The spindle-assembly checkpoint in space and time

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Abstract | In eukaryotes, the spindle-assembly checkpoint (SAC) is a ubiquitous safety device that ensures the fidelity of chromosome segregation in mitosis. The SAC prevents chromosome mis-segregation and aneuploidy, and its dysfunction is implicated in tumorigenesis. Recent molecular analyses have begun to shed light on the complex interaction of the checkpoint proteins with kinetochores — structures that mediate the binding of spindle microtubules to chromosomes in mitosis. These studies are finally starting to reveal the mechanisms of checkpoint activation and silencing during mitotic progression.

Anaphase-promoting complex/cyclosome (APC/C). A multiprotein complex with ubiquitin-ligase activity that is responsible for the ubiquitylation of several key cell-cycle regulators, including cyclin B and securin. Also known as the cyclosome.

26S proteasome
A multiprotein complex endowed with protease activity. It is responsible for the proteolytic degradation of substrates tagged by polyubiquitin chains, including those created by the APC/C.

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In 1991, two independent screens identified various genes, mutation of which bypassed the ability of wild-type *Saccharomyces cerevisiae* cells to arrest in mitosis in the presence of spindle poisons[1,2]. The genes identified in these screens included the *MAD* (mitotic-arrest deficient) genes *MAD1*, *MAD2* and *MAD3* (*BUBR1* in humans) and the *BUB* (budding uninhibited by benzimidazole) gene *BUB1* (REFS 1,2) (FIG. 1). Another *BUB* gene, *BUB3*, was identified as an extra-copy suppressor of the *bub1-1* mutant[1]. These genes are conserved in all eukaryotes, and they are collectively involved in a pathway that is active in prometaphase and which prevents the precocious separation of sister chromatids[3,4] (FIG. 2). This pathway constitutes the spindle-assembly checkpoint (SAC).

The SAC targets CDC20, a co-factor of the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C)[5,6] (FIG. 2). Specifically, the SAC negatively regulates the ability of CDC20 to activate the APC/C-mediated polyubiquitylation of two key substrates, cyclin B and securin, thereby preventing their destruction by the 26S proteasome (FIG. 2). Securin is a stoichiometric inhibitor of a protease known as separase. Separase is required to cleave the cohesin complex that holds sister chromatids together, and cohesin cleavage is required to execute anaphase[7] (FIG. 2). On the other hand, the proteolysis of cyclin B inactivates the master mitotic kinase, CDK1, which promotes exit from mitosis[7]. By keeping CDC20 in check, the SAC prevents this chain of events, prolonging prometaphase until all chromosomes have become bi-orientated between separated spindle poles on the metaphase plate (FIG. 3). Chromosome bi-orientation finally extinguishes the checkpoint, relieving the mitotic arrest and allowing anaphase to proceed. A mitotic checkpoint complex (MCC) that contains three SAC proteins, MAD2, BUBR1/Mad3 and BUB3, as well as CDC20 itself[8], has emerged in recent years as a possible SAC effector. The MCC binds the APC/C and seems to render it unable to exercise its ubiquitin-ligase activity on securin and cyclin B[8–19].

Besides MCC, other 'core' SAC components include MAD1 and the kinases BUB1, multipolar spindle-1 (MPS1) and Aurora-B (Ipl1 in *S. cerevisiae*). These proteins are required to amplify the SAC signal and the rate of MCC formation (for example, see REFS 18,20–30). Additional proteins that regulate SAC activity in higher eukaryotes include: constituents of the ROD (rough deal)–ZW10 (zeste white-10)–ZWILCH (RZZ) complex (reviewed in REF. 31); p31^{comet} (previously known as CMT2)[32–34]; several protein kinases including mitogen-activated protein kinase (MAPK), CDK1–cyclin-B, NEK2 and polo-like kinase-1 (PLK1) (REFS 19,35–42); the microtubule motors centromere protein (CENP)-E (also known as kinesin-7)[43,44] and dynein; and dynein-associated proteins such as dynactin, cytoplasmic linker protein (CLIP)170 and lissencephaly-1 (LIS1) (REFS 45–47) (BOX 1; FIG. 1).

During prometaphase, CDC20 and all SAC proteins concentrate at kinetochores[48,49] (FIG. 2). Kinetochore localization of CDC20 and of its binding partners in the MCC is dynamic, which indicates that kinetochores provide a catalytic platform to accelerate the production of the MCC[50–54]. So far, most evidence indicates that the SAC monitors only one aspect of the spindle, the interaction between kinetochores and spindle microtubules. In this respect, 'kinetochore checkpoint' would describe the spatial and functional requirements for this checkpoint more faithfully than 'spindle-assembly checkpoint'[55]. The reference to spindle assembly[38] reflects the original observation that the SAC is activated by interference with spindle assembly, as it might occur, for example, in the presence of spindle poisons. 'Spindle-assembly

Mitotic checkpoint complex
(MCC). A complex that contains the APC/C activator CDC20 and the spindle-assembly checkpoint proteins MAD2, BUBR1/Mad3 and BUB3. The MCC is regarded as the effector of the spindle-assembly checkpoint.

Kinetochore
A large protein assembly that mediates the attachment of chromosomes to spindle microtubules. Kinetochores assemble, specifically during mitosis, around specialized chromosomal regions known as centromeres, and disassemble at the end of mitosis.

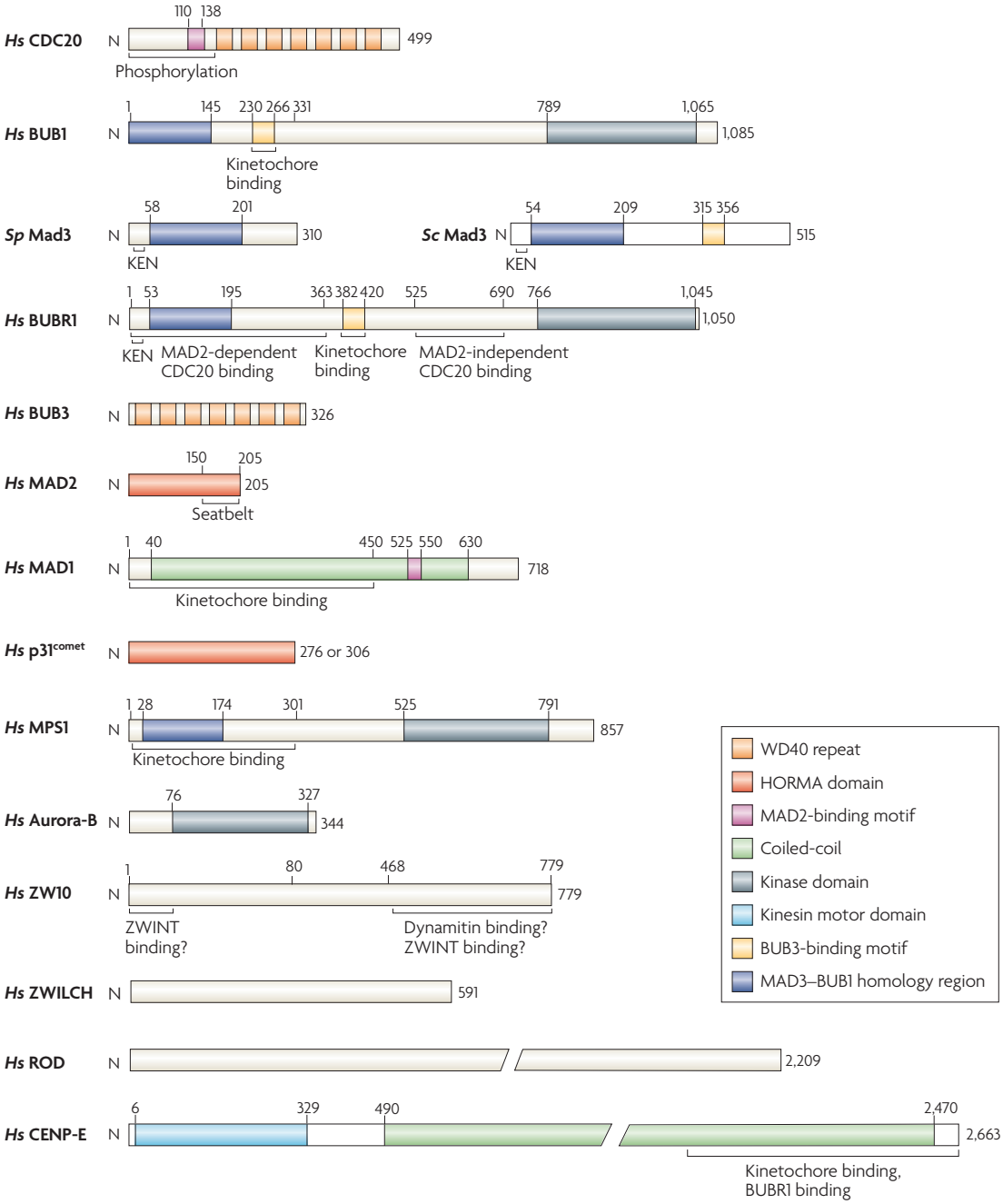

Figure 1 | **Domain organization of SAC proteins.** CDC20 and budding uninhibited by benzimidazole (BUB)3 contain WD40 domains (orange) and fold as 7-bladed β-propellers. The mitotic-arrest deficient-2 (MAD2)-binding site in the N-terminal region of CDC20 was first identified genetically[5,6], and subsequently mapped biochemically[110,111]. BUB1, BUBR1 and multipolar spindle-1 (MPS1) share a similar organization, with an N-terminal helical domain[69,70,139] (blue) and a C-terminal kinase domain (grey). The BUB3-binding regions of BUB1 and BUBR1 (yellow) have also been described[69,171]. Note that *Saccharomyces cerevisiae* (*Sc*) Mad3 and *Homo sapiens* (*Hs*) BUBR1 differ by the presence of a kinase domain in BUBR1. The identity of the region within Mad3 and BUBR1 that is required to bind CDC20 remains controversial[12,14,99]. A kinase domain with short N- and C-terminal extensions is also present in Aurora-B. MAD2 and p31^{comet} contain the HORMA (HOP1p, REV7p, MAD2) domain. The 'seatbelt' motif in the C-terminal region of MAD2 is a mobile element that holds different conformations in O-MAD2 (open MAD2) and C-MAD2 (closed MAD2; see FIG. 4). MAD1 and centromere protein (CENP)-E are predominantly coiled-coil proteins. CENP-E also contains a kinesin-type motor domain. The kinetochore-binding domains of MAD1 and CENP-E have been defined in REFS 27,121. The domain structures of ROD (rough deal), ZW10 (zeste white-10) and ZWILCH have not been studied in detail. There are contradictory reports on the localization of the ZWINT (ZW10 interactor)-binding site of ZW10 (REF. 31). Like ROD and ZWILCH, ZW10 lacks discernible sequence similarity to other proteins. The abundance of CDC20, MPS1, Aurora-B, CENP-E, BUB1 and possibly BUBR1 is regulated during the cell cycle, reaching a peak in mitosis. Proteins are drawn to scale. *Sp*, *Schizosaccharomyces pombe*.

checkpoint' has now become the generally used term and we will not attempt to change this habit here.

