Review

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Joined at the hip: kinetochores, microtubules, and spindle assembly checkpoint signaling

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Error-free chromosome segregation relies on stable connections between kinetochores and spindle microtubules. The spindle assembly checkpoint (SAC) monitors such connections and relays their absence to the cell cycle machinery to delay cell division. The molecular network at kinetochores that is responsible for microtubule binding is integrated with the core components of the SAC signaling system. Molecular-mechanistic understanding of how the SAC is coupled to the kinetochore–microtubule interface has advanced significantly in recent years. The latest insights not only provide a striking view of the dynamics and regulation of SAC signaling events at the outer kinetochore but also create a framework for understanding how that signaling may be terminated when kinetochores and microtubules connect.

The SAC at a glance

Achieving and maintaining proper interactions between chromosomes and spindle microtubules is the be-all and end-all of faithful chromosome segregation. Proper interactions are obtained by embedding the plus ends of microtubules into the microtubule-attachment sites of chromosomes, known as kinetochores. Such 'end-on' attachments enable chromosome biorientation, a state in which the two sister chromatids of a chromosome are attached to opposing spindle poles allowing them to be pulled to opposite sides during cell division. Improper attachments are corrected by the error-correction machinery, orchestrated by the Aurora B kinase. Aurora B phosphorylates microtubule-binding proteins at the outer kinetochore, triggering changes in the dynamics of the microtubules and weakening the affinity of the kinetochore for microtubules [1]. Thus, during mitosis, kinetochore–microtubule interactions are frequently created and destroyed until finally all chromosomes are bioriented and attachments stabilized. Throughout, the lack of stable attachments needs to be communicated to the cell cycle machinery, which cannot be permitted to initiate chromosome segregation (anaphase) and cell division. The messenger is the SAC (also known as the mitotic checkpoint) (Figure 1).

The transition to anaphase is triggered by the E3 ubiquitin ligase APC/C, which tags inhibitors of mitotic exit (CYCLIN B) and of sister chromatid disjunction (SECURIN) for proteasomal degradation [2]. The SAC has a one-track mind, inhibiting APC/C as long as incorrectly attached chromosomes persist. It goes about this in the most straightforward way possible: it assembles a direct and diffusible inhibitor of APC/C at kinetochores that are not connected to spindle microtubules. This inhibitor is named the mitotic checkpoint complex (MCC) (Figure 1).

The kinetochore-derived SAC signal is generated at a supercomplex called the KMN network, formed by three different subcomplexes: KNL1-C, MIS12-C, and NDC80-C. This network is the pre-eminent kinetochore interface for contacting microtubules and the main target of the error-correction machinery (Box 1). Thus, the attachment site at kinetochores is intimately connected with the SAC machinery. The connection is a two-way street: many SAC components can alter the microtubule affinity of the KMN network and improve error correction, thereby ensuring that error correction and SAC activities are coordinated in space and time (Box 2).

MCC assembly is orchestrated by the kinase MPS1, which associates with the NDC80-C subcomplex of the KMN network (Figure 2) [3,4]. There, it orchestrates the recruitment of an interlinked network of SAC proteins including the components initially identified in budding yeast genetic screens: BUB1, BUB3, MAD1, MAD2, and Mad3/BUBR1 (human protein nomenclature is used unless otherwise stated). MAD2, BUB3, and BUBR1 constitute the final effector of the pathway (the MCC) and its assembly critically depends on BUB1 and MAD1, along with some additional auxiliary proteins [5,6]. Since the discovery of this pathway more than 20 years ago, the field is progressing rapidly, and several recent excellent reviews have compiled the history, basics, and principles of the SAC [5–9]. The aim of this review is to expand on these with recent insights and a particular focus on the integration of the KMN and SAC networks. This is a narrative in reverse, from effector back to initial and local response, to ensure focus on the events that matter for SAC effector assembly.

