversine and IN-1/2 [54,58,75]. Mps1 has also proved amenable to chemical genetics, whereby mutation of specific residues in the ATP-binding pocket render it sensitive to ATP-analogue inhibitors [55–57].

The phenotypes observed following Mps1 inhibition can be distilled into the following two-step model (Figures 4D and 5). Early on in mitosis, concomitant with kinetochore assembly, Mps1 activity promotes recruitment of the RZZ complex, which in turn recruits Mad1–C-Mad2 [54–56,58]. Once in mitosis, Mps1 activity is no longer required to maintain RZZ and Mad1–C-Mad2 at kinetochores. It is, however, continuously required to recruit O-Mad2 to the Mad1–C-Mad2 core complex [58]. Specifically, when Mps1 is inhibited after mitotic entry, Mad1–C-Mad2 remains bound to kinetochores but O-Mad2 is no longer recruited [58]. Supporting this notion, kinetochore localisation of a Mad2 mutant locked in the open conformation was completely abolished when Mps1 was inhibited. By contrast, $Mad2^{RQ}$, a mutant that can only bind kinetochores via a direct interaction with Mad1 [73], was unaffected [58]. Thus, in mitotic cells, Mps1 is continuously required to promote the template reaction. Consistently, when Mps1 was inactivated in cells already arrested in mitosis, assembly of Mad2–Cdc20 complexes was blocked [55].

How Mps1 activity promotes the template reaction is unclear. Although Mps1 can directly phosphorylate Mad1 and Mad2 [76,77], the template reaction can be reconstituted *in vitro* using recombinant proteins without the need for additional kinase activity [69]. One possibility therefore is that, in cells, Mps1 inhibits an activity that blocks the template reaction [57,58]. An attractive option is that $p31^{comet}$, which can 'cap' the Mad1–C-Mad2 core complex *in vitro* [69], is phosphorylated by Mps1, thereby ejecting it from the core complex and allowing the recruitment of O-Mad2 [5]. While there is evidence both for and against $p31^{comet}$ acting as an inhibitory cap on kinetochore-bound Mad1–C-Mad2 [78,79], it is interesting to note that Mps1 itself has the properties predicted of a Mad1–C-Mad2 cap. Specifically, when Mps1 activity is inhibited during mitosis, its own levels at kinetochores increase dramatically due to reduced exchange [54,58,80]. Moreover, O-Mad2 is not recruited despite the fact that Mad1–C-Mad2 remains bound to the kinetochore [58], and the SAC is inactive. While the biochemical mechanism by which inactivated Mps1 blocks the template reaction remains to be defined, it is also interesting to note that Mps1 can dimerise and transphosphorylate [58,81,82]. So one possibility is that early on in mitosis Mps1 activity sets up the template reaction by promoting recruitment of RZZ and Mad1–C-Mad2, and then, by stimulating its own release from the kinetochore, Mps1 activity allows recruitment of O-Mad2, thereby kick-starting the template reaction [58] (Figure 5B).

Mps1's role in the SAC also appears to be promoted by myosin-like proteins (MLPs), namely Mlp1/2 in budding yeast, Megator in flies and Tpr in human cells [83–85]. In mitosis, these proteins form part of the 'spindle matrix', a non-microtubule entity that contributes to mitotic spindle function [86,87]. While the spindle matrix concept remains controversial, Megator/Tpr is required for kinetochore localisation of Mps1, Mad1 and Mad2. These proteins also appear to be part of a complex, so perhaps MLPs facilitate assembly of the Mad1–C-Mad2 core complex, a reaction that is otherwise relatively slow [83]. An alternative, but not necessarily mutually exclusive possibility is that by binding the spindle matrix, Mps1, Mad1 and Mad2 are kept in close proximity to the chromosomes, thereby facilitating efficient kinetochore loading [83]. Indeed, when microtubules are removed from aligned chromosomes, Mps1 activity is required to reload Mad1–C-Mad2 to the now unattached kinetochores [58]. This role for the spindle matrix in localising SAC proteins close to the chromosomes may explain why the SAC signal is diffusible but at the same time restricted to the vicinity of the spindle [88].

How Does the SAC Inhibit Anaphase Onset?