The reconstitution of checkpoint reactions *in vitro* is providing a molecular understanding of the interplay between checkpoint proteins, kinetochores and the mitotic cytosol in the generation of the MCC. Here we summarize recent advances, concentrating in particular on the controversial role that kinetochores have in the generation of the MCC[56]. Owing to space limitations, our analysis will refer exclusively to the mitotic cycle. Readers interested in the role of the SAC in meiosis are referred to REF. 57 (BOX 2).

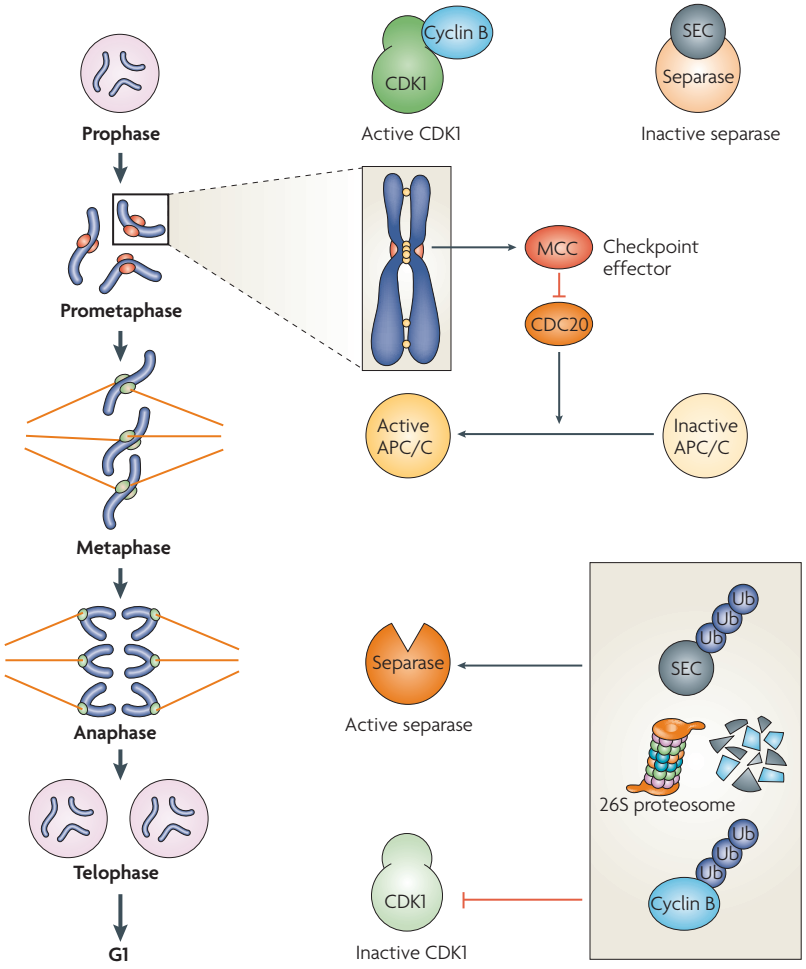

Figure 2 | **Relationship of the SAC with the cell-cycle machinery.** Mitosis is traditionally subdivided into five consecutive and morphologically distinct phases: prophase, prometaphase, metaphase, anaphase and telophase. To enter mitosis, the cell requires the activity of the master mitotic kinase, cyclin-dependent kinase-1 (CDK1), which depends strictly on the binding of cyclin B to CDK1 (REF. 7). Separase is a protease, the activity of which is required to remove sister-chromatid cohesion at the metaphase-to-anaphase transition (cohesin is indicated in yellow on the expanded view of the chromosome). Prior to anaphase, separase is kept inactive by the binding of a protein known as securin (SEC). Unattached kinetochores (red hemi-circles) contribute to the creation of the mitotic checkpoint complex (MCC), which inhibits the ability of CDC20 to activate the anaphase-promoting complex/cyclosome (APC/C). The attachment of all sister-kinetochore pairs to kinetochore microtubules, and their bi-orientation — which produces congression to the spindle equator — negatively regulates the spindle-assembly checkpoint (SAC) signal. This releases CDC20, which can now activate the APC/C. This results in the polyubiquitylation of anaphase substrates such as cyclin B and securin, and their subsequent proteolytic destruction by the proteasome. The degradation of SEC results in the activation of separase, which targets the cohesin ring that is holding the sister chromatids together, thus causing the loss of sister-chromatid cohesion and the separation of sister chromatids. The degradation of cyclin B at this stage also inactivates the master mitotic kinase CDK1–cyclin B, initiating cytokinesis and the mitotic-exit programme. Attached kinetochores are shown in green.

SAC basics

As mentioned above, the function of the SAC is intimately linked to kinetochores. Here, we introduce the fundamental concepts of attachment and tension to explain how kinetochores sense conditions that activate the SAC.

The source of SAC activity. Shortly after the discovery of the *BUB* and *MAD* genes, the hypothesis that kinetochores control the SAC started to gain strength[58,59]. Crucial discoveries that supported this hypothesis were that mutations in centromeric DNA and impaired kinetochore function activate the SAC in *S. cerevisiae*[60–64]. Furthermore, the onset of anaphase in vertebrate cells was shown to be blocked by a single unattached kinetochore[65–68]. This condition (known as mono-orientation or monotelic attachment; FIG. 3) occurs normally during mitosis as sister kinetochores become bi-orientated. The connection between SAC and kinetochores was strengthened further when the vertebrate homologues of the SAC proteins were shown to concentrate at unattached kinetochores in mitosis and become depleted from kinetochores by microtubule attachment and chromosome bi-orientation[69–73]. Conditions that affect the structural integrity of kinetochores, and thereby prevent binding of the 'core' SAC proteins, generally inhibit SAC activity (for example, see REFS 74–77).

Kinetochore localization of SAC components. The histone variant CENP-A and additional constitutive proteins mark the inner plate of mammalian kinetochores, which is located at the periphery of centromeric chromatin[48,78,79] (FIG. 4). Before mitosis, other evolutionarily conserved proteins, including Spc105 (KNL-1 in *Caenorhabditis elegans*) and the Mtw1 (MIS12 in humans), Ndc80 (HEC1 in humans) and minichromosome maintenance protein-21 (MCM21) complexes, are recruited to the kinetochore outer plate to form attachment sites for bundles of spindle microtubules, which are known as the kinetochore microtubules[48,78–81] (FIG. 4). Several other proteins, including the SAC and motor proteins, bind within a region of the kinetochore that is known as the fibrous corona and is found at the periphery of the outer plate. The temporal order and specific requirements for the recruitment of kinetochore and SAC proteins are complex and will not be reviewed here in detail (for example, see REFS 4,46,49,56,77,82,83).

SAC inactivation by attachment. The SAC monitors the attachment of kinetochores to microtubule plus-ends during an unperturbed mitosis (BOX 1). Ideally, SAC activity at sister kinetochores should remain active until bi-orientation, the only condition that ensures accurate segregation at anaphase (FIG. 3). Cyclin B and securin start

Monotelic attachment
A condition in which only one sister kinetochore in a pair of sister kinetochores is attached to kinetochore microtubules. Monotelic attachment is a normal stage during the process of microtubule–kinetochore attachment and chromosome bi-orientation in prometaphase. The unattached kinetochore of a mono-orientated chromosome is a potent checkpoint signal.

Histone variant
A non-allelic variant of the histone proteins that has specific expression and localization patterns.

to be degraded after the last chromosome has aligned, and they become largely depleted prior to anaphase[84,85]. The addition of spindle poisons early during this process stops proteolysis immediately and blocks anaphase onset in a SAC-dependent manner[84]. Interference with kinetochore assembly, impairment of microtubule motors (for example, dynein or CENP-E) and interference with microtubule dynamics also activate the SAC (reviewed in REF. 86).

Certain SAC proteins are immediately removed after the attachment of microtubule plus-ends and formation of kinetochore microtubules. For example, MAD2 localizes to unattached kinetochores in prometaphase or in cells treated with nocodazole to induce microtubule depolymerization and prevent formation of microtubule–kinetochore end-on attachments[72,87]. Conversely, the amount of MAD2 becomes highly reduced at metaphase kinetochores (50–100-fold compared with unattached prometaphase kinetochores). Therefore, kinetochore localization of MAD2 decreases substantially as kinetochores fill more of their attachment sites with kinetochore microtubules (occupancy).

Because MAD1 and MAD2 accumulate at unattached kinetochores and are removed on attachment, their kinetochore localization is interpreted to signify lack of attachment[45,87,88]. In metazoans, MAD2 depletion from kinetochores depends not only on microtubule attachment but also on cytoplasmic dynein motility along microtubules. Inhibiting dynein at metaphase kinetochores results in the return of MAD2 to ~25% of the level at unattached kinetochores without a loss in kinetochore-microtubule number[45,46]. This indicates that kinetochore-microtubule formation promotes MAD1 and MAD2 dissociation by providing high local concentrations of microtubules to promote the ATP-dependent motility of dynein along microtubules[45,46].