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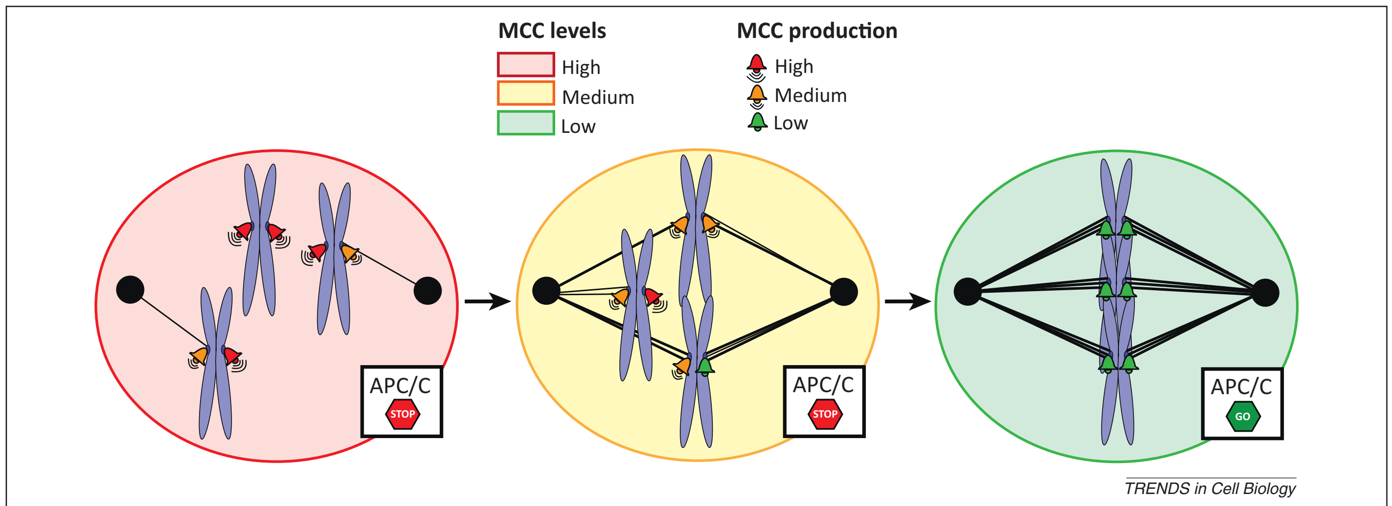

Figure 1. The spindle assembly checkpoint (SAC) response. Unattached kinetochores activate the SAC response (bells), which culminates in assembly of the APC/C-inhibitory mitotic checkpoint complex (MCC). Total MCC levels (indicated by background colors) are determined by the rate of MCC production at kinetochores, which is initially high (red bells) but declines as kinetochores connect with increasing numbers of microtubules (yellow bells), until finally production is halted altogether (green bells). The SAC must nevertheless maintain MCC levels above an undefined threshold to ensure sufficient APC/C inhibition (STOP signals) and prevent anaphase onset and mitotic exit. Only when all kinetochores have met the conditions for correct chromosome segregation is the block on APC/C released (GO signal), allowing cells to proceed to anaphase.

Inhibiting anaphase: a matter of MCC dynamics

Assembly of the MCC delays anaphase onset by preventing CDC20 from activating APC/C (Figure 2). Recent evidence suggests that the MCC is also continuously disassembled [10–13], possibly to allow rapid APC/C activation when the last chromosome has achieved proper attachments. The ability to maintain mitotic delay is thus likely to depend on higher MCC assembly rates compared with disassembly rates.

For proper SAC function, stable association between the MCC and APC/C is required [14]. Despite continuing uncertainty regarding the exact roles of each MCC subunit, it is clear that MAD2 and BUBR1 are indispensible for APC/C inhibition. BUBR1 and its orthologs harbor a KEN box near their N termini, a motif normally recognized by APC/C coactivators as a degron [2]. The KEN box of BUBR1, however, competes with other substrates for

Box 1. The KMN network: Velcro for microtubules

A fully attached human kinetochore is bound by approximately 20 microtubules. In the recently proposed 'lawn' model, hundreds of microtubule-binding protein complexes on a human kinetochore cooperate to maintain attachment [96]. The predominant microtubule-binding complex is known as the KMN network [7,97]. It is assembled from three subcomplexes: KNL1-C (KNL1 and Zwint-1), MIS12-C (NNF1, MIS12, DSN1, and NSL1/MIS14), and NDC80-C (HEC1, NUF2, SPC24, and SPC25) (see Figure 2 in main text). MIS12-C connects the inner kinetochore with KNL1-C and NDC80-C [7,97], which extend to the cytosol to directly contact microtubules. NDC80-C interacts with microtubule filaments through binding the interface between two tubulin subunits [98]. For this, it utilizes two globular CH domains present near the N termini of HEC1 and NUF2.

An important role is reserved for an 80-amino acid N-terminal sequence of HEC1 known as the tail. It is suggested to promote end-on attachments by enhancing NDC80-C oligomerization or by directly contacting the microtubule lattice [97]. In addition, it is the key target of the error-correction machinery. Through multisite phosphorylation of the HEC1 tail, Aurora B is proposed to create electrostatic repulsion between tail and microtubule and hence lower the microtubule-binding affinity of kinetochores (see Figure 2 in main text) [1,7]. In addition, a long coiled-coil region that follows the CH domain of Hec1 is disrupted by a loop that is involved in the recruitment of multiple factors that help in the formation of stable kinetochore–microtubule interactions, including the SKA complex in human cells and the Dam1 complex in yeast [99].

