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Regulation of mitotic progression by the spindle assembly checkpoint

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Abbreviations: APC/C, Anaphase Promoting Complex/Cyclosome; CH, Calponin Homology; MCC, Mitotic Checkpoint Complex; MELT, Met-Glu-Leu-Thr sequence; PP2A, Protein Phosphatase 2A; SAC, Spindle Assembly Checkpoint; TPR, Tetratricopeptide repeat.

Equal segregation of sister chromatids during mitosis requires that pairs of kinetochores establish proper attachment to microtubules emanating from opposite poles of the mitotic spindle. The spindle assembly checkpoint (SAC) protects against errors in segregation by delaying sister separation in response to improper kinetochore–microtubule interactions, and certain checkpoint proteins help to establish proper attachments. Anaphase entry is inhibited by the checkpoint through assembly of the mitotic checkpoint complex (MCC) composed of the 2 checkpoint proteins, Mad2 and BubR1, bound to Cdc20. The outer kinetochore acts as a catalyst for MCC production through the recruitment and proper positioning of checkpoint proteins and recently there has been remarkable progress in understanding how this is achieved. Here, we highlight recent advances in our understanding of kinetochore–checkpoint protein interactions and inhibition of the anaphase promoting complex by the MCC.

Introduction

The kinetochore is a large protein structure assembled at the centromere region. For accurate sister chromatid segregation it is essential that kinetochores bind microtubules in an end-on manner and that the sisters biorient in the center of the cell (**Fig. 1**).[1] Incorrect attachments often occur, but luckily the cell has mechanisms in place that provide the time and tools to fix erroneous attachments. Time is provided by the spindle assembly checkpoint (SAC) that prevents anaphase entry until all kinetochores have made proper attachments whereas the tools are provided by the error correction machinery.[2] Importantly, upstream components of the SAC are also part of the error correction machinery, thus providing a tight link between these activities. How the checkpoint discriminates proper from improper attachments is not clear but proper attachments generate tension that results in intrakinetochore stretching and this kinetochore state is unable to bind SAC proteins.[3] A particular type of improper attachments that is not detected by the checkpoint is merotelic attachment, in which the same kinetochore binds to microtubules from both poles. Merotelic attachments generate tension and are therefore not sensed as erroneous.

Conserved components of the SAC that were originally identified by genetic screens in yeast are the Mad (mitotic-arrest deficient) proteins Mad1, Mad2, and Mad3 (BubR1 in humans; we will use the human name throughout) and the Bub (budding uninhibited by benzimidazole) proteins Bub1 and Bub3 (**Fig. 2**).[2] Bub1 and BubR1 both exist in a stable complex with Bub3, which binds a conserved GLEBS motif in the proteins, whereas Mad1 and Mad2 form a stable tetrameric complex. In addition, a large fraction of Mad2 exists as a soluble unliganded form. In addition, the Aurora B and Mps1 kinases are essential for SAC signaling and their kinase activity is required for a functional SAC.

The final inhibitory complex consists of Mad2 and BubR1–Bub3 bound to Cdc20, the mitotic co-activator of the anaphase promoting complex/cyclosome (APC/C), and is referred to as the mitotic checkpoint complex (MCC). This complex can bind stably to the APC/C thereby inhibiting its E3 ubiquitin ligase activity (**Fig. 1**).[4,5] The APC/C is a large ubiquitin ligase that targets multiple proteins for degradation through the attachment of ubiquitin chains.[6] Its substrates contain short destruction motifs of different kinds, the most common being D-boxes and KEN-boxes. Binding of substrates to the APC/C depends on a combined binding pocket between Cdc20 and the APC10 subunit; in addition Cdc20 activates the APC/C.[7] Thus, by inhibiting Cdc20, the SAC efficiently inhibits the APC/C and halts mitotic progression by preventing the degradation of 2 key substrates, securin and cyclin B1. Securin is an inhibitor of separase, a protease that cleaves a cohesin subunit allowing sister chromatid separation, and cyclin B1 is an activator of CDK1, the major mitotic kinase. Pioneering work by Conly Rieder showed that the SAC signal is generated by the kinetochore, and consistent with this