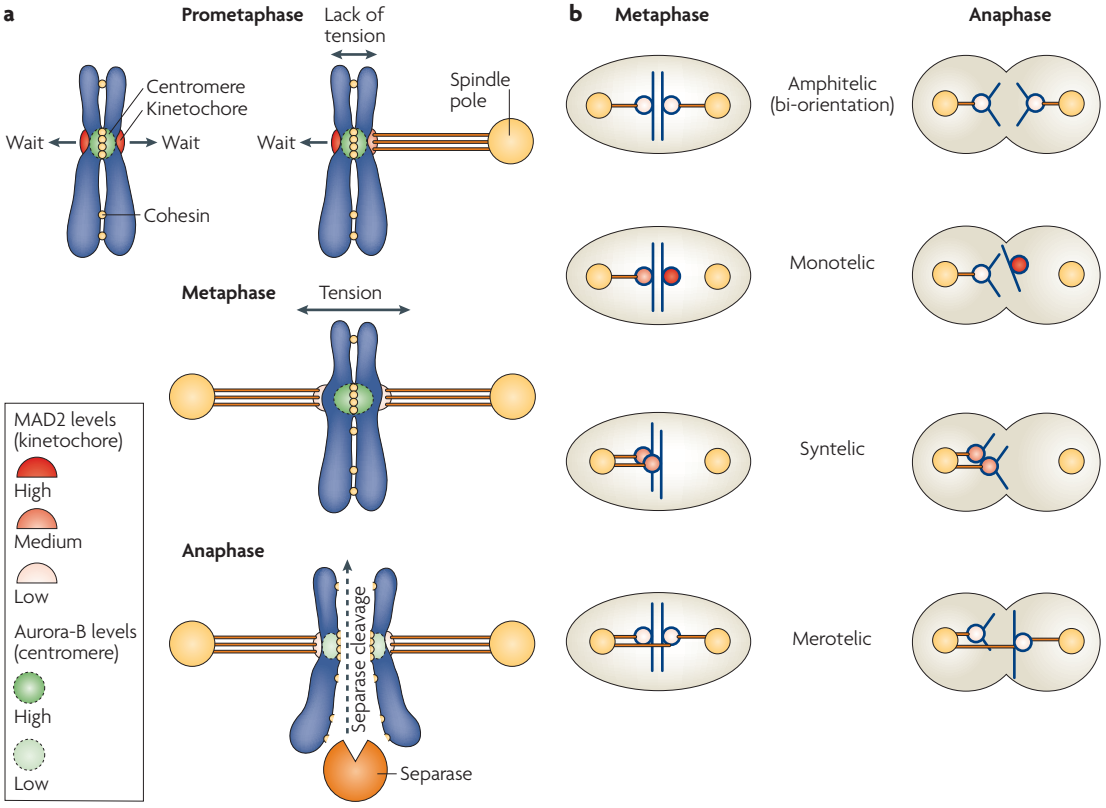

Figure 3 | **The attachment process. a** | Unattached kinetochores generate a 'wait' signal and recruit the spindle-assembly checkpoint (SAC) proteins. The levels of mitotic-arrest deficient homologue-2 (MAD2) are high at unattached kinetochores (left) and moderately high at attached kinetochores in a monotelic pair (right). Under these conditions, the Aurora-B kinase concentrates at centromeres and is believed to be activated by the lack of tension between the sister chromatids. Bi-orientation depletes MAD2 (and budding uninhibited by benzimidazole (BUB)R1) from kinetochores and promotes the acquisition of tension in the centromere area, which is visualized as an increase in the inter-kinetochore distance between sister chromatids. When all chromosomes have achieved this situation, the SAC signal is extinguished and anaphase ensues thanks to the activation of separase, which removes sister-chromatid cohesion by proteolysing cohesin[7]. At entry into anaphase, Aurora-B is also depleted from the centromere region. **b** | Correct and incorrect attachments can occur during mitosis, and correction mechanisms exist to prevent incorrect chromosome inheritance, which would occur if improper attachment persisted until anaphase. Monotelic attachment is a normal condition during prometaphase before bi-orientation. In syntelic attachment, both sisters in a pair connect to the same pole. A mechanism that corrects this improper attachment depends on the Aurora-B/Ipl1 kinase (reviewed in REF. 94). Merotelic attachment occurs quite frequently and is also corrected by the Aurora-B kinase[172,173]. The SAC might be able to sense syntelic attachment, but it is unable to detect merotelic attachment[94].

Syntelic attachment
A type of incorrect microtubule–kinetochore attachment in which both sister kinetochores become attached to microtubules from the same spindle pole. Syntelically attached kinetochores often reside near a pole and do not congress to the spindle equator. The correction of syntelic attachment requires the Aurora-B/Ipl1 kinase.

Merotelic attachment
A type of incorrect microtubule–kinetochore attachment in which a kinetochore becomes attached to microtubules from both spindle poles. Merotelic attachment by one sister kinetochore does not prevent chromosome bi-orientation by monotelic attachment of the other sister kinetochore and it does not activate the spindle-assembly checkpoint. However, a mechanism of correction based on the Aurora-B/Ipl1 kinase exists.

SAC inactivation by tension. In addition to microtubule–kinetochore attachment, tension is important for SAC inactivation[68,89]. Stretching of centromeric chromatin on bi-orientation increases kinetochore-to-kinetochore distance and kinetochore tension (FIG. 3a). Microtubule–kinetochore attachment is normally destabilized at low kinetochore tension and stabilized by high tension between bi-orientated sister kinetochores[89,90]. Tension might therefore provide a fundamental criterion to discriminate against incorrect attachments. For example, if both sister kinetochores attach to microtubules from the same pole (syntelic attachment; FIG. 3b), not enough tension is generated and microtubule–kinetochore attachment is destabilized to correct the problem. This destabilization depends on Aurora-B/Ipl1 kinase[23,91–93].

Aurora-B/Ipl1 is also critical for correcting merotelic attachments (FIG. 3b), which are not sensed by the SAC. Merotelic attachment occurs when one sister kinetochore becomes attached to microtubules from opposite poles. Bi-orientation of chromosomes with merotelic kinetochores produces sufficient occupancy and tension to turn off SAC activity. As a result, merotelic kinetochores, if left uncorrected, can produce lagging chromatids and potential chromosome mis-segregation in anaphase[94]. Tension and centromere stretch also seem to have a significant role in turning off SAC activity by inhibiting the association rate of SAC proteins at kinetochores. For example, SAC activity is turned on by adding taxol or low doses of vinblastine to metaphase HeLa cells to stop kinetochore-microtubule dynamics and reduce tension. (Taxol decreases inter-kinetochore tension in bi-orientated sisters while stabilizing the number of metaphase kinetochore microtubules.) In cells undergoing these treatments, SAC activation correlates with a significant increase in the concentration of BUBR1 and BUB1 at kinetochores, as well as the phosphorylation by PLK1 kinase of an unknown phosphoepitope that is detected by the antibody 3F3/2 (REFS 65,87,89). This type of experiment does not prove that lack of tension alone is sufficient to activate the SAC, as metaphase cells treated with taxol were shown to contain one or a few MAD2-positive kinetochores (possibly owing to low occupancy), which could be sufficient to trigger a SAC arrest[87]. However, 3F3/2 phosphorylation and re-recruitment of BUBR1 and BUB1, but not MAD2, to all kinetochores imply that the reduction in tension changed the kinetochore in a significant way, independently of any change in occupancy. Therefore, irrespective of whether the checkpoint is indeed regulated by tension independently of occupancy, kinetochore localization of checkpoint components is differentially regulated by occupancy and tension.

Even so, it must be remembered that distinguishing the relative contributions of tension and attachment when manipulating spindles is difficult, as interfering with the creation of tension probably affects attachment[91,95]. We remain unaware of any SAC or SAC-related protein for which activity is directly regulated by tension in an adequately reconstituted system. SAC activity during the successive phases of bi-orientation (FIG. 3a) that macroscopically define the attainment of attachment and tension seems to result in the generation of the same cell-cycle inhibitor, the MCC[18]. Consistently, MAD2 is required to sustain the SAC even after its substantial depletion from kinetochores[13,45]. Similarly, although kinetochore MAD2 remains low in taxol-treated cells, MAD2 is critically required to maintain the taxol-dependent arrest[13,45,87,88]. To what extent attachment and tension are separable at the molecular level will be clarified through a better understanding of the physical connections at the kinetochore between the microtubule-binding machinery and the SAC.

MCC as a SAC effector

In *S. cerevisiae*, the MCC contains at least Mad2, Mad3, Bub3 and Cdc20. The complex accumulates in mitosis, when it seems to associate with the APC/C[14,15,17]. Within the MCC, both Mad2 and Mad3 bind Cdc20 directly[5,14]. The interaction of Mad3 with Cdc20 requires all SAC proteins, whereas only Mad1 and Mps1 are required to form Mad2–Cdc20 (REFS 5,14,15). The Mad2–Cdc20 subcomplex (which lacks Mad3 and Bub3) also accumulates in mitotic cells[17]. Cdc20 stability during SAC activation is thought to be regulated[96]. Furthermore, the activation of Cdc20 requires the chaperonin CCT, but this precedes inhibition by the SAC[97].