While generating C-Mad2–Cdc20 complexes may be sufficient to alert the cell to the presence of unattached kinetochores, it is not sufficient to block anaphase onset. When BubR1/Mad3 is depleted, unattached kinetochores still produce C-Mad2–Cdc20, yet SAC function is abolished. Thus, BubR1/Mad3 acts downstream of Mad1–C-Mad2 to block anaphase onset [89,90]. Indeed, it appears that the generation of C-Mad2–Cdc20 is only an initial step in the assembly of the cytosolic APC/C inhibitor, namely the MCC [18]. Importantly, when the SAC is active, the MCC can be found bound to APC/C [18,20,91], suggesting that the SAC on signal does not simply sequester Cdc20 away from the APC/C. Thus, key questions include how is the MCC assembled, and how does it then bind and inhibit the APC/C?

How Is the MCC Assembled?

The MCC is assembled from two subcomplexes, the Mad2–Cdc20 complex and the Mad3/BubR1–Bub3 complex [5] (Figures 5 and 6A). While formation of Mad2–Cdc20 is catalysed by unattached kinetochores (see above), BubR1–Bub3 exists throughout the cell cycle and does not seem to be regulated [89,92]. The BubR1–Bub3 and Mad2–Cdc20 interactions are well characterised, thanks to the elucidation of 3D structural models for these proteins [64,67,93]. More recently, the crystal structure of the *S. pombe* Cdc20–Mad2–Mad3 complex has been solved [52], significantly enhancing our understanding of the protein–protein interactions involved in MCC assembly.

It is well established that Mad2 is necessary for BubR1/Mad3 to bind Cdc20 [20,89,90,94,95]. In addition, it has been shown in yeast, flies and human cells that the

cyclin B1.) See text for more details. Key questions for the future include the following: (Q#1) how does $p31^{comet}$ influence MCC dynamics? (Q#2) What is the contribution of the BubR1–Bub3–Cdc20 (BBC) complex to APC/C inhibition? (Q#3) How does BubR1 binding to the APC/C block substrate recruitment? (Q#4) How does CUEDC2 contribute to disassembly of APC/C-bound MCC? (Q#5) How does APC15 dissociate checkpoint complexes from the APC/C? (Q#6) Is ubiquitylation of other SAC proteins required for silencing? (Q#7) Are disassembly reactions post-APC/C release different from MCC disassembly mechanisms upstream of APC/C binding? (Q#8) What is the source of the Cdc20 that finally activates the APC/C; is it derived from *de novo* synthesis or is it liberated from SAC complexes?

amino-terminal KEN box in Mad3/BubR1 is essential for Mad2–Cdc20 binding [53,94–100] (Figure 6A). The KEN boxes in Mad3/BubR1 have attracted considerable attention as in other proteins these motifs often serve as APC/C degrons [13]. The MCC crystal structure reveals that the KEN box adopts a helix-loop-helix structure which establishes direct interactions with both Mad2 and Cdc20 [52]. This provides a clear explanation for why Mad2 is required for the Mad3/BubR1–Cdc20 interaction. In addition, the TPR domains in Mad3 also directly interact with Cdc20, consistent with the observation that mutating these domains in BubR1 disrupts its ability to bind Cdc20 [53]. Importantly, only the closed conformation of Mad2 is compatible with Mad3 binding. Interestingly, C-Mad2 bound to Cdc20 interacts with Mad3 using the same surface that dimerises with O-Mad2 [52]. Therefore, when Mad2 forms part of the MCC, it cannot bind O-Mad2 and is therefore unlikely to catalyse formation of additional Mad2–Cdc20 complexes.

Considering the similarity between Mad3 and BubR1, it is likely that the structure of the MCC is highly conserved. However, BubR1 has a carboxy-terminal kinase domain. The significance of its catalytic activity was unclear until a recent study demonstrated that human BubR1 is not an active kinase due to the accumulation of several inactivating mutations throughout evolution [101]. While some organisms appear to have lost the kinase domain altogether, yielding Mad3-like proteins, others — including humans — retained it as it is crucial for protein stability [101]. This revelation provides a rationale for why the amino terminus of BubR1 is sufficient for SAC function in cells and APC/C inhibition *in vitro* [53,97].