The third component, KNL1-C, also exhibits affinity for microtubules, via a basic patch near the N terminus of KNL1, but the importance of this for the stability of kinetochore–microtubule connections is less well defined [42,89]. KNL1 does play a critical role as a scaffold for the assembly of error-correction and SAC signaling modules (see main text for details). Finally, via a C-terminal coiled-coil region, KNL1 interacts with Zwint-1, thereby recruiting the RZZ complex and Spindly – and thus dynein – to kinetochores [39,43,82].

Box 2. Feedback regulation of error correction by the SAC

The error-correction and SAC machineries are coordinately activated on unattached kinetochores. Unsurprisingly, therefore, the two pathways feed back on each other. As outlined in the main text, Aurora B potentiates MPS1 activation in prophase, ensuring maximal SAC function at the onset of mitosis. Conversely, various SAC proteins modulate error correction by regulating either Aurora B or its targets. BUB1 ensures inner-centromere localization of Aurora B by phosphorylating histone H2A in centromeric nucleosomes to indirectly create a docking site for the Aurora B-associated protein Borealin [100]. BUBR1 harbors a KARD motif that constitutes a docking site for the phosphatase PP2A-B56 (see Figure 2 in main text) [101,102]. BUBR1-associated PP2A-B56 helps to stabilize microtubule attachments by (partially) dephosphorylating Aurora B substrates within the KMN network [98,101]. MPS1 promotes both the activity and the localization of Aurora B [103,104] and impacts on error correction indirectly via the localization of BUB1 and BUBR1 [52]. These examples illustrate the complexity of signaling feedback at kinetochores: MPS1 and BUB1 promote Aurora B actions at kinetochores while simultaneously – by localizing BUBR1/PP2A-B56 – counteracting those actions. It seems likely that such networks have evolved to install features like robustness, switchability, and/or multilevel regulation on the system. Detailed spatiotemporal analysis of signaling events coupled with mathematical modeling may be required to capture the intricacies of this network and predict its behavior under various conditions. Such an approach may help to incorporate added levels of complexity such as the impact of CDK1 and PLK1 on some of these signaling connections.