Box 1 | **What is a SAC gene?**

Checkpoints are regulated transition points where progression to the next cell-cycle phase can be arrested by negative signals if the correct conditions arise. The protein components that are responsible for such controls are also often defined as a checkpoint. Indeed, in this article we often resort to this second interpretation. In *Saccharomyces cerevisiae*, the spindle-assembly checkpoint (SAC) is a non-essential device that meets the definition of a checkpoint, in that it only becomes essential in response to 'damage'; that is, perturbations in the microtubule–kinetochore attachment process. In metazoans, on the other hand, the SAC is an essential pathway, the integrity of which is required to prevent chromosome mis-segregation and cell death. At the beginning of mitosis, when kinetochores are unattached, the SAC is activated to coordinate the variable duration of microtubule–chromosome attachment and chromosome alignment — an intrinsically stochastic process — with the subsequent loss of sister-chromatid cohesion at the metaphase-to-anaphase transition. Cells that have an impaired SAC enter anaphase prematurely and suffer an intolerable level of genomic instability. Indeed, SAC abrogation generally results in early lethality in metazoans[164]. Studies with hypomorphic SAC alleles in the mouse uncovered an involvement of certain SAC proteins in ageing, which is possibly indicative of participation in other cellular functions[164].

The non-essential versus essential character of the SAC in *S. cerevisiae* and metazoans probably reflects specific differences in the way chromosomes attach to the spindle. Because the SAC is essential in metazoans, the definition of what a SAC gene is must be rather liberal. For example, there are several structural components of the kinetochore, the functions of which are required to sustain the checkpoint, such as the Ndc80 (known as HEC1 in humans), minichromosome maintenance protein-21 (Mcm21) and Mtw1 (known as MIS12 in humans) complexes (for example, see REFS 74,75). These protein complexes are probably important for the localization of the SAC proteins to the kinetochore and are therefore required for the SAC. *CDC20*, the abrogation of the protein product of which results in a mitotic arrest, would also escape the classical definition of a checkpoint gene, which defines checkpoint genes as negative regulators of a cell-cycle transition (see above). An operational definition would describe a SAC component as one that is directly involved in sensing the state of attachment of kinetochores to spindle microtubules and/or that participates in relaying this information to the cell-cycle machinery. The limits of such a definition are inevitably rather arbitrary.

Box 2 | **Chromosome mis-segregation, aneuploidy and cancer**

Discrepancies in chromosome number (aneuploidies) during meiosis are responsible for miscarriages and for human diseases, such as Down's syndrome. Aneuploidy also occurs frequently in cancer cells[164]. The attractive hypothesis that chromosomally unstable colon cancer cells might be generated as a consequence of impaired spindle-assembly checkpoint (SAC) activity (reviewed in REF. 164) has not been fully corroborated by data so far[165]. The available database of mutations of SAC genes in tumours is growing only slowly. Indeed, the essential character of the SAC (BOX 1) can hardly be reconciled with the idea that the mutational inactivation of the SAC might be causative of tumorigenesis. It is possible, however, that a weakening of the SAC and a moderate rate of chromosomal instability will facilitate tumorigenesis. Rather than resulting from a drastic inactivation of the SAC, these effects are more likely to derive from a mild suppression of SAC function or from mild perturbations of chromosome-segregation function that escape the SAC (for example, see REFS 164,166). On the other hand, the essential character of the SAC implies that its complete inhibition will be deleterious for normal cells as well as cancer cells. It was shown that cancer cell lines undergo apoptosis by a cell-autonomous pathway when they are depleted of SAC components[167]. This leads to a different model, in which SAC inactivation might become a desirable way to kill rapidly dividing cancer cells[168]. This model might lie behind the success of the Aurora-kinase inhibitors in the treatment of certain tumour types[169,170]. The molecular links between the components of the SAC and the apoptotic machinery have not yet been uncovered, and therefore represent an interesting field for future investigations.

A partially purified MCC preparation from mitotic HeLa cells contains MAD2, BUB3, BUBR1 (which is orthologous to *S. cerevisiae* Mad3) and CDC20 in apparently equal stoichiometries[8]. This enriched fraction of the human MCC was a 3,000-fold stronger APC/C inhibitor *in vitro* compared with recombinant MAD2 (REF. 8). BUBR1 has a distinct binding site on CDC20 from MAD2 and, together, MAD2 and BUBR1 have a synergistic effect on APC/C inhibition[11,12,18,98,99]. At present, it is unclear how and when MAD2, CDC20 and BUBR1–BUB3 intersect to create the MCC (FIG. 5a). It is possible that a MAD2–CDC20 subcomplex functions as a seed to promote MCC formation. In human cells, binding of the N-terminal region of BUBR1 to CDC20 requires previous binding of MAD2 to CDC20 (REF. 99). Furthermore, Mad2 is required for the accumulation of a Mad3–Cdc20 complex in *S. cerevisiae*, whereas the converse is not true (see above). Similarly, BubR1 depletion leads to an only modest reduction in the levels of Mad2–Cdc20 in *Xenopus laevis* extracts, whereas Mad2 is indispensable for the binding of BubR1–Bub3 to Cdc20 (REF. 100). The MAD2–CDC20 subcomplex by itself is not sufficient to sustain checkpoint arrest, as BUBR1 is also needed for checkpoint activation under all conditions. Similarly, Mad3 is required for arrest on Mad2 overexpression in *Schizosaccharomyces pombe*[16]. There is also evidence that at least three kinases, BUB1, MAPK and CDK1, phosphorylate CDC20 to modulate its binding by MAD2 or BUBR1 (REFS 11,12,19,30,35,37,96,98,101). How the MCC inhibits APC/C activity is also poorly understood. The MCC might bind to the APC/C as a pseudosubstrate thanks to a KEN-box motif in BUBR1 (REFS 8–19). This indicates that the MCC needs to disassemble from the APC/C at metaphase to elicit anaphase[18,98].

KEN-box motif
A sequence motif (KENXXXN) that is present in several substrates of the APC/C.

MCC formation away from kinetochores

Although the evidence that kinetochores are necessary to sustain SAC signalling is overwhelming, their contribution to the production of the MCC is controversial. In the next two sections, we will discuss the pros and cons of kinetochore-dependent and kinetochore-independent theories of MCC formation.

The case against kinetochores. Even though all SAC proteins can be recruited to mitotic kinetochores in *S. cerevisiae*[102], the MCC is detectable in checkpoint-defective cells in which Ndc10 — a protein that is required for core kinetochore assembly — has been mutated[15,17]. The MCC is also detectable in normal metaphase-arrested cells in which the SAC is inactive. This indicates that MCC formation does not require checkpoint activation in *S. cerevisiae*[17]. Therefore, the MCC (and its subcomplexes) might form in mitosis independently of kinetochores, and its accumulation *per se* is insufficient to sustain a checkpoint arrest.

A detailed analysis of mitotic timing in mammalian cells that are depleted of different SAC and kinetochore proteins also supports the idea that the MCC might form in a kinetochore-independent manner[74]. The main conclusion from this analysis is that MAD2 or BUBR1 depletions affect the overall timing of mitosis in a kinetochore-independent manner. Conversely, the depletion of MAD1, BUB3 and certain kinetochore proteins, such as the Ndc80/HEC1 complex, results in SAC deficiency without significantly altering the duration of mitosis. Therefore, SAC control in mitosis might be seen as consisting of two phases. In phase I, a kinetochore-independent, cytosolic timer containing MAD2 and BUBR1 controls the average duration of mitosis. In phase II, a 'genuine' SAC signal that requires unattached kinetochores, MAD1 and BUB3 in addition to MAD2 and BUBR1, extends prometaphase if, after extinction of the timer, cells retain one or more unattached kinetochores[74].

The existence of two distinct phases must be reconciled with the consideration that a cytosolic timer containing MAD2 and BUBR1 (REF. 74) is biochemically similar to the MCC and probably coincides with it. If unattached or incorrectly attached kinetochores contribute to generating the MCC late in prometaphase when the timer is close to extinction, there is no obvious reason why they should not do so earlier in prometaphase, synchronizing creation and repression of the MCC with microtubule attachment. Therefore, we suspect that the reason why the depletion of MAD2 and BUBR1 has a stronger effect on mitotic timing, even relative to BUB3, is that these two SAC proteins bind CDC20 directly, possibly causing a temporary inhibition of CDC20 through sequestration, even when checkpoint signalling is compromised. It might be argued that a mitotic timer would be required to prevent mitotic exit prior to full kinetochore maturation and the establishment of a strong SAC signal. Such a timer, however, can already be identified in cyclin A. In HeLa cells, the degradation of cyclin A is required for anaphase onset independently of the SAC[103,104]. As cyclin A degradation starts in prometaphase[103,104], cells are expected to have sufficient time to complete kinetochore assembly and start checkpoint signalling in early prometaphase.