Several observations have alluded to a second Cdc20 binding site in BubR1, downstream of the Bub3 binding domain [19,94,97]. This may account for why, in *in vitro* binding assays, BubR1 can bind Cdc20 in the absence of Mad2 [19,94]. It may also explain why overexpression of BubR1 mutants in cells can yield KEN-box-independent Cdc20 binding [94,96,97]. However, the significance of this interaction remains unclear and it may not be required for SAC function [53,96,97].

Does MCC Assembly Depend on Unattached Kinetochores?

Despite the widely accepted view that the SAC delays anaphase onset in response to unattached kinetochores, some observations argue that the SAC signalling network can operate independently of kinetochores. For example, budding yeast strains mutated in Ndc10, a protein essential for kinetochore function, still assemble MCC during mitosis [102,103]. Also, in *S. pombe*, Bub3 is not required for SAC activation [104–106], even though it is required for kinetochore localisation of Bub1 and Mad3, as well as Mad1 and Mad2 [106–108], leading to the notion that SAC proteins need not be enriched at the kinetochores for SAC function [106].

In mammalian cells, similar ideas have been proposed [30,97]. Although BubR1 is found bound to Bub3 throughout the cell cycle, Bub3 is not necessary for the binding of BubR1 to Mad2–Cdc20 [20]. Indeed, Bub3 is not necessary for the ability of BubR1 and Mad2 to inhibit APC/C^{Cdc20} activity *in vitro* [19,20,53,109]. Also, overexpressing an amino-terminal BubR1 mutant lacking the Bub3 binding site in BubR1-deficient MEFs partially restores SAC function [97]. Because BubR1's Bub3 binding site is required for its ability to localise to kinetochores, such observations have led to suggestions that kinetochore localisation of BubR1 is not necessary for SAC function [97].

While Bub3 may not be required for *in vitro* binding of BubR1 to C-Mad2–Cdc20, or APC/C inhibition, in a cellular context the ability of BubR1 to bind Bub3 is required for an efficient SAC response [53,96]. Consistent with the *in vitro* data, a BubR1 mutant that cannot bind Bub3 can still bind C-Mad2–Cdc20 and the APC/C in cells, but with a drastically reduced efficiency [53]. Accordingly, this mutant can sustain SAC function in cells but again, very inefficiently [53,96]. We propose a relatively simple explanation for these observations; by virtue of binding Bub3, BubR1 is concentrated at unattached kinetochores, i.e. at the site where C-Mad2–Cdc20 complexes are generated, thereby promoting efficient MCC assembly (Figure 5). Accordingly, while BubR1 mutants incapable of kinetochore localisation are still capable of capturing C-Mad2–Cdc20 in the cytosol, they do so inefficiently, explaining why they are only partially SAC-proficient.

So, just as the MCC can be assembled *in vitro* without kinetochores — albeit slowly [109] — it may be that BubR1/Mad3 can bind C-Mad2–Cdc20 in cells independently of kinetochores. This probably only results in a low level of MCC, but in certain settings, e.g. in some organisms and/or following experimental manipulation such as overexpression of BubR1, reasonable levels may accumulate. Presumably, upon mitotic entry, this residual MCC has only limited potential and kinetochores then become essential to rapidly catalyse the generation of C-Mad2–Cdc20 and the subsequent binding of BubR1–Bub3. Thus, while the MCC may well assemble to some degree in the absence of kinetochores, both steps of the MCC assembly pathway are greatly facilitated by kinetochores.

What Is the Nature of the APC/C Inhibitor?

Early observations led to the notion that kinetochore-activated Mad2 was an important anaphase inhibitor, most likely by directly binding to Cdc20 and thus preventing it from activating the APC/C [16,17]. Consistently, in several systems, overexpression of Mad2 was shown to activate the SAC [16,40,110]. Despite discovery of the MCC [18] and the demonstration that BubR1 potentiates Mad2-mediated inhibition of the APC/C *in vitro* [20], the view that Mad2 was the ultimate downstream APC/C inhibitor remained. Indeed, in a recent experiment, artificially tethering Mad2 to Cdc20 in budding yeast delayed mitosis in a manner that was only partially dependent on Mad3 [111], consistent with the Mad2–Cdc20 complex being sufficient to inhibit the APC/C.