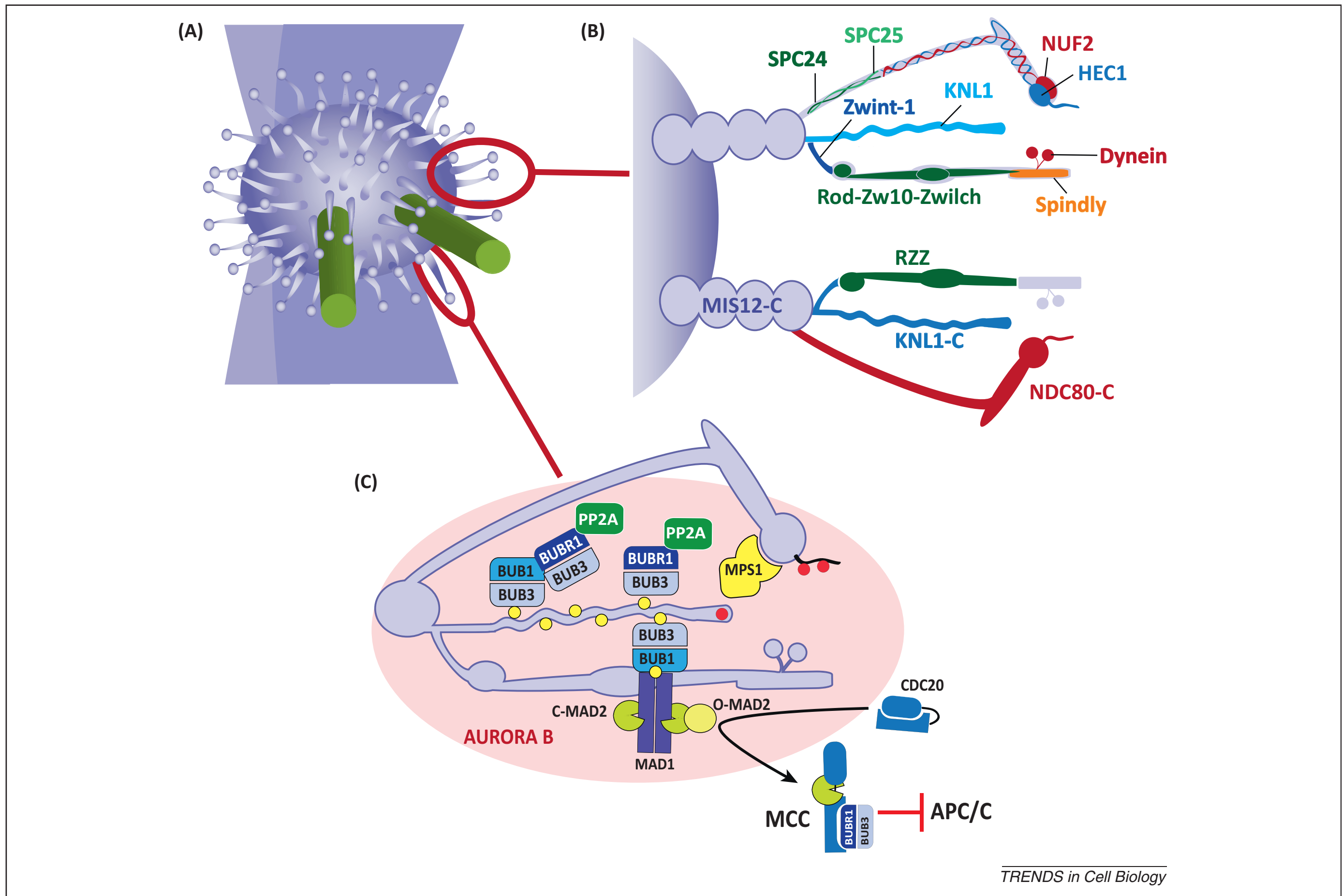

Figure 2. Spindle assembly checkpoint (SAC) signaling at the KMN network. **(A)** The outer kinetochore is decorated with KMN network complexes, which cooperate to establish microtubule interactions. **(B)** A single KMN network complex comprises the three subcomplexes MIS12-C, NDC80-C, and KNL1-C (lower part of panel). Individual components of the NDC80 and KNL1 complexes are indicated in the KMN network in the upper part of the panel. KNL1 contains an unstructured region (depicted as the squiggly middle region) that harbors the repeat motifs that bind BUB3–BUB1 complexes. The Zwint-1 component of KNL1-C recruits the Rod–Zw10–Zwilch (RZZ) complex, which recruits the dynein adaptor Spindly. **(C)** Assembly of SAC protein complexes onto a single KMN network [depicted as purple outlines of structures in (B)]. This assembly is aided by Aurora B kinase activity (region of activity indicated by red ellipse), which regulates MPS1 binding to NDC80-C and counteracts PP1 binding to KNL1. Aurora B additionally phosphorylates the HEC1 tail to decrease the microtubule-binding affinity of NDC80-C (Aurora B-dependent phosphosites mentioned in the text are represented by red dots). MPS1 phosphorylates, among others, KNL1 and BUB1 (phosphosites represented by yellow dots) to recruit the BUBs (BUB1/BUB3/BUBR1) and MAD1–MAD2, respectively. O-MAD2 is then converted to C-MAD2, which binds to CDC20 to expose a binding site for BUBR1, eventually resulting in mitotic checkpoint complex (MCC) production and APC/C inhibition. BUBR1 in turn recruits the phosphatase PP2A-B56, activity of which promotes stability of kinetochore–microtubule interactions by dephosphorylating Aurora B targets in the KMN network.

CDC20 binding and as such acts as a pseudosubstrate inhibitor [15,16]. Structural studies of APC/C^{MCC} showed that the MCC is likely to obstruct substrate recognition by CDC20 and displaces CDC20 away from the APC10 subunit, preventing the formation of a recognition site for ubiquitination [17,18]. MAD2 acts to stabilize the MCC and correctly position the BUBR1 KEN box [17]. It further competes with APC/C for binding to a KILR motif on CDC20, which is essential for the activation of APC/C [12,19]. Such a direct role for MAD2 in APC/C inhibition is supported by an elegant study showing that an artificial Mad2–Cdc20 fusion protein was able to arrest the budding-yeast cell cycle in a manner independent of any other SAC protein, including Mad3/BUBR1 [20].

Conditional protein depletion experiments in human cells verified that BUBR1 is essential for persistent mitotic arrest imposed by unattached kinetochores, thus supporting a direct role for BUBR1 in APC/C inhibition [21]. Surprisingly, and in contrast to what was predicted from the experiments outlined in the previous paragraph, MAD2 was dispensable for SAC maintenance. Instead, MAD2 was argued to enable a stable association between CDC20 and BUBR1 by exposing a BUBR1-binding site on CDC20, but to become dispensable after this matchmaking [21]. This is in agreement with observations that MAD2 is not stably associated with the MCC *in vitro* and that partial proteolytic removal of MAD2 from MCC-bound APC/C did not release inhibition [21,22]. In addition, MCC complexes isolated from cells contain substoichiometric amounts of MAD2 [23,24].