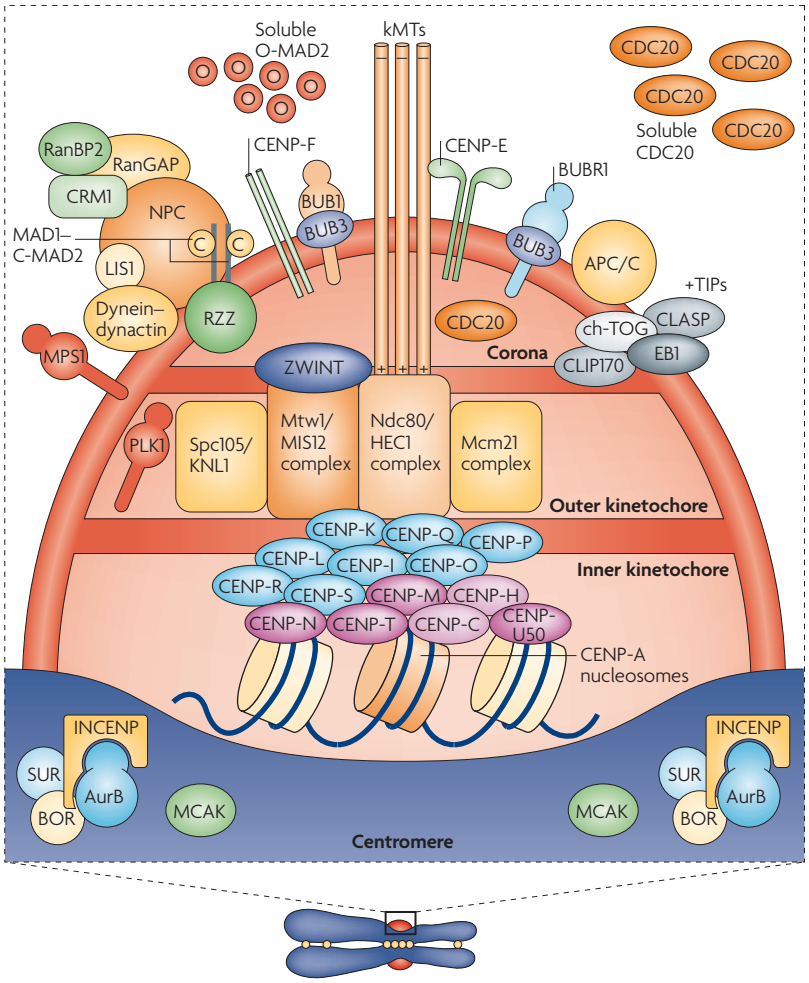

Figure 4 | **The centromere–kinetochore region.** At the heart of the kinetochore is a specialized nucleosome that contains centromere protein (CENP)-A, a histone H3 homologue[48]. Several inner-kinetochore components (cyan and purple ovals) associate with kinetochores throughout the cell cycle[48,174,175]. Many other proteins, including those in multiprotein complexes that contain the Ndc80/HEC1, Mtw1/MIS12, minichromosome maintenance protein-21 (Mcm21) and spindle pole component (Spc)105/KNL-1 proteins, are recruited to the outer kinetochore specifically in mitosis. They provide a landing platform for the spindle-assembly checkpoint (SAC) proteins[56]. The Ndc80/HEC1 complex seems to be directly involved in microtubule binding[80,81]. Several microtubule-plus-end-binding proteins (+TIPs) are important for microtubule–kinetochore attachment[176]. Borealin (BOR), survivin (SUR), Aurora-B (AurB), inner centromere protein (INCENP) and mitotic centromere-associated kinesin (MCAK) preferentially populate the centromere region and regulate the stability of microtubule–kinetochore attachments. They have been implicated in the correction of attachment errors[94]. Several subunits of the nuclear pore complex (NPC) also localize to mitotic kinetochores, but their kinetochore function is unclear. The anaphase-promoting complex/cyclosome (APC/C) is recruited to mitotic kinetochores in a SAC-dependent manner[77,107]. The ROD (rough deal)–ZW10 (zeste white-10)–ZWILCH (RZZ) complex mediates kinetochore recruitment of mitotic-arrest deficient homologue (MAD)1–MAD2 (REF. 31). Large cytosolic pools of MAD2 and CDC20 exist besides the populations that are recruited to the kinetochore. This might be true for other SAC proteins, including budding uninhibited by benzimidazole (BUB)R1 and BUB3, and for the APC/C itself. As discussed in the main text, MAD2 adopts two conformations, O-MAD2 (open MAD2) and C-MAD2 (closed MAD2), which are depicted as red circles (O-MAD2) and yellow circles (C-MAD2). Most proteins indicated in this drawing are present at kinetochores in all metazoans. CLASP, CLIP-associating protein-1; CLIP170, cytoplasmic linker protein-170; EB1, end-binding protein-1; kMTs, kinetochore microtubules; LIS1, lissencephaly-1; MPS1, multipolar spindle-1; PLK1, polo-like kinase-1; RanBP2, Ran-binding protein-2; RanGAP, Ran-GTPase-activating protein; ZWINT, ZW10 interactor.

Kinetochore sensitization. In vertebrates, mature kinetochores only exist in mitosis, whereas the MCC might exist throughout the cell cycle[8], which questions the role of kinetochores in MCC generation. One report has shown that active MCC can be isolated from either mitotic or interphase extracts; however, MCC inhibits only the mitotic, and not the interphase, APC/C[8]. Rather than being involved in the generation of the MCC, unattached prometaphase kinetochores might therefore 'sensitize' the APC/C to inhibition by the MCC[8,56,105,106]. The model predicts that certain components of the SAC, probably those that are not part of the MCC, are important to recruit and sensitize the APC/C at mitotic kinetochores. Indeed, there is some evidence that APC/C is recruited to kinetochores and that recruitment requires a functional SAC. The functional consequences of this recruitment are potentially important, but they are currently unclear[77,107].

Despite the attractiveness of the sensitization model, the presence of an interphase form of the MCC has not been confirmed[18,27,98,108]. Furthermore, recombinant Mad2 and BubR1 inhibit the *X. laevis* interphase APC/C *in vitro*[98], which is in contrast to the idea that interphase APC/C is not susceptible to inhibition by the MCC. Finally, the MCC does not form in *X. laevis* meiosis II extracts — despite the presence of its constituents — unless sperm nuclei are added together with nocodazole to prevent spindle assembly[100].

A kinetochore-based theory of MCC formation

Localization of all SAC proteins at unattached kinetochores in mitosis strongly hints that kinetochores contribute to MCC formation. Investigations of the mechanisms of SAC recruitment to kinetochores are leading to detailed molecular models of SAC function. Here we discuss one model, the 'MAD2-template model', which stems from investigations of kinetochore dynamics of SAC proteins by fluorescence recovery after photobleaching (FRAP) and from structural studies[28,50–53,109–111].

Kinetochore dynamics of MAD2. All MCC constituents cycle on and off kinetochores with high turnover rates, which is consistent with the idea that the MCC is created dynamically at or near kinetochores[50–53]. The kinetochore dynamics of MAD2 are well understood. Kinetochore MAD2 consists of two roughly equal-sized pools: a more stably bound pool and a mobile, high-turnover pool[53]. MAD2 binds tightly to MAD1 (FIG. 1), a protein that is required for kinetochore localization of MAD2 and that has been shown to be a rather stable kinetochore resident during prometaphase (REFS 27,28,51,54,73,112). When bound to MAD1, MAD2 adopts a conformation known as closed-MAD2 (C-MAD2, or N2-MAD2) (REFS 110,111) (FIG. 5b). As C-MAD2 does not seem to dissociate from MAD1 during checkpoint activation, MAD1–C-MAD2 accounts for the more stable kinetochore pool of MAD2 (REFS 28,51,53,54). C-MAD2 bound to MAD1 is the kinetochore receptor for a different conformer of MAD2, open-MAD2 (O-MAD2, or N1-MAD2)[28,109–111] (FIG. 5b). Interestingly, this interaction can be reproduced *in vitro* with distinct conformers of an otherwise chemically