However, a growing body of evidence now indicates that BubR1, not Mad2, is the key APC/C inhibitor. Firstly, Mad2 in isolation can only inhibit the APC/C *in vitro* when added in very high amounts with respect to Cdc20 [17,19,20,109]. Secondly, in some cases the mechanism by which Mad2 inhibits the APC/C *in vitro* seems to involve the sequestration of Cdc20 [19], an observation that is at odds with the fact that Mad2 is found bound to the APC/C during a SAC response [16,91]. Thirdly, Mad2 can bind APC/C^{Cdc20} *in vitro* without significantly affecting its activity [53]. Finally, Mad2 is not always a stoichiometric component of the MCC during mitosis [79,90].

Indeed, the stoichiometry of Mad2 bound to BubR1–Bub3–Cdc20 may reflect the number of unattached kinetochores (Figure 6A). In nocodazole-arrested cells, when

many kinetochores are unattached, Mad2 is easily detectable bound to BubR1–Cdc20 [79]. However, in taxol-arrested cells, when only a few kinetochores are signalling, much less Mad2 is bound to BubR1–Cdc20. This observation implies that as well as the MCC, the BubR1–Bub3–Cdc20 complex is also a viable APC/C inhibitor. Moreover, during a normal mitosis, the nature of the inhibitor might shift according to the number of attached kinetochores, with the MCC dominating early on, and the BubR1–Bub3–Cdc20 complex later (Figure 6A). Thus, the identification of the MCC as a tetrameric APC/C inhibitor could reflect the fact that nocodazole was used to arrest the cells in mitosis [18], which in turn reinforced the notion that Mad2 was part of the final APC/C inhibitor. Taken together, these more recent data suggest that Mad2 may in fact not be an APC/C inhibitor *per se*, but rather its role is to promote the interaction between BubR1 and Cdc20 in order to create the final inhibitor [79,90,109]. Therefore, understanding how the SAC works ultimately boils down to knowing how BubR1 inhibits the APC/C.

How Does BubR1 Inhibit the APC/C?

When the APC/C is engaged by the MCC, its ability to recruit cyclin B1 and securin is reduced [112], suggesting that the MCC, and therefore by extension BubR1, somehow interferes with substrate binding. Recent structural studies of the APC/C show that D-box substrates bind at the interface between the co-activator and the small APC/C subunit Apc10 [113,114]. Therefore, BubR1 might block substrate binding to the APC/C by either inducing a conformational change on the APC/C that impairs its ability to form the bi-partite substrate receptor, or by directly occupying the substrate binding site, possibly as a 'pseudosubstrate'.

The 'pseudosubstrate model' was initially supported by the observation that Mad3 binds to Mad2–Cdc20 via its amino-terminal KEN box (KEN1) [95]. Accordingly, Mad3 was shown to compete with Cdc20 for substrate binding in a KEN1-dependent manner [95]. Consistent with this notion, the fission yeast MCC structure shows that by interacting with Cdc20, Mad3's KEN1 occupies the KEN-box binding site on Cdc20 [52]. In other words, Mad3 does indeed appear to act as a pseudosubstrate, at least for KEN box-containing substrates.

Interestingly, BubR1/Mad3 contains a second conserved KEN box (KEN2) [95,98]. Even though this motif is not required for binding of BubR1 to Cdc20, Mad2 or the APC/C, it is essential for SAC function [53,95–99]. Furthermore, *in vitro*, BubR1 inhibits the binding of D-box-containing substrates to APC/C^{Cdc20}, and importantly this is dependent on KEN2 [53] (Figure 6B). Thus, while KEN1 blocks the binding of KEN-box substrates to Cdc20, KEN2 might occupy the bi-partite substrate receptor, and thus act as a pseudosubstrate for D-box substrates. However, although KEN2 is missing from the recently described MCC structure, the data suggest that KEN2 is unlikely to be able to contact the D-box binding site [52].