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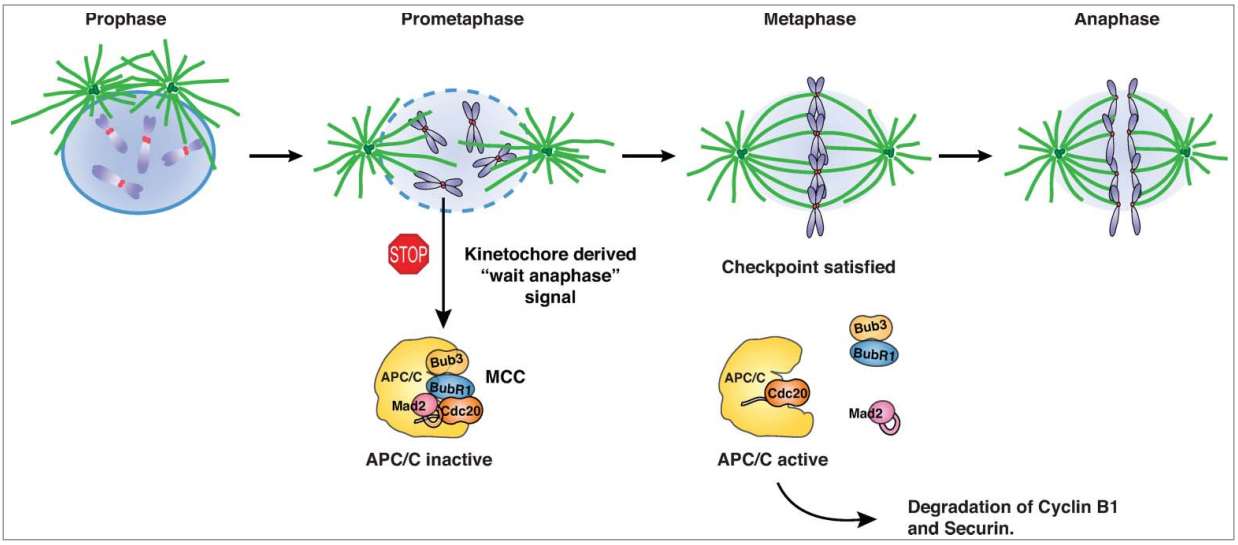

Figure 1. Regulation of chromosome segregation by the spindle assembly checkpoint. Entry into mitosis is marked by nuclear envelope breakdown, at which stage the SAC becomes active because of the presence of unattached kinetochores (prometaphase state). The unattached kinetochores recruit SAC proteins resulting in the generation of the mitotic checkpoint complex (MCC), which can bind stably to the anaphase promoting complex (APC/C) and inhibit it. At metaphase, when all kinetochores have attached and are under tension, the checkpoint turns off and the MCC disassembles thus freeing Cdc20 for APC/C activation. The active APC/C-Cdc20 complex targets securin and cyclin B1 for degradation resulting in sister chromatid separation and mitotic exit, respectively.

all SAC components are recruited to unattached kinetochores where they exhibit rapid turnover.[8-10] In addition, Cdc20 is recruited to kinetochores, strongly suggesting that Cdc20 is incorporated into inhibitory complexes at the kinetochore.[9,11] Once all kinetochores are correctly attached to microtubules, the MCC disassembles and the APC/C–Cdc20 complex becomes active and triggers anaphase entry.

Given the central role of the kinetochore in the SAC there has been a strong interest in understanding how the checkpoint proteins interact with the kinetochore, the order in which they are recruited, and how this is regulated in response to microtubule attachment. With our increased understanding of kinetochore architecture and function it has become clear that the microtubule binding activity of the kinetochore constituted by the KNL1–Mis12–Ndc80 complex (KMN network) is the major binding site for SAC proteins at the outer kinetochore.[12,13] There has recently been an explosion in our understanding of the interaction between checkpoint proteins and the KMN network and how these interactions are dynamically regulated. We will begin our tour of the SAC by introducing the KMN network before discussing how checkpoint proteins are recruited to assemble a platform for MCC production.