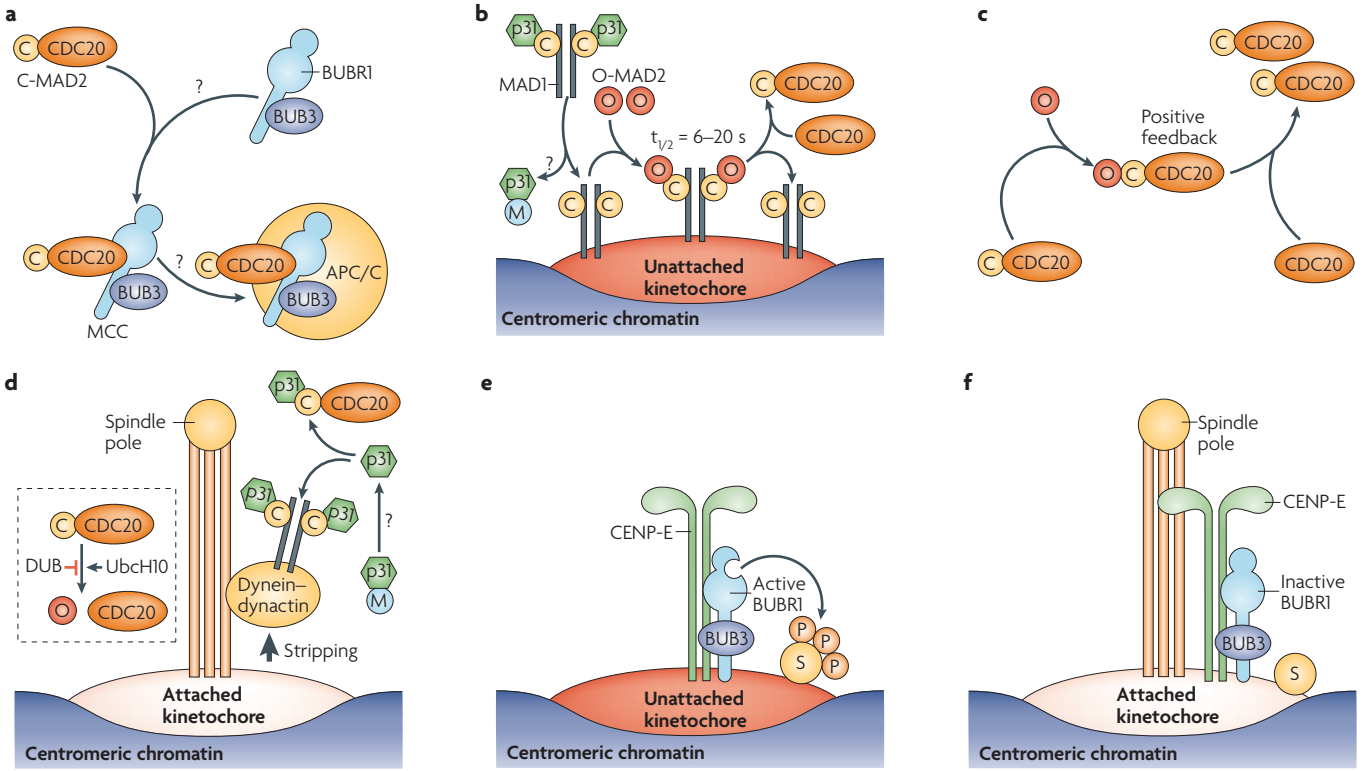

Figure 5 | **Model for the molecular basis of SAC function. a** | The mitotic checkpoint complex (MCC) forms from mitotic-arrest deficient homologue (MAD)2–CDC20 and budding uninhibited by benzimidazole (BUB)R1–BUB3 subcomplexes. MAD2 bound to CDC20 has the C-MAD2 (closed MAD2) conformation. How C-MAD2–CDC20 and BUBR1–BUB3 create the MCC is unclear. A KEN-box motif in BUBR1 might mediate MCC binding to the APC/C. **b** | The MAD2-template model[28,115] proposes a mechanism for creating C-MAD2–CDC20. Unattached kinetochores bind MAD1–C-MAD2–p31^{comet}, and p31^{comet} is then released through an unknown modification (M). MAD1–C-MAD2 (the template) recruits O-MAD2 (open MAD2) to kinetochores and facilitates its structural conversion. This explains the fluorescence recovery after photobleaching (FRAP) cycle of MAD2 (REFS 28,50,51,53,54). O-MAD2 binds CDC20 and turns into C-MAD2, creating a structural copy of C-MAD2–MAD1. **c** | C-MAD2–CDC20 might be involved in a cytosolic auto-amplification reaction based on the same interaction. **d** | At least three regulatory aspects might favour C-MAD2–CDC20 dissociation at anaphase. First, disappearance of unattached kinetochores might result in reactivation of the capacity of p31^{comet} to inhibit the C-MAD2–O-MAD2 interaction and thereby inhibit the catalytic amplification of the SAC signal that is predicted by the template model. Second, non-degradative ubiquitylation of CDC20 in a reaction that involves the E2 enzyme UbcH10 and the de-ubiquitylating protein (DUB) protectin might accelerate the dissociation of C-MAD2–CDC20 (REF. 159). Third, the dynein–dynactin complex 'strips' MAD1–C-MAD2 and other proteins from kinetochores on formation of kinetochore microtubules, decreasing the ability to form new C-MAD2–CDC20 complexes[45]. **e** | In prometaphase, CENP-E activates the kinase activity of BUBR1 at unattached kinetochores[44,119]. The substrates of BUBR1 (S) are unknown. **f** | On microtubule attachment, BUBR1 activity is switched off[44].

identical molecule[28,113] (this is known as conformational dimerization). Therefore, the mobile and immobile fractions of kinetochore MAD2 consist, respectively, of rapidly cycling O-MAD2 and the MAD1–C-MAD2 receptor. In support of this model, mutations in MAD2 that prevent C-MAD2–O-MAD2 dimerization disrupt O-MAD2 recruitment to kinetochores and inactivate the SAC[28,34,114]. In addition, the existence of stable and dynamic fractions of MAD2 can be demonstrated in an *in vitro* FRAP assay where purified recombinant MAD1–C-MAD2 is tightly bound to the surface of beads[54].

The MAD2-template model. CDC20 triggers the same conformational change in MAD2 as the one triggered by MAD1, which is a consequence of the fact that MAD1 and CDC20 have a similar MAD2-binding motif (FIGS 1,4a,b). Therefore, O-MAD2 changes its conformation to C-MAD2 after binding CDC20 (REFS 110,111,113) (FIG. 5a–c). The rationale of the network of interactions that emerges from these studies is that an empty O-MAD2 conformer turns into a CDC20-bound C-MAD2 conformer, and this conversion is facilitated by a stable MAD1–C-MAD2 complex. MAD2 dimers do not form from homogeneous O-MAD2 or homogeneous C-MAD2. Instead, MAD2 dimerization occurs for O-MAD2 plus MAD1-bound C-MAD2 or O-MAD2 plus CDC20-bound C-MAD2. In the cell, most or all MAD1 is bound to C-MAD2, and there is a large excess of O-MAD2 (REF. 27). These premises justify the name 'template model', which reflects the fact that MAD1–C-MAD2 can be viewed as a template for the formation of a C-MAD2–CDC20 structural copy (FIG. 5b).

Fluorescence recovery after photobleaching
(FRAP). An imaging technique that measures the kinetics and extent of fluorescence recovery in small volumes that have been subjected to a short high-energy laser pulse to irreversibly bleach a fluorophore. The recovery curves measure labelled-protein diffusion rates in the cytosol and dissociation rate constants from binding sites.

The K_d of the interaction of O-MAD2 with CDC20 is ~100 nM and cellular concentrations of these proteins predict that binding occurs spontaneously[54,110,111]. The forward rate constant (K_{on}) of this interaction, however, is 3–4 orders of magnitude slower than normal protein-association rates (M. Vink, M. Simonetta, R. Manzoni, A. Ciliberto and A.M., unpublished observations). Therefore, a large catalytic barrier slows down the binding of O-MAD2 to CDC20, which indicates that the conformational change of MAD2 that is needed to promote binding to CDC20 is the rate-limiting step in SAC activation. MAD1-bound C-MAD2 at kinetochores might lower this barrier (FIG. 5b), although how this would take place is currently unclear. Because C-MAD2 bound to CDC20 has the same conformation as C-MAD2 bound to MAD1, its formation might contribute an important autocatalytic prion-like reaction to produce global firing of the checkpoint with a small amount of C-MAD2–CDC20 at the kinetochore, thereby triggering a positive-feedback loop whereby C-MAD2–CDC20 promotes the formation of more C-MAD2–CDC20 (FIG. 5c). This strategy for amplification of the checkpoint signal away from kinetochores, which remains speculative, might provide a molecular mechanism to explain the observation that a single kinetochore is sufficient to activate the SAC[67].

Model predictions. The MAD2-template model predicts that MAD1–C-MAD2 at kinetochores is 'activated' to trigger the conformational rearrangement of O-MAD2 that is required to bind CDC20 (REFS 28,115). The implication is that the cytosolic pool of MAD1–C-MAD2 must be unable to catalyse the structural rearrangement of MAD2, as the latter should be triggered exclusively at unattached kinetochores to which MAD1–C-MAD2 is bound. This predicts that the interaction of O-MAD2 with the cytosolic MAD1–C-MAD2 complex might be inhibited. A second implication is that cells must contain a device to avoid firing the positive-feedback loop based on MAD2–CDC20 in the absence of signals from kinetochores. The non-catalysed binding of MAD2 to CDC20 will ignite the positive-feedback loop predicted by the template model, albeit slowly, as soon as even a few C-MAD2–CDC20 molecules have formed.

These predictions have in common that the catalytic function of C-MAD2 must be under tight kinetochore control. p31^{comet}, a MAD2-binding protein, functions as a negative regulator of the SAC[32,33,54]. *In vitro*, p31^{comet} competes with O-MAD2 for binding to C-MAD2 that is bound to either MAD1 or to CDC20 (REFS 33,54) (FIG. 5b,d). By binding to C-MAD2 with high affinity, p31^{comet} prevents O-MAD2–C-MAD2 dimerization[54]. Kinetochores might negatively regulate the ability of p31^{comet} to bind C-MAD2 in their vicinity, thereby allowing the interaction of O-MAD2 with C-MAD2, which results in the accumulation of MAD2–CDC20 and SAC activation. The reactivation of p31^{comet} at metaphase would then trigger SAC inactivation. While this model awaits confirmation, it must also be said that there are no obvious orthologues of p31^{comet} in lower eukaryotes. Therefore, there may be additional control mechanisms that have not been yet discovered.

The role of SAC kinases

The implications of the mechanism of MAD2 recruitment to kinetochores are far-reaching. Although the precise identity of the kinetochore receptors of the other SAC proteins remains unknown, epistatic relationships for kinetochore localization of SAC proteins have started to emerge, mainly from RNA interference (RNAi) studies. These show that MPS1, BUB1, BUBR1, PLK1, NEK2, MAPK and Aurora-B kinases are important for recruiting SAC proteins to kinetochores and for generating robust SAC activity (for example, see REFS 18,22,23,41, 76,77,83,116–118).

BUBR1, CENP-E and microtubule attachment. There have been contradictory reports as to whether the kinase activity of BUBR1 is required for SAC function[100,119]. The BUBR1 orthologue in *S. cerevisiae*, MAD3, lacks kinase domains (FIG. 1). Because MAD3 and BUBR1 seem to have equivalent roles in the MCC, the catalytic activity of BUBR1 in the MCC might not be required. BUBR1 kinase activity might be required to control other aspects of kinetochore signalling or chromosome alignment[22,120].

BUBR1 interacts with, and phosphorylates, CENP-E, a microtubule-plus-end-directed motor that contributes to chromosome alignment[108,121,122]. In *X. laevis*, the loss of CENP-E overrides the SAC, which indicates that CENP-E is a *bona fide* component of the SAC[43]. The effects of CENP-E depletion have been more difficult to interpret[121–124]. Substantial depletion of CENP-E in human cells or CENP-E-null mutations in mouse embryo fibroblasts (MEFs) cause the alignment of almost all chromosomes at the spindle equator, but a few have difficulty in alignment[125]. This is probably due to CENP-E having a role in kinetochore motility towards the spindle equator[126]. In the absence of CENP-E, a few unattached kinetochores are unable to sustain SAC activity; however, adding nocodazole or taxol to disrupt attachment or tension at most chromosomes activates robust SAC activity[125]. This implies that CENP-E-mediated amplification of the SAC signal becomes indispensable for checkpoint activity when there are only one or a few unattached kinetochores.

CENP-E binds BUBR1 and activates its kinase activity *in vitro*[44,119,121]. This activity is repressed when CENP-E binds to microtubules, which indicates that BUBR1 kinase activity is high before kinetochore-microtubule formation and inactivated following microtubule–kinetochore attachment. This model was tested with a truncated form of CENP-E that lacks the microtubule-binding domain; when substituted for the endogenous CENP-E, it caused chronic activation of the SAC[44,119]. A possible caveat is that the constitutive activation of the SAC that is observed with a construct that lacks the CENP-E microtubule-binding domain — but retains the BUBR1 activation domain — might be caused by a genuine perturbation of the attachment process.