Indeed, docking of the yeast MCC structure onto the APC/C reveals that Mad3 induces a displacement of Cdc20 from Apc10, thus affecting the ability of these proteins to form the bi-partite D-box receptor [52]. These data are in agreement with cryo-EM studies showing that MCC binding induces a change in the position of Cdc20 within the APC/C [112,114]. Furthermore, they agree with the observation that the binding of Cdc20 to the APC/C is mediated by different APC/C subunits, depending on whether the SAC is on or off [115]. Also, lysine 92 on Mad3's TPR domain might partially interfere with the ability of Cdc20 to recognise the arginine residue of the D-box [52]. Thus, the mechanism by which Mad3/BubR1 inhibits substrate binding to the APC/C appears to be indirect, by preventing the formation of the substrate-binding site.

One possibility therefore is that KEN2 inhibits substrate binding indirectly, by stabilising the MCC–APC/C structure. Since KEN1 binds the WD40 repeats of co-activators [52], perhaps KEN2 binds another WD40 domain-containing APC/C subunit, such as Apc4 [112]. Indeed, Apc4 undergoes a major conformational change upon MCC binding [112], which in turn might disrupt the bi-partite substrate receptor. In summary, therefore, the two KEN boxes and the TPR domain in BubR1 cooperate to efficiently inhibit APC/C^{Cdc20} activity.

How Is the SAC 'On' Signal Extinguished?

While it is essential that the APC/C is inhibited when kinetochores are unattached, the ability to alleviate APC/C inhibition once the SAC is satisfied is equally important. How does microtubule attachment satisfy the SAC? Once satisfied, how is the production of inhibitory complexes blocked? And, how are existing inhibitors inactivated? Exactly how microtubule binding satisfies the SAC is still unclear, and whether it is actual microtubule attachment or the ensuing tension remains controversial. Recent observations show that kinetochores stretch when under mechanical tension [116,117], and that Aurora B, a well established 'tension-sensor', may play a direct role in the SAC [118]. Rather than revisit these issues, we refer the reader to a recent review [119]. Once attached to microtubules, several mechanisms contribute to SAC silencing. Firstly, the Mad1–C-Mad2 template is removed from attached kinetochores via stripping, also known as streaming. Secondly, phosphatases counterbalance the activity of mitotic kinases (the role of phosphatases has recently been reviewed in [120], so will not be discussed here). And finally, mechanisms that result in MCC disassembly have a significant role in alleviating APC/C inhibition.

Stripping

The shape and composition of kinetochores dramatically changes upon microtubule attachment [121]. While some shape changes are due to tensile forces, in higher eukaryotes stripping removes a subset of kinetochore components upon stable microtubule attachment. Relevant to the SAC, stripping removes Mad1 and Mad2 from kinetochores. This appears to be essential for efficient SAC inactivation, as artificially tethering Mad1 to bioriented kinetochores delays anaphase onset [70]. Stripping is mediated by the minus-end directed microtubule motor dynein as inhibiting dynein function blocks removal of Mad1 and Mad2 from attached kinetochores [121].

The kinetochore module subjected to dynein-mediated stripping is the RZZ complex [27], which is also required to recruit Spindly to kinetochores. While the precise role of Spindly remains unclear, possibly due to differences in its function across model systems, in all organisms studied Spindly depletion impairs kinetochore recruitment of dynein [122–126]. Interestingly, human cells depleted of Spindly can still remove Mad1 and Mad2 from attached kinetochores, despite the lack of dynein recruitment [123,125,126]. However, if Spindly-deficient cells are reconstituted with a mutant

lacking the 'Spindly-box', dynein is still not recruited but the persistence of Spindly at kinetochores now results in a stripping defect [125,126]. Therefore, dynein-mediated removal of Spindly from attached kinetochores is the critical step for the stripping of RZZ, Mad1 and Mad2 from attached kinetochores. These results suggest that Spindly acts by inhibiting another unknown, dynein-independent stripping mechanism, which might be evolutionarily conserved.

Upon microtubule binding, blocking the formation of new MCC complexes may be achieved simply by stripping Mad1–C-Mad2 from kinetochores. However, emerging evidence indicates that other aspects of kinetochore–microtubule binding influence the SAC, independent of stripping. While phosphatases play a key role [120], other mechanisms appear to be phosphatase-independent, for example with microtubule binding to KNL1 directly influencing SAC silencing [127].