The KMN Network and Regulation of Kinetochore–Microtubule Interactions

The KMN network is the core microtubule binding activity of the outer kinetochore and is composed of 3 protein complexes, namely the large KNL1 protein in complex with Zwint (Zwint is only present in metazoans), the 4-subunit Mis12 complex (Mis12, Nnf1, Dsn1, Nsl1), and the 4-subunit Ndc80 complex (Ndc80, Nuf2, Spc24 and Spc25).[1,14] The Mis12 complex links the KMN network to the inner kinetochore whereas the Ndc80 complex and KNL1 can directly bind to microtubules. As discussed below, the Ndc80 complex and KNL1 are docking sites for checkpoint proteins. The Ndc80 complex, and in particular the Ndc80 protein, is essential for end-on attachment of microtubules to kinetochores.[15] The Ndc80 protein contains an N-terminal basic tail followed by a calponin homology (CH) domain that both contribute to microtubule binding (**Fig. 2**). The basic tail of Ndc80 contains numerous phosphorylation sites for the Aurora B kinase and these are phosphorylated in response to improper kinetochore–microtubule interactions, thereby destabilizing the interaction.[16,17] As Aurora B is concentrated at the centromere region this establishes a gradient of Aurora B activity, and proper kinetochore–microtubule attachments move the KMN network away from Aurora B activity thus stabilizing the binding.[16,18] In addition to this spatial regulation of kinetochore–microtubule interactions dictated by the Aurora B gradient, protein phosphatase 2A (PP2A) in complex with B56 regulatory subunits directly counteracts Aurora B at the outer kinetochore.[19] Indeed, BubR1 recruits B56–PP2A to kinetochores, and the binding of BubR1 to B56–PP2A is stimulated by Cdk1- and Plk1-mediated phosphorylation of BubR1 specifically at kinetochores.[20-23] A complex interplay between major mitotic kinases and phosphatases thus regulates microtubule binding to kinetochores.

Recruitment of Mps1 to Kinetochores

The Mps1 kinase is a master regulator of the SAC and its kinase activity is required for kinetochore recruitment of all