Multiple roles of BUB1. Slow FRAP recovery rates indicate that BUB1 is a more stable kinetochore resident than BUBR1 or BUB3 (REF. 51). BUB1 kinetochore levels are downregulated after kinetochore attachment and are also sensitive to the establishment of inter-kinetochore tension[127].

In various organisms, BUB1 contributes to the centromere–kinetochore recruitment of MAD1, MAD2, BUBR1, BUB3, MPS1, CDC20, CDC23, CENP-F, protein phosphatase (PP)2A, MEI-S332 (also known as shugoshin), mitotic centromere-associated kinesin (MCAK, also known as KIF2C) and possibly CENP-E[29,69,77,82,83,128–132]. For at least a subset of these proteins, BUB1 might participate in their recruitment by contributing to the structural stability of the centromere–kinetochore[29,128,133]. BUB1 also contributes directly to the formation of a complex of the MCC with the APC/C, and it contributes this function synergistically with Aurora-B/Ipl1 (REF. 18). Whether the catalytic activity of BUB1 — possibly stimulated by phosphorylation at unattached kinetochores[29,127,134,135] — is important for this is contentious[18,82,129,134]. However, BUB1 binds and phosphorylates CDC20 to sensitize it to SAC inhibition[14,30,136].

A role for BUB1 in chromosome alignment has also emerged[83,129–131,137]. This could be linked to the function of BUB1 in the localization of a complex comprising MEI-S332/shugoshin and the PP2A phosphatase to the centromere[132]. Loss of this complex on inhibition of BUB1 causes the loss of sister-chromatid cohesion at centromeres[132]. Whereas MEI-S332/shugoshin has been reported to be important for the detection of tension in *S. cerevisiae*[138], removal of MEI-S332/shugoshin results in the activation of a stable mitotic arrest in vertebrate cells[132].

MPS1 and kinetochore recruitment. MPS1, a dual-specificity kinase, is a core component of the SAC[24,26]. In vertebrates, MPS1 is required for kinetochore localization of MAD1, MAD2, PLK1 and CENP-E[25,77,118,139,140]. As measured by FRAP, MPS1 exchanges completely with kinetochores with a half-life of 10 seconds (REF. 51). As this is much faster than for MAD1, MPS1 probably has a regulatory role rather than a structural role in recruiting MAD1–MAD2 to kinetochores. Interestingly, MAD1, MAD2 and MPS1 all localize to the nucleoplasmic side of the nuclear pore complex during interphase[139,141]. The potential connection between the nuclear pore complex and kinetochores has recently been reviewed[142].

Aurora-B/Ipl1: a tension sensor? If a tension sensor exists, the chromosomal passenger complex (CPC) that contains the Aurora-B/Ipl1 kinase is an appealing candidate (reviewed in REFS 143,144). Aurora-B is not required to sustain a mitotic arrest that is caused by the lack of kinetochore–microtubule interactions (for example, by nocodazole depolymerization of the spindle). However, it is required to arrest the cell cycle temporarily under conditions that prevent the creation of inter-kinetochore tension — for example, during the division of non-replicated chromosomes[145]. Aurora-B/Ipl1 controls the microtubule-severing KINI kinesin MCAK, the DASH complex and the Ndc80/HEC1 complex at the microtubule–kinetochore interface[143,144]. It is believed that these interactions allow Aurora-B/Ipl1 to detect and destabilize faulty microtubule–kinetochore attachments. This generates unattached kinetochores that are detected by the SAC[91]. Indeed, the SAC activation that has been observed with several kinetochore mutants in *S. cerevisiae* might require the creation of unattached kinetochores by Aurora-B/Ipl1[92,146].

In this model, the involvement of Aurora-B/Ipl1 in the SAC is indirect, as it is limited to the resolution of faulty microtubule–kinetochore connections, which, in turn, results in the generation of unattached kinetochores that activate the SAC. Other observations, however, directly implicate Aurora-B/Ipl1 in the SAC. For example, Aurora-B is required to support a taxol-induced SAC arrest[22,23,145] when, at metaphase, almost all kinetochores maintain their full microtubule occupancy[87]. Furthermore, if the role of Aurora-B/Ipl1 in the SAC were limited to creating unattached kinetochores, the addition of a spindle poison such as nocodazole in the absence of Aurora-B/Ipl1 activity would be expected to promote MAD2 and BUBR1 recruitment to kinetochores. On the contrary, inhibition of Aurora-B significantly reduces MAD2 and BUBR1 localization to kinetochores in nocodazole[18,21,22]. Finally, the kinase activity of Aurora-B is essential to maintain a nocodazole-induced SAC arrest on depletion of BUB1 (REF. 18), the activity of which is required for the recruitment of SAC proteins to kinetochores. All this implies that Aurora-B/Ipl1 is directly involved in the SAC response, presumably by promoting MCC assembly.

Localization of other SAC proteins to kinetochores. Kinetochore recruitment of the MAD1–MAD2 complex requires normal levels of CENP-I, Ndc80/HEC1, MPS1, Aurora-B, the RZZ complex and other kinetochore proteins[74,75,82,102,140,147–149]. Another relationship recently identified concerns a requirement for the ZW10 interactor (ZWINT) in kinetochore recruitment of the RZZ complex and of MAD1–MAD2. Ablation of ZWINT results in a checkpoint defect, presumably because the RZZ, and consequently MAD1–MAD2, cannot be recruited to kinetochores[150,151]. There is also evidence that HEC1 binds ZWINT[152], which is consistent with the observation that HEC1 is also required for the recruitment of MAD1–MAD2 (FIG. 4).

In general, RNAi studies have often failed to provide clear correlations between kinetochore localization of SAC proteins and functional integrity of the SAC. For example, depletion of CENP-I or Ndc80/HEC1, or even of a SAC component such as BUB1, can result in a MAD2-dependent mitotic arrest, despite levels of MAD2 and/or BUBR1 at kinetochores being 'undetectable' by immunofluorescence microscopy[83,140,148,149,153]. It is important to recognize that residual amounts of the SAC components at damaged kinetochores can be sufficient to catalyse the formation of the MCC. Consistently, more complete depletions of Ndc80/HEC1 or BUB1 (~99% or greater) result in a checkpoint defect[74,129]. This indicates that there is an inherent danger of reaching the wrong conclusions about protein function in the SAC on the basis of RNAi experiments. It is important to remember that a single, normal, unattached kinetochore can maintain SAC activity[67]. A perturbation that affects all kinetochores and causes them to recruit undetectable amounts of SAC proteins could also produce SAC activation if the integrated activity from a large number of kinetochores is equal to, or greater than, that produced by a single, normal, unattached prometaphase kinetochore[13].

Chromosomal passenger complex
(CPC). A protein complex that shares a characteristic pattern of association with chromatin in prophase, centromeres in metaphase and early anaphase, and the midzone and midbody in late anaphase and telophase, respectively.

Turning the checkpoint off

Several mechanisms contribute to the inactivation of the SAC after chromosome bi-orientation during normal cell cycles.

Role of dynein motility. A key process in turning off the SAC is the 'stripping' of MAD1, MAD2, the RZZ complex, MPS1, CENP-F and other protein components from the kinetochore, which results in their redistribution to the poles[49] (FIG. 5d). In metazoans, this process depends on dynein motility along microtubules[45,46,154–156]. Whereas dynein has an important role in inactivating the SAC by stripping off MAD1–MAD2 from attached kinetochores[45,46], the regulation of BUBR1 activity by CENP-E depends on CENP-E binding to microtubules (FIG. 5f). Both these 'inactivation pathways' seem to depend critically on kinetochore-microtubule formation and motor activity, as discussed above. There is no nuclear dynein or CENP-E homologue in *S. cerevisiae*. Mutants for minus-directed motor protein Kar3 are synthetically lethal with SAC protein mutants in *S. cerevisiae* and, therefore, Kar3 might have the equivalent function to dynein in checkpoint inactivation[157].

The checkpoint kinases BUB1, MPS1 and Aurora-B promote the recruitment of SAC proteins to kinetochores (see above). Dynein–microtubule-mediated stripping of proteins from kinetochores antagonizes this association[45,46]. Inhibiting ATP to micromolar levels with azide-deoxyglucose will suppress kinases but allow dynein motility. Under these conditions, most peripheral proteins from the outer domain of kinetochores (for example, MAD2, BUBR1, CENP-E, dynein, 3F3/2 phosphoepitope and CDC20) are removed from kinetochores and concentrate at the poles[45]. This can be reversed by raising the levels of ATP[45]. Treatment with nocodazole to remove microtubules, or the addition of dynein inhibitors to block stripping, prevents the loss of these peripheral kinetochore proteins on ATP depletion. This could help to explain why the inhibition of Aurora-B compromises the SAC on taxol treatment, when stripping is not inhibited, but does not do so after nocodazole treatment[22,23]. Nevertheless, it is important to recognize that steady-state concentrations of SAC proteins at kinetochores are subject to different regulatory steps that have profound effects on the overall outcome of SAC activation.

Inactivation by p31^{comet}. Another mechanism of inactivation is centred on p31^{comet}. As explained above, p31^{comet} might function as a brake for the positive-feedback loop that is based on C-MAD2 (REF. 54), and it is possible (but has not been shown as yet) that kinetochores modify this protein to temporarily prevent it from carrying out this function. As part of the MAD2-template model, it is speculated that the reactivation of p31^{comet} on disappearance of unattached kinetochores silences the SAC by turning down the catalytic activation of MAD2 (FIG. 5). Consistent with this prediction, reduction of p31^{comet} delays mitotic exit after release from nocodazole-induced arrest in HeLa cells[32,33]. Concomitantly with the predicted reactivation of p31^{comet}, there seems to be an inhibitory phosphorylation of MAD2 by unknown kinases[158]. As the SAC is regulated by phosphorylation, phosphatases are likely to be important for SAC downregulation, but little is known about this possible mechanism of regulation at present.