How do we reconcile these findings? A diplomatic answer is that both MAD2 and BUBR1 are essential components of the MCC during normal SAC activity. It is important to highlight that in the above-mentioned human-cell study showing dispensability of MAD2 for SAC maintenance, a protein known as p31^{comet} was depleted [21]. p31^{comet} participates in MCC disassembly, possibly by extracting MAD2 from the complex [24] and favoring CDC20 turnover [11]. Its depletion allowed the authors to cleanly assess the role of MAD2 and BUBR1 in MCC function. Conceivably, therefore, MAD2's role of stabilizing the MCC and inhibiting CDC20's KILR motif becomes less

relevant in the face of the hyperstable MCC due to p31[comet] depletion. Conversely, in the budding yeast experiments, fusion of Mad2 to Cdc20 may have created a hyper-inhibited Cdc20 that no longer needed Mad3's contribution to restrain it further.

Assembling the MCC at kinetochores: localized MAD2 activation

How does an unattached kinetochore cause assembly of the MCC? A large part of the answer revolves around the ability of MAD2 to alternate between an active and an inactive conformation, referred to as closed (C-MAD2) and open (O-MAD2), respectively [25,26]. MAD2 conversion is spontaneous but slow and is thus in need of acceleration [25]. This is enabled by MAD1, a protein that links the transition of MAD2 to unattached kinetochores. Two molecules of MAD1 form a stable complex with two molecules of C-MAD2 throughout the cell cycle [27] and this tetramer is recruited specifically to kinetochores devoid of microtubule attachments (Figure 2). In the so-called template model, it is proposed that, by their ability to homodimerize, the MAD1-bound C-MAD2 molecules recruit soluble O-MAD2 and accelerate their transition to the closed conformation. These newly formed C-MAD2 molecules are subsequently released to interact with CDC20 and initiate assembly of the MCC [28]. Interestingly, while the response time of MAD2 recruitment to kinetochores is fast, the response time to APC/C inhibition appears to be slow (Box 3) [29–31]. Insight into this discrepancy is likely to provide valuable insights into the kinetics of MAD2-dependent MCC assembly. Of note, recent studies have indicated that the role of MAD1 is not simply to localize and activate soluble MAD2 molecules [32–34], but the additional function of MAD1 remains to be discovered.

How the MAD1–C-MAD2 tetramer is recruited to the kinetochore has been a long-standing mystery that is now starting to take focus. In budding yeast, Mad1 directly binds Bub1 in a manner dependent on Bub1 phosphorylation by Mps1 [35] (Figure 2). Bub1 kinase activity is dispensable for this binding and the Mad1–Bub1 interaction is mediated by the C-terminal domain of Mad1 and a poorly defined region between the GLEBS motif of Bub1 and its kinase domain [35]. Although the requirement of Bub1 for Mad1 localization seems conserved, details differ between species. In *Caenorhabditis elegans*, for instance, BUB-1 binds MAD-1 via its kinase domain rather than the sequences preceding it [36] and this interaction does not require MPS1, as *C. elegans* has discarded an MPS1-like gene from its genome [6]. In human cells, BUB1 clearly impacts MAD1 kinetochore binding, but it is not the straightforward connection that is seen in budding yeast. Penetrant BUB1 depletion cannot prevent significant loading of MAD1 to kinetochores [37,38]. The involvement of other players has been suggested, specifically a trimeric complex known as Rod–Zw10–Zwilch (RZZ) [39] and NDC80-C [40,41]. There is currently no evidence that any of these players interact stably with MAD1 in human cells, suggesting either that they cooperate in MAD1 targeting or that the MAD1 receptor in human cells remains to be identified.

Box 3. Not so fast: the SAC response paradox

To ensure that APC/C will not initiate ubiquitination of its metaphase substrates directly at mitosis onset, the SAC response at kinetochores needs to be switched on rapidly. This is achieved at least in part by kick-starting MPS1–Aurora B feedback, which achieves rapid and localized activation of the SAC responses [63,64,104]. Similar feedback-activation mechanisms may operate during mitosis: controlled detachment of microtubules from one of the sisters of a bioriented chromosome caused detectable accumulation of MAD2 at that kinetochore within 30 s [30]. Paradoxically, however, the time to inhibition of APC/C was significantly longer (~5 min) [30,31]. The lag between kinetochore response and APC/C inhibition may not be problematic in prometaphase, when at any given moment multiple kinetochores are signaling. It does, however, create problems if, at metaphase, a kinetochore detaches while degradation of APC/C substrates has already advanced significantly [30]. A similar risk can be envisioned in the earliest phases of mitosis, when delayed inhibition of APC/C may lead to less robust protection against premature sister separation and mitotic exit during the remainder of mitosis. A solution to this conundrum was suggested by recent findings that an interphasic pool of MCCs, assembled at nuclear pores, inhibits APC/C sufficiently until the kinetochore-derived MCC pool takes over [105]. Premitotic MCC assembly adheres, at least to a large extent, to the same rules as assembly of kinetochore-generated MCC. It depends on MPS1 and MAD1/2, which are localized to nuclear pores by virtue of interacting with the large scaffold Tpr [24,106–108]. Interaction with Tpr additionally protects the SAC proteins from proteasomal degradation [109]. Interestingly, some factors important for MCC function (BUBR1, BUB3, and O-MAD2) are not found at the nuclear envelope, while p31[comet], an inhibitor of MCC, is found there [24]. Examining interphase MCC assembly may thus provide answers to important questions such as where does MCC assembly occur and how many MCCs are needed for sufficient APC/C inhibition?