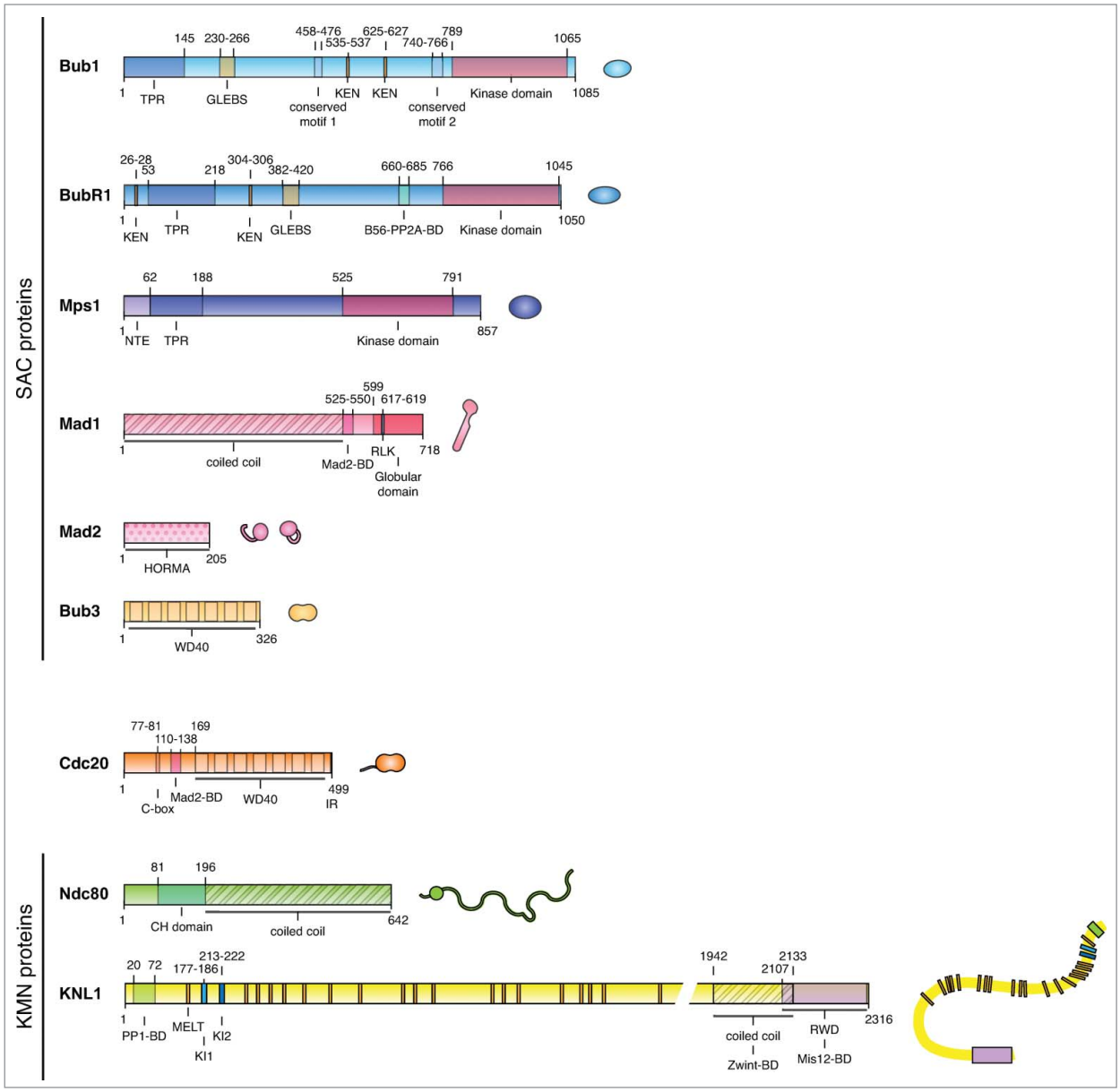

Figure 2. Schematic of SAC proteins and KMN network components. A schematic of checkpoint proteins, Cdc20, and the KMN network components Ndc80 and KNL1. The primary structure is shown with important domains and motifs indicated. Next to the primary structure is an illustration of the schematic used for that protein in subsequent figures. BD, binding domain.

downstream components. Mps1 and the Aurora B kinase constitute the most upstream components of the checkpoint.[24-26] Aurora B is part of the chromosomal passenger complex (CPC) and is concentrated at the centromere; this concentration depends on interactions between the survivin and borealin CPC components and histone H3 phosphorylated on Thr3 and histone H2A phosphorylated on Thr120, respectively.[27] Aurora B activity is required for the SAC and stimulates the rapid recruitment of Mps1 to the kinetochore, resulting in the recruitment of the Bub1 kinase that phosphorylates H2A on Thr120, thus creating a positive feedback loop stimulating further Mps1 recruitment.[24-26,28] The major function of Aurora B is to stimulate the recruitment of Mps1 as artificial recruitment of Mps1 to the kinetochore bypasses the need for Aurora B in the checkpoint.[24,26]

The N-terminal 200 amino acids of Mps1 are critical for its kinetochore localization and contain an N-terminal extension (NTE) followed by a tetratricopeptide repeat (TPR) domain.[29,30] TPR domains are found at the N-terminus of Mps1, Bub1, and BubR1 and are α-helical protein–protein interaction modules (**Fig. 2**). Both the NTE and TPR domains of Mps1 contribute to kinetochore localization, and the ability of Aurora B to stimulate Mps1 recruitment depends on this domain.[29] Indeed, in the absence of the TPR domain Mps1 localization is no longer stimulated by Aurora B. Based on this finding, it was proposed that the TPR domain could inhibit the ability of the NTE to bind

kinetochores and that this inhibition is relieved by Aurora B.[29] Interestingly, autophosphorylated Mps1 accumulates to lower levels on kinetochores suggesting that Mps1 autophosphorylation might negatively regulate the NTE-TPR kinetochore-targeting module. Understanding the details of how Aurora B and Mps1 activity regulates Mps1 kinetochore localization will be important given that this is the initiating event in the SAC.

Mps1 kinetochore localization also depends on the Ndc80 complex. A direct interaction between Mps1 and the CH domain of the Ndc80 protein has been observed in budding yeast and appears to be conserved in humans.[29,31] Moreover, the role of the Ndc80 CH domain in localizing Mps1 to kinetochores is in agreement with the observed requirement of this domain in the checkpoint.[32] The fact that the Ndc80 CH domain directly contacts microtubules suggests that upon microtubule attachment the Mps1 binding site is blocked. Testing and validating this model requires that we understand the interaction between Ndc80 and Mps1 in more detail.