Energy-dependent complex dissociation. A third mechanism of SAC inactivation has emerged recently. As indicated above, MAD2–CDC20-complex formation is slow, but it occurs spontaneously. Even if p31^{comet} arrests the positive-feedback loop that is described in FIG. 5c, an input of energy might be required to recreate free MAD2 and CDC20 after accumulation of the MCC and of the MAD2–CDC20 subcomplex, by speeding up the slow dissociation rate of CDC20 from C-MAD2 (see above). The source of energy is unknown, but non-degradative ubiquitylation of CDC20 has been proposed as a possible active mechanism of dissociation of the MAD2–CDC20 complex[159]. Consistently, the de-ubiquitylating (DUB) enzyme protectin antagonizes this reaction and is required to sustain the SAC[159] (FIG. 5d). Therefore, SAC maintenance might be a dynamic state in which the MCC and MAD2–CDC20 subcomplexes are continuously actively dissociated and recreated by unattached kinetochores.

APC/C-induced proteolysis. A fourth mechanism that contributes to SAC inactivation at anaphase is proteolysis induced by APC/C activation. At anaphase, tension is lost at kinetochores because of the loss of cohesion between sister chromatids. This loss of tension does not reactivate the SAC, which indicates that the SAC might be inhibited at anaphase. Anaphase proteolysis of cyclin B and the inactivation of CDK1–cyclin-B kinase activity probably has an important role in inhibiting SAC activity[19,36]. In *S. cerevisiae*, Mps1 is also degraded at anaphase in a Cdc20-dependent manner[160]. Overexpression of Mps1 during anaphase or removal of Cdc20 in anaphase-arrested cells is sufficient to reactivate the SAC, which indicates that removing Mps1 at anaphase might be essential to avoid SAC activation after removal of sister-chromatid cohesion[160]. Whether non-degradable Mps1 expressed at physiological levels also impairs the anaphase downregulation of the SAC is unclear[160]. In vertebrates, anaphase is only triggered when securin and cyclin B are almost entirely degraded[84,85], which indicates that SAC reactivation in anaphase would have no consequences for cell-cycle progression. Bub1 is degraded after anaphase and the expression of a non-degradable form of Bub1 has no effect on the cell cycle[161].

Checkpoint adaptation. The term 'adaptation' (or 'slippage' or 'leakage') describes an escape from mitosis under the continued presence of conditions that normally activate the SAC. For example, adaptation occurs on prolonged treatments with nocodazole[86]. The causes of adaptation are generally unclear but might differ in different species[86]. In rat kangaroo cells and human cells, adaptation correlates with the degradation of cyclin B, but SAC proteins are retained at kinetochores, which indicates that the escape is not caused by a depletion of the SAC signal[162].

Outstanding issues and future directions

The complexity of the SAC network is daunting (FIG. 6). *In vitro* reconstitution studies and the identification of crucial dynamic parameters of the network represent important progress in the SAC field. For example, the MAD2-template model provides a useful rationalization of MAD2 function in the SAC. Many aspects of this model, however, remain purely speculative and an assessment of their validity will require further investigations. For example, it is unclear how the interaction of O-MAD2 with C-MAD2 facilitates the binding of O-MAD2 to CDC20. The hypothesis that C-MAD2–CDC20 functions as a functional copy of C-MAD2–MAD1 in the amplification of the SAC signal will also require adequate testing. Understanding how the MCC forms from its constituent subunits and how it binds and inhibits the APC/C is also important. For this, it will be essential to gain a better understanding of the APC/C itself. Evidently, structural analysis will be instrumental to elucidate these problems.

From a more distant vantage point, it is crucial to understand how kinetochores coordinate the formation

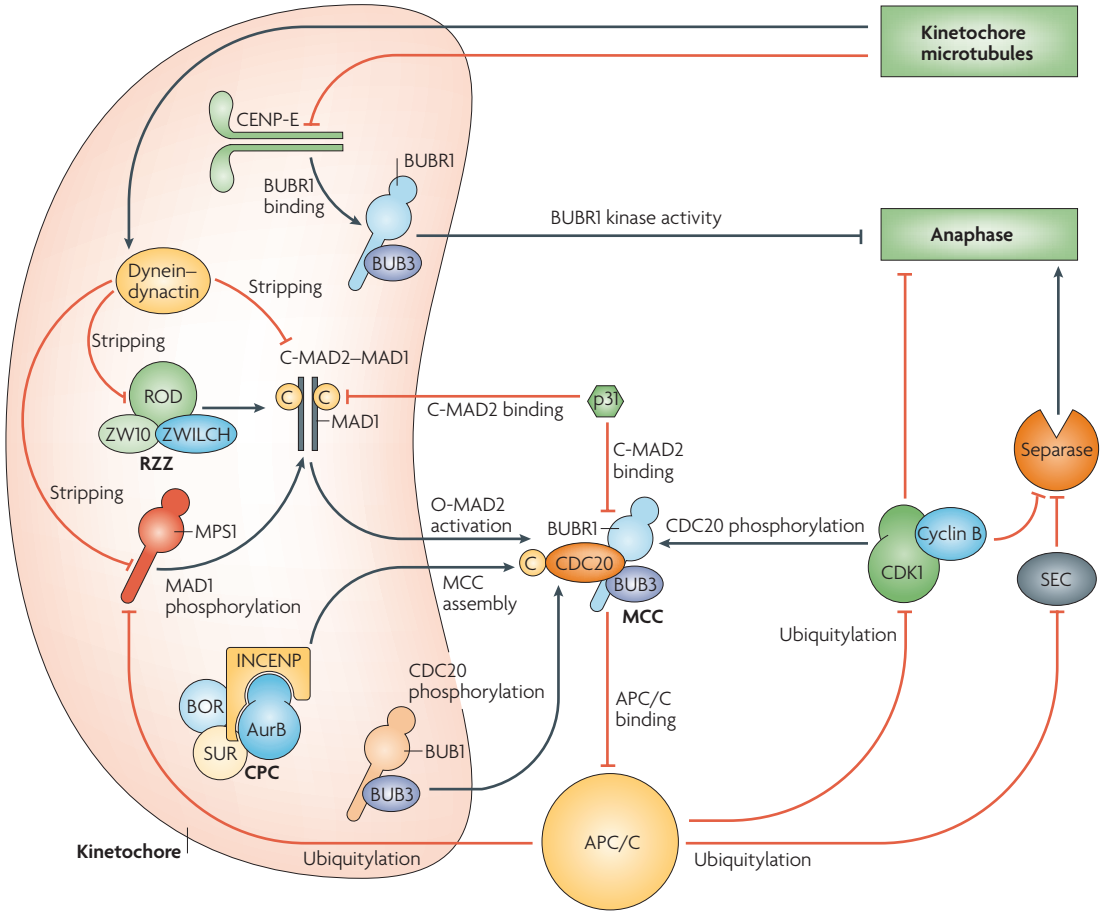

Figure 6 | **'Feedback' pathways in the SAC network.** The spindle-assembly checkpoint (SAC) network is complex. In the absence of kinetochore microtubules in prometaphase, several activities converge on the creation of the mitotic checkpoint complex (MCC), which is an anaphase-promoting complex/cyclosome (APC/C) inhibitor. The Aurora-B (AurB), cyclin-dependent kinase-1 (CDK1) and budding uninhibited by benzimidazole (BUB)1 kinases might stimulate directly the formation of the MCC. Although the mechanistic details for this are still unclear, phosphorylation of CDC20 by BUB1 and CDK1 (and possibly mitogen-activated protein kinase (MAPK); not shown) might contribute to the formation of the MCC. The closed MAD2 (C-MAD2)–MAD1 complex is recruited to kinetochores thanks to the ROD (rough deal)–ZW10 (zeste white-10)–ZWILCH (RZZ) complex and is regulated by the multipolar spindle-1 (MPS1) kinase. C-MAD2–MAD1 activates what is probably the rate-limiting step of MCC formation, the binding of open MAD2 (O-MAD2) to CDC20. In a separate branch of the SAC response, centromere protein (CENP)-E binds and activates BUBR1 at unattached kinetochores. The kinase activity of BUBR1 is directed against unknown substrates. We have depicted BUBR1 kinase activity as having a direct inhibitory effect on anaphase. On microtubule–kinetochore attachment, a mechanism of 'stripping' based on the poleward-directed microtubule-motor activity of the dynein–dynactin complex starts removing SAC proteins, including the RZZ complex, MPS1 and C-MAD2–MAD1, from kinetochores. p31^{comet} might also be reactivated at this stage to inhibit the interaction of C-MAD2 with O-MAD2. The ability of CENP-E to activate BUBR1 also subsides on formation of stable microtubule–kinetochore attachments. All this results in the activation of the APC/C-dependent polyubiquitylation of cyclin B and securin (SEC) and their subsequent proteolysis, which then allows execution of anaphase through activation of separase. The ubiquitylation of MPS1 by the APC/C also seems to be required to irreversibly inactivate the SAC at anaphase. BOR, borealin; CPC, chromosomal passenger complex; INCENP, inner centromere protein; SUR, survivin.

of kinetochore microtubules and SAC downregulation in the cytosol. The physical linkages between the microtubule-binding machinery at kinetochores and the SAC proteins must be identified and understood at the structural level. One such link is the Ndc80/HEC1 complex, which is essential for proper kinetochore recruitment of MAD1, MAD2 and MPS1 (REFS 74,140, 149,153,163). This complex is directly involved in microtubule attachment[80,81], and is therefore an excellent candidate to regulate the state of SAC activation.

Another good candidate to regulate the state of SAC activation is CENP-E, as this protein is also involved in microtubule binding and in its coordination to BUBR1 kinase activity[44,119,121]. Identifying the kinase regulatory pathways at the kinetochore that are involved in SAC activation or inactivation, MCC formation and APC/C inhibition will also be crucial to future progress. For this, a comprehensive list of substrates of the checkpoint kinases, as well as a characterization of their function, is urgently required.

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Competing interests statement
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