Recruiting the MCC assembly factors: the KNL1 connection

If the SAC is sensitive to the microtubule-binding state of the kinetochore, logic predicts that the protein complexes that bind microtubules are the same ones that recruit factors needed for assembly and regulation of the MCC. Evidence is mounting that this indeed is the case. The most intriguing example is the scaffold KNL1 (also known as CASC5 or Blinkin) [42]. KNL1 is predicted to be largely unstructured, with the exception of a C-terminal tandem of RWD domains involved in its targeting to kinetochores and a coiled-coil region that mediates the interaction with Zwint-1, which connects RZZ to the outer kinetochore (Figure 2) [42–44]. The unstructured N-terminal half of KNL1 is decorated with an array of large repeat motifs (hereafter referred to as MELT motifs) that show striking evolutionary divergence. Although most eukaryotic KNL1 homologs contain multiple repeats, their sequence and number can vary extensively [6]. The core of the repeats in most species comprises (variants of) a MELT-like sequence. This core is flanked by motifs that differ widely between species but are consistent within the repeats of a single species [6].

The MELT motifs are recruitment hubs for SAC signaling [45–50]. When phosphorylated by MPS1, they form phospho-docking sites for BUB3 (Figure 2) [51]. BUB3 carries with it BUB1 or BUBR1 [52] and thus bridges KNL1 to these two BUB paralogs. This simple recruitment pathway explains BUB1 kinetochore binding, but the

mechanism of BUBR1 recruitment is more complex because BUBR1 requires BUB1 to correctly localize while the reciprocal dependency does not exist [37,53,54]. Perhaps BuGZ, a recently identified zinc finger-containing protein that interacts with BUB3 through a BUB1/R1-like GLEBS motif, is involved in this, but more work is needed to clarify its role in BUB protein loading [55,56].

In addition to the MELT-like motifs, KNL1 exhibits a pair of motifs that interact *in vitro* with BUB1 and BUBR1 [57,58]. These 'KI' motifs are located close to the N-terminal-most MELT and are found only in KNL1 homologs of some subclasses of vertebrates [6,43]. The KI motifs interact with the convex surface of the TPR domains of BUB1 (KI-1) and BUBR1 (KI-2) [57,58] but these interactions are not required for faithful chromosome segregation, at least as measured by current assays in the field [48,50,58]. A role for the KI motifs does become apparent, however, in the context of suboptimal KNL1 function. An N-terminal fragment of KNL1 encompassing the first MELT motif and the KI motifs recruits low levels of BUB1 and supports SAC signaling in a KI-dependent manner [48,49]. Moreover, grafting the KI motifs onto other, KI-less MELT repeats enhances their ability to recruit BUB1 and activate the SAC [48]. Interestingly, while the N-terminal MELT module is sufficient for SAC signaling in cells, the efficiency of chromosome biorientation is proportional to the number of MELT motifs and thus to the amount of BUB1 that KNL1 is able to recruit (Box 2) [48,50]. The reason for this difference is unknown, but perhaps error correction needs toning down as kinetochores engage more and more microtubules while the SAC is needed at maximal strength until it is allowed to switch off. It will be interesting to investigate whether error correction and the SAC are affected when too much BUB1 is localized to kinetochores; for instance, by adding additional MELT repeats to the KNL1 protein.

Recruiting the engine that moves the SAC: MPS1 and the NDC80 complex

As reflected above, MPS1 is the master regulator of the SAC. By phosphorylating the MELTs of KNL1, it ensures BUB1 and, by proxy, BUBR1 and MAD1 kinetochore binding (Figure 2). The influence of MPS1 on the SAC is even larger, however, as it is also involved in kinetochore binding of RZZ and CDC20, in MAD2 conformational activation, and in the maintenance of MCC stability [9].