The KNL1 MELTing pot

Once Mps1 is located and active at kinetochores, it stimulates recruitment of the Bub1–Bub3 complex, which is needed for recruitment of BubR1–Bub3 and Mad1–Mad2. Bub1 kinase activity is not required for the SAC but is needed for proper chromosome segregation.[2] Recent elegant work from a number of laboratories has clarified how Mps1 phosphorylation of the outer kinetochore protein KNL1 stimulates Bub1–Bub3 recruitment and thus BubR1–Bub3.[33-35] KNL1 has previously been identified as the kinetochore receptor of Bub1 and BubR1, and earlier studies showed that 2 distinct KI motifs located in the N-terminal region of human KNL1 make contact specifically with the TPR domains of Bub1 or BubR1 (**Fig. 3** box 1).[36,37] The KI motifs are named after their consensus sequence (Lys-Ile-(Asp/Asn)-X-X-X-Phe-(Leu/Ile)-X-X-Leu-Lys) and bind a ridge on the convex side of the Bub1 and BubR1 TPR domains.[38,39] It is, however, clear that the KI motif interactions are dispensable for Bub1 and BubR1 kinetochore localization, suggesting that additional mechanisms contribute.[38-40] The breakthrough came when it was shown that Mps1 phosphorylates so-called Met-Glu-Leu-Thr (MELT) motifs in KNL1 and that this event is required for Bub1 kinetochore localization (**Fig. 3**).[33-35] Binding to phosphorylated MELT motifs (MELTp) depends on Bub3, explaining why Bub1 and BubR1 need to bind to Bub3 in order to localize.[41] The role of Bub3 became crystal clear when the structure of a ternary complex composed of budding yeast Bub1–Bub3 in complex with a phosphorylated form of the second MELT motif of Spc105 (the budding yeast homolog of KNL1) was solved.[42] This structure showed that the residues of the MELTp motif interact almost exclusively with Bub3 and that the phospho-threonine residue of the MELTp motif directly faces the positive charge of 2 arginine residues of Bub3, thus explaining the increased affinity for phosphorylated MELT motifs. However, even though the interaction with the MELTp motif of KNL1 mainly relies on Bub3, Bub1 strongly increases the affinity of this interaction.[33,42] The structure shows that this increase in affinity provided by Bub1 is due to stabilizing effects on the Bub3 MELTp binding region and direct contact between MELTp and Bub1.[42]

Human KNL1 contains 12 MELT motifs and at least 7 MELT-like sequences, all located in the N-terminal half of the protein.[43-45] The fact that a defining feature of KNL1 proteins appears to be the presence of numerous MELT motifs immediately poses the question of why there are so many. Recent studies indicate that KNL1 can recruit multiple Bub1–Bub3 and BubR1–Bub3 complexes and that the number recruited correlates with the number of MELT motifs.[44-46] This argues that several, if not all, of the MELT motifs are capable of binding Bub3-containing complexes. Surprisingly, there appears to be a large degree of redundancy and flexibility since a KNL1 protein with a limited number of MELT motifs or engineered MELT motifs is fully functional in supporting chromosome segregation and the SAC.[44,46] Chromosome alignment appears to be much more sensitive to the level of Bub protein recruited than the SAC and thus very low levels of Bub1 and BubR1 have to be present at kinetochores to generate a functional SAC signal. Although the collective conclusion appears to be that KNL1 might not need all its binding sites for the Bub proteins, it remains to be determined whether the individual MELT motifs have distinct roles under certain conditions.

The Bub Balance

Given the fact that Bub1 stimulates the recruitment of Aurora B whereas BubR1 recruits the B56–PP2A phosphatase to counteract Aurora B activity, it is likely that the exact amount and ratio of Bub1 and BubR1 proteins have to be precisely controlled to fine-tune Aurora B activity.[20-22,27,28] Regulating the ratio of Bub1 to BubR1 would be difficult if both proteins used exactly the same mechanism of kinetochore localization. However, BubR1 is subjected to a higher level of regulation, as it requires Bub1 for its localization to the kinetochore.[47,48] The exact reason for this is unclear but it is possible that BubR1, in contrast to Bub1, does not contribute to MELTp binding, making the affinity for MELTp too low for kinetochore recruitment. BubR1 can bind to the N-terminal region of KNL1 containing the first MELT motif only if both KIs are present.[44,45] However, its binding to the other MELT motifs of KNL1 does not seem to rely on the KI motifs but instead requires the kinetochore environment (**Fig. 3**).[44,45] Given that only one MELT motif is located in proximity to the KI domains, it has been proposed that Bub1–Bub3 makes contact with the MELTp motif and Bub1 stabilizes this interaction by binding to KI1. The bound Bub1–Bub3 can in turn allow subsequent loading of the BubR1–Bub3 complex, with BubR1 interacting with KI2 and possibly Bub1.[45] Whether BubR1 needs the priming loading of Bub1 to be able to interact with the other MELTp repeats is not clear although some insight into this is provided by the protein BuGZ, a recently described Bub3 chaperone that regulates Bub3 stability.[49,50] Similar to Bub1 and BubR1, BuGZ can directly bind Bub3 through its GLEBS motif and its knockdown reduces Bub3 protein levels, resulting in less Bub3 at the kinetochore. Interestingly, this leads