Inactivation of APC/C Inhibitors

In addition to preventing the assembly of new MCC, extinguishing the SAC on signal requires that existing inhibitory complexes are disassembled in order to liberate Cdc20 so that it can activate the APC/C. Indeed, several mechanisms contribute to MCC disassembly, both in terms of free MCC and APC/C-bound MCC.

Mad2 Recycling

As alluded to above, several recent studies highlight the dynamic nature of the MCC, including studies focussed on p31^{comet}. Originally identified in a yeast two-hybrid screen as a Mad2 interactor [128], p31^{comet} is a SAC antagonist: overexpression overrides the SAC while RNAi-mediated inhibition delays anaphase onset [79,129,130]. The function of p31^{comet} is intimately related to Mad2 — mutants of p31^{comet} that cannot bind Mad2 do not influence the SAC [71,79]. Elegant structural and *in vitro* studies revealed that p31^{comet} binds C-Mad2 at its dimerisation interface, and that this interferes with O-Mad2 recruitment [68,71,131]. This led to the proposal that p31^{comet} 'caps' Mad1–C-Mad2 to prevent C-Mad2–Cdc20 production (Figure 5) [5,69]. Consistent with this, p31^{comet} interacts and co-localises with Mad1–C-Mad2 throughout the cell cycle [79,130]. FRAP shows that p31^{comet} is stable at the nuclear envelope but is highly dynamic at unattached kinetochores [130], consistent with the idea that when the Mad1–C-Mad2 core complex is at unattached kinetochores, p31^{comet} rapidly traffics on and off.

However, a number of observations suggest that p31^{comet} does not regulate the SAC by capping Mad1–C-Mad2 at kinetochores. Specifically, p31^{comet} RNAi or overexpression does not affect levels of O-Mad2 bound to kinetochores [79,130], and inhibition of Mps1 in mitosis, which prevents O-Mad2 recruitment, does not cause an increase in p31^{comet} at unattached kinetochores [79]. Indeed, several recent studies have highlighted a role for p31^{comet} in MCC disassembly. p31^{comet} associates with the MCC during mitosis, and p31^{comet} RNAi increases MCC levels [79,132,133], which transiently delays anaphase [79,130,132,133]. Crucially, *in vitro*, p31^{comet} is able to disrupt the MCC [79,134] and this depends on its ability to bind Mad2 [79]. Consistent with these data, expressing a Mad2 mutant that cannot bind p31^{comet} delays mitotic exit [79].

The exact mechanism by which p31^{comet} promotes MCC turnover remains obscure. One report suggests that p31^{comet} disrupts the interaction between BubR1 and C-Mad2–Cdc20 [134]. This appears consistent with structural data which show that Mad3 and p31^{comet} both bind C-Mad2 at the C-Mad2/O-Mad2 dimerisation interface [52]. However, depletion of p31^{comet} in cells markedly increases the amount of Mad2 bound to BubR1–Cdc20 [79,132,133]. Moreover, another report showed that addition of recombinant p31^{comet} to preassembled MCC results in less Mad2 bound to BubR1–Cdc20 [79]. Therefore, we favour the option whereby p31^{comet} extracts Mad2 from the MCC, leaving behind a BubR1–Bub3–Cdc20 complex [79] (Figure 6A,C). How p31^{comet} does this remains unclear, but it may explain why under some circumstances Mad2 is a substoichiometric MCC component relative to BubR1, Bub3 and Cdc20 [90]. Indeed, several studies show that whereas the amount of BubR1 bound to Cdc20 is consistent under a variety of conditions, levels of bound Mad2 can vary considerably [79,129,132,133].

An important observation is that p31^{comet} only extracts Mad2 from free MCC, not APC/C-bound MCC [79,133]. How then is APC/C-bound MCC disassembled? Recently, a novel APC/C subunit, APC15, has been shown to promote turnover of MCC bound to the APC/C [133,135] (Figure 6B). APC15 RNAi causes a similar phenotype to p31^{comet} RNAi, and also increases Mad2–Cdc20 levels. Importantly, simultaneous depletion of p31^{comet} and APC15 has a synergistic effect on the delay in anaphase onset, suggesting that p31^{comet} and APC15 act in partially redundant parallel pathways [133]. Note that, in contrast to p31^{comet}, APC15 is conserved in lower eukaryotes, including yeasts. Indeed, Mnd2, the budding yeast APC15 homologue, is also required for spindle checkpoint silencing [136]. Thus, disassembly of the MCC on the APC/C appears to be the more evolutionarily ancient mechanism. Potentially relevant to this pathway is CUEDC2, which has recently been shown to be required for SAC silencing; CUEDC2 binds to Cdc20 and promotes its release from Mad2 [137] (Figure 6B), although the mechanism remains unclear.