MPS1 accumulates on kinetochores in early mitosis and is subsequently activated by autophosphorylation [59,60]. Following this initial activation, MPS1 displays rapid kinetics, with a half-life at kinetochores in the range of seconds [61,62]. The association of MPS1 with the kinetochore is dependent on the NDC80 complex [3,4] and regulated by Aurora B (Figure 2) [4,63,64]. Although Aurora B activity is not an absolute requirement for MPS1 localization and SAC activation, it critically impacts on the efficiency of MPS1 kinetochore binding in early mitosis and the extent to which the SAC is initially established [63]. An obvious candidate target of MPS1 regulation by Aurora B is the tail of HEC1, phosphorylation of which is critically involved in destabilizing kinetochore–microtubule interactions [1,7]. Many studies, however, have reported no involvement of the tail region of HEC1 in either MPS1 localization or SAC activation [4,65–67], although this was recently challenged [68]. Rather, the binding of MPS1 to kinetochores involves the microtubule-binding calponin homology (CH) domain of HEC1 [4]. How does Aurora B impact this? Localization of MPS1 is mediated by an N-terminal bipartite module comprising a TPR domain preceded by a 62-amino acid N-terminal extension (NTE). The NTE is the predominant localization signal but its ability to localize is inhibited when Aurora B is inactive. Deleting the TPR domain causes MPS1 localization to be insensitive to Aurora B inactivation, suggesting that Aurora B lifts an inhibitory constraint imposed on the NTE by the TPR domain [4]. Whether the TPR directly inhibits the NTE and how Aurora B permits NTE function, as well as the contribution of other kinases like mitogen-activated protein kinase (MAPK) or checkpoint kinase 2 (CHK2) to MPS1 localization, remain to be uncovered [69,70]. Since artificially tethering MPS1 bypasses the need for Aurora B in SAC regulation [63,71,72], insights into the mechanism by which Aurora B regulates MPS1 localization are crucial, as they will describe how the main error-correction kinase communicates with the master regulator of the SAC.

Digital or analog: the graded response of the kinetochore SAC signal

Laser-ablation experiments in the mid-1990s showed that a single unattached kinetochore was responsible for delaying anaphase for several hours [73]. This prompted the notion that the SAC has a digital, all-or-none response. If this is true, either a single unattached kinetochore would have to produce enough MCC to inhibit all relevant APC/C complexes or the kinetochore-derived signal is amplified to saturating levels in the cytoplasm. The latter seems unlikely given recent insights that the amount of MCC formed and the strength with which cellular APC/C activity is inhibited correlate with the number of unattached kinetochores [30,74]. In addition, it seems that the amount of MAD2 recruited per kinetochore depends on how many microtubules are connected to that kinetochore [74]. This raises two intriguing questions: how much MCC is required to achieve sufficient APC/C inhibition and how many microtubules need to bind to a kinetochore before MCC production dips below that threshold?

Elegant quantitative studies in *Schizosaccharomyces pombe* have uncovered another surprising side to the SAC: it appears to be quite fragile, relying on tight regulation of the relative amount of the Cdc20 homolog Slp1 with respect to MCC components [75]. In human cells, too, relatively slight reductions in MAD2 significantly weaken the SAC responses [74,76,77]. It is unclear why this fragility is tolerated given the severe consequences of aneuploidization for both single-celled and multicellular organisms [78]. Perhaps it is related to benefits that such a system provides with respect to evolutionary adaptability and/or the ability to rapidly release APC/C activity once all chromosomes are bioriented.

Stopping the assembly line: how microtubules block kinetochore-dependent MCC production

Attached kinetochores are devoid of MAD1 and MAD2 and have much lower levels of MPS1 than unattached ones. Removal of these proteins is critical for extinguishing the

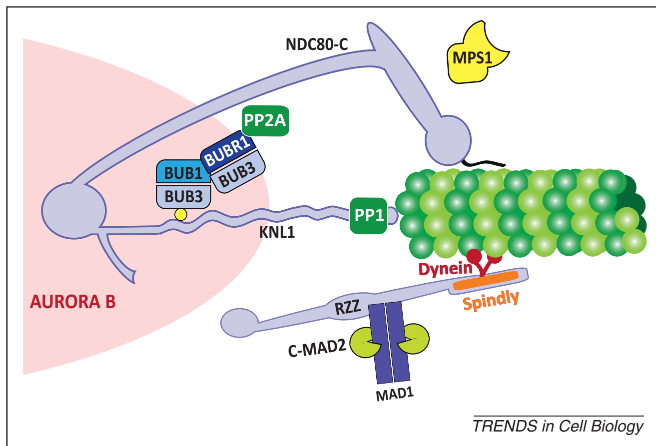

Figure 3. Spindle assembly checkpoint (SAC) silencing at kinetochores. Many SAC components are completely or partially displaced from the kinetochore when microtubules bind to the KMN network. Removal is accomplished by dynein-dependent 'stripping' of Rod–Zw10–Zwilch (RZZ) complex–MAD1 and Spindly and by dephosphorylation of key phosphosites by PP1 (and possibly PP2A-B56) phosphatase. Microtubule binding to NDC80-C and to a basic patch near the N terminus of KNL1 may also more directly contribute to SAC silencing.