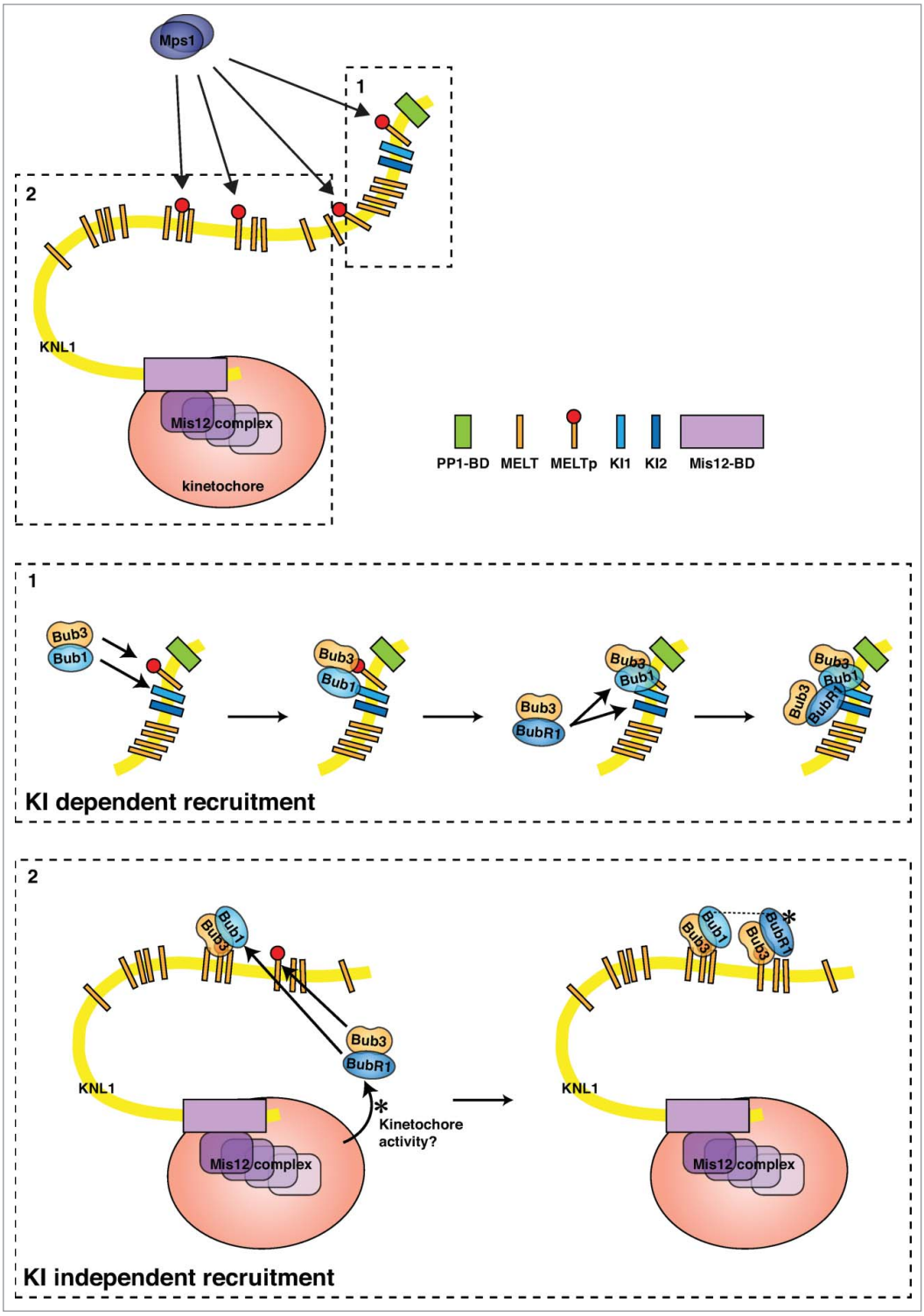

Figure 3. Bub protein interactions with KNL1. A schematic of the KNL1 protein showing the KI motifs, MELT motifs, and PP1 binding site (PP1-BD) as well as its domain for interaction with the Mis12 complex (Mis12-BD). Section 1 highlights the interactions centered on the N-terminal KI motifs and the phosphorylated MELT motif next to them. Bub1 interacts specifically with KI1, with Bub3 binding to the phosphorylated MELT motif; this facilitates BubR1-Bub3 binding through a BubR1–KI2 interaction. Section 2 highlights interactions involving phosphorylated MELT motifs that do not have neighboring KI motifs. Here, Bub1–Bub3 is recruited through an interaction between Bub3 and phosphorylated MELT motifs and similar interactions are also involved in BubR1–Bub3 recruitment. However, additional kinetochore-localized activities as well as Bub1 are required for kinetochore localization of BubR1.

to a reduction in the level of Bub1, but not of BubR1, at kinetochores.[49] As a consequence, cells with reduced levels of BuGZ show alignment defects as a result of the reduced Aurora B activity at the kinetochore but have a functional checkpoint. It will be important to clarify whether BuGZ differentially regulates Bub1 and BubR1 localization and whether there is an excess of Bub1 to BubR1 on kinetochores that allows BubR1 levels to be unaffected despite decreasing Bub1 levels. There is clearly more to be learned about the role of BuGZ and Bub1 in kinetochore recruitment of BubR1.

Getting the Mads on Board

The loading onto kinetochores of the tetrameric Mad1–Mad2 complex, composed of a stable Mad1 dimer with each member bound to a Mad2 molecule, is the event that finally engages the SAC. The kinetochore provides a unique environment for activation of the complex and indeed artificial re-recruitment of the complex to kinetochores after the SAC has been silenced is sufficient to re-engage the checkpoint.[51-53] This re-engagement of the checkpoint, which could normally occur at anaphase when kinetochore-microtubule tension is lost, is prevented by making Mad1–Mad2 localization dependent on high levels of cyclin B1-Cdk1 activity.[54,55] The exact role of cyclin B1-Cdk1 activity in the SAC is yet to be determined but it is