Cdc20 Turnover, the Role of Ubiquitylation and Proteolysis

Ubiquitylation by the APC/C and/or proteasome-mediated degradation have been proposed as mechanisms that participate in MCC disassembly and/or SAC silencing [129,132,133,135,136,138–142]. Consistent with this, inhibition of proteasome activity following a nocodazole release impairs MCC disassembly [129,140]. Furthermore, cells treated with TAME, a small molecule APC/C inhibitor, undergo a prolonged mitotic arrest that is dependent on a functional SAC, leading to the suggestion that APC/C-mediated ubiquitylation promotes SAC silencing [139]. However, TAME was recently shown to induce cohesion fatigue [143], a phenomenon whereby persistent microtubule-dependent pulling forces experienced during a protracted metaphase arrest eventually cause a loss of sister chromatid cohesion, [144,145]. Because separated sisters cannot biorient, the SAC is re-activated. Cohesion fatigue can be induced by a variety of treatments that lead to a prolonged metaphase, regardless of their mechanism of action. Thus, in light of the cohesion fatigue phenomenon, the notion that APC/C and/or proteasome activity are required to inactivate the SAC needs to be treated with caution. Indeed, experiments that are not complicated by cohesion fatigue suggest that proteasome activity is not required for dissociation of the MCC from the APC/C. Specifically, when the SAC is satisfied

with Aurora B inhibitors, the MCC can still disassemble even if the proteasome is inhibited [90,91,112,129,140].

Despite the cohesion fatigue problem, other lines of evidence suggest that ubiquitylation, independently of proteolysis, promotes MCC disassembly. For instance, one study showed that UbcH10, an E2 ubiquitin conjugating enzyme, promotes dissociation of MCC components from the APC/C [142] (Figure 6B). Importantly, proteasome activity was not present in these experiments. Another study showed that a different E2, Ube2S, is also required for checkpoint silencing, possibly by elongating ubiquitin chains initiated by UbcH10 [146].

Potential ubiquitylation substrates involved in SAC silencing are Cdc20 and BubR1, both of which are APC/C substrates [90,146–148]. Cdc20 is continuously synthesised, ubiquitylated and degraded during prometaphase, with turnover dependent on its ability to assemble into the MCC [90,98,136,147]. Cdc20 ubiquitylation, which impairs Mad2 binding *in vitro*, may therefore promote SAC silencing by inducing MCC disassembly [129,132,142]. Accordingly, the APC/C subunit APC15/Mnd2 is responsible for both Cdc20 ubiquitylation and SAC silencing [135,136]. A complicating factor is that Cdc20 mutants that cannot be ubiquitylated can still dissociate from MCC components [90,133]. Consequently, whether Cdc20 ubiquitylation is required for SAC silencing is unclear.

Recent data suggest that BubR1 is regulated by both ubiquitylation and acetylation [146,148]. Acetylation of BubR1 at lysine 250 protects it from being ubiquitylated by the APC/C in prometaphase [148]. At metaphase, BubR1 is de-acetylated and thus converted to an APC/C substrate. Moreover, mutation of BubR1 lysine 250 to an acetylation-mimicking residue impairs mitotic exit [148]. This suggests a mechanism whereby active deacetylation of BubR1 at metaphase converts it into an APC/C substrate, with subsequent ubiquitylation silencing the SAC, possibly by promoting disassembly of BubR1–Cdc20 complexes.

In summary, while current evidence suggests that APC/C activity promotes MCC disassembly and SAC silencing, the exact mechanism remains to be determined.