SAC signal, as artificially maintaining any of them on attached kinetochores causes sustained SAC activation [33,62,79]. Moreover, chemical retargeting of MAD1 to bioriented kinetochores after APC/C activation in metaphase is sufficient to reactivate SAC signaling [34,80]. An important contribution to the removal of the MPS1–MAD1–MAD2 axis is made by the microtubule motor complex dynein, which carries SAC proteins including MAD1 and MAD2 poleward on microtubule capture by the kinetochore (Figure 3) [81]. This process, known as 'stripping', may not, however, be the predominant means of blocking MCC production on microtubule attachment. Depletion of the kinetochore dynein adaptor Spindly did not prevent the removal of SAC proteins on attachment or SAC silencing [82,83], and kinetochore dynein is not widely conserved in eukaryotic species [6]. Other, more ancient silencing mechanisms are therefore likely to exist. It should be noted that a silencing mechanism involving inactivation of BUBR1 kinase activity on microtubule capture by the kinesin CENP-E was proposed [84,85]. Since CENP-E is not essential for the human SAC and is not widely conserved ([86] and our unpublished data), and since virtually all eukaryotic BUBR1 homologs are devoid of kinase activity [87], we do not consider this mechanism a likely candidate for quenching the kinetochore SAC signal.

The intimate connection between the KMN network and the SAC machinery provides a basis for understanding how production of MCCs is locally inhibited when kinetochores engage microtubules. The dependency of MPS1 kinetochore binding on the same protein domain in HEC1 that also contacts microtubules provides a straightforward hypothesis of mutual exclusivity between MPS1 and microtubules in binding to NDC80-C. *In vitro* reconstitution of these interactions should be able to test this hypothesis directly.

The N-terminal region of KNL1 harbors a basic patch that contributes to microtubule interactions [88] (Box 1) but that may also be directly involved in SAC silencing (Figure 3). Mutation of this patch in *C. elegans* did not prevent formation of load-bearing attachments but did delay SAC silencing [89]. A mechanistic understanding of this is lacking, but it is of interest to note that a docking site for the PP1 phosphatase is adjacent to the basic patch (Figure 3) [42]. While preventing the binding of PP1 to KNL1 in human cells destabilizes kinetochore–microtubule attachments [90], it delays SAC silencing in *S. pombe* and *Saccharomyces cerevisiae* [91–94]. The budding yeast PP1 homolog Glc7 dephosphorylates the MELTs of Spc105/KNL1, thus providing a mechanistic basis for SAC silencing [47]. Although a mutant of human KNL1 deficient in PP1 binding increases BUB1 and BUBR1 localization [50], it remains to be formally demonstrated that KNL1-bound PP1 is important for SAC silencing in human cells. Verification of this awaits ways in which the role of kinetochore PP1 in stabilizing microtubule attachments can be experimentally uncoupled from a potential role in SAC silencing. Nevertheless, one can imagine that the interaction between the basic patch of KNL1 and the incoming microtubule enhances PP1 binding, activation, and/or proximity to substrates. Alternatively, microtubule binding by the basic patch could more directly impact the SAC machinery. For instance, an intriguing possibility unsubstantiated by any published observations is that force exerted on KNL1 may alter the affinity of repeat motifs for the BUB proteins.

Concluding remarks

As recently reviewed in [8], SAC research has just entered its third decade. The first two provided an amazing number of insights, from identification of all components of the SAC machinery to mechanistic insights into their localization, activation, and mode of action. However, to paraphrase Albert Einstein, 'the more we learn, the more we realize what we don't yet know'. Various intriguing questions have been scattered throughout this review, but some additional ones are worth mentioning. Does all APC/C need to be inhibited or is it sufficient to inhibit specific pools, such as the one localized to chromosomes [95]? How are the many localized feedback mechanisms regulated in space and time and how is the network wired to achieve speed and robustness? Is there a role for biorientation in SAC silencing? How is the MCC disassembled and is that disassembly actively coupled to attachment/biorientation of the final chromosome? Answers to these and other questions will require systematic mapping of relevant modifications, biochemical reconstitution of complexes and their regulation, sophisticated single-cell analyses, and biosensors of, for instance, MCC assembly and kinase activities. Judging by the speed of new discoveries over the past years, the SAC is bound to reveal some of its remaining mysteries soon.

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