Dynamic Control of the Mitotic State

Interestingly, not only is Cdc20 continuously synthesised and degraded in mitosis, but so is cyclin B1 [149–151], with *de novo* synthesis arising via cytoplasmic-polyadenylation-dependent translation [151]. The significance of this is unclear but raises an interesting possibility. Both Cdc20 and cyclin B1 are specifically targeted for proteolysis during the cell cycle; cyclin B1 is degraded in order to trigger mitotic exit [152] while continued Cdc20 degradation during G1 inactivates the APC/C [153]. Interestingly, even when the SAC is fully on, there is some residual APC/C activity, resulting in the slow degradation of cyclin B1 and eventual mitotic exit, a phenomenon known as 'slippage' [154]. This is potentially dangerous because exiting mitosis before SAC satisfaction can lead to chromosome missegregation.

De novo synthesis of cyclin B1 during mitosis may be a way of counterbalancing its degradation, topping up cyclin B1 levels to sustain Cdk1 activity. Indeed, if cells are treated with cyclohexamide to prevent *de novo* protein synthesis, slippage is accelerated [132,139]. Cdc20 degradation is also APC/C-dependent [90,147] and presumably therefore there is a risk that, during a prolonged mitosis, residual APC/C activity could deplete Cdc20. This is presumably equally dangerous as Cdc20 depletion causes mitotic arrest [155]. Moreover, once chromosomes are correctly aligned, it is important that the cell rapidly segregates its chromosomes and exits mitosis in order to re-start transcription and DNA damage repair processes.

Thus, maybe the evolutionary advantage of *de novo* cyclin B1 and Cdc20 synthesis is maintenance of the mitotic state, and the ability to exit mitosis rapidly following SAC satisfaction, respectively. A complication of this is that overproduction of Cdc20 may well overwhelm the SAC, triggering premature exit. To some extent, the SAC is capable of dealing with excess Cdc20; note that in the presence of MG132, MCC levels increase as Mad2 and BubR1 mop up the elevated Cdc20 [132]. However, elevating MCC levels is not inconsequential; e.g. inhibition of $p31^{comet}$ or APC15 shows that excess MCC prolongs the mitotic state despite the chromosomes being ready for segregation [79,130,132,133,135]. The notion that the SAC can be swamped is indicated by the fact that Cdc20 overexpression or preventing Cdc20 ubiquitylation overrides the SAC [15,90,136,147].

Thus, maintaining Cdc20 levels via a dynamic 'production–destruction' mechanism allows dynamic control of the mitotic state; Cdc20 is continuously synthesised to ensure that there are sufficient levels to drive anaphase and mitotic exit, while at the same time Cdc20 is continuously degraded to ensure that the MCC assembly pathways are not overwhelmed, thereby allowing the cell to stay in prometaphase (Figure 6D). In this scenario, Cdc20 ubiquitylation and degradation is not a SAC-silencing mechanism that is specifically activated at metaphase but rather acts as a governor to limit the amount of Cdc20, thereby capping MCC levels so that the APC/C is readily activated once kinetochores stop generating C-Mad2–Cdc20 complexes.

Conclusions

During the last five years, considerable progress has been made in terms of further dissecting the SAC network. Advances in defining kinetochore structure mean that understanding how the SAC module is assembled is just around the corner, with the Bub1–KNL1 interaction being an obvious area for focus. The Mad2 template model is now well established, and the realisation that Mps1 regulates this in cells is an important advance. However, the molecular mechanism requires further attention. An important new concept is the notion that the MCC is dynamic, highlighted by new insights into $p31^{comet}$ function, the role of APC15 and Cdc20 turnover. Structural studies continue to provide key insights; in particular, the crystal structure of a partial MCC complex has yielded significant insight into how the MCC inhibits the APC/C. How stable microtubules initiate SAC silencing is still mysterious. In principle, satisfaction of the SAC could simply be down to stable microtubules stripping away the Mad1–C-Mad2 core complex, but it is unlikely to be this simple. Consequently, despite satisfactory progress, the questions outlined early on in this review remain only partially answered and as such there is more work ahead.

Acknowledgements

P.L.G. is funded by CONICYT Chile and the University of Manchester; F.G.W. was funded by the Wellcome Trust; and S.S.T. is a Cancer Research UK Senior Fellow. Due to space constraints we have not been able to discuss all aspects of SAC signalling and we apologise to our colleagues whose work we have not been able to cite.

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