possible that the complex phosphorylates kinetochore or checkpoint proteins to allow Mad1–Mad2 recruitment.

How the Mad1–Mad2 complex interacts with the kinetochore is still somewhat enigmatic and it appears that different organisms might use different recruitment mechanisms. Two recent studies have pointed to Bub1 as the direct receptor for the Mad1–Mad2 complex. This is in line with original observations from the Hardwick laboratory that a larger Mad1–Mad2–Bub1–Bub3 complex forms during an active checkpoint in budding yeast.[56] Building on this, London and Biggins found that in budding yeast, the middle part of Bub1 is the direct kinetochore receptor for Mad1–Mad2 and that the interaction depends on Mps1 phosphorylation of this region of Bub1.[57] This region of Bub1 encompasses the conserved motif 1 (cm1) that is required for Mad1 localization and the SAC in human cells and fission yeast.[48,58] Whether the cm1 of Bub1 directly binds the Mad1–Mad2 complex, potentially by binding the conserved Arg-Leu-Lys (RLK) motif in the C-terminus of Mad1 that is required for Mad1 kinetochore localization, is not clear.[56,59] Moreover, the interaction between Mad1–Mad2 and Bub1 depends on Mad2, raising the possibility that Bub1 might also contact and regulate Mad2.[56,57] A direct role of Bub1 in Mad1–Mad2 recruitment is also supported by recent work from the Desai laboratory in *Caenorhabditis elegans*.[60] Here, the interaction depends on the C-terminal region of Bub1 encompassing the kinase domain and residues in the central part of the coiled-coil region of Mad1. As *C. elegans* lacks an Mps1 homolog, it is not clear whether the interaction is regulated by a different kinase or a different mechanism.

The kinetochore localization of Mad1–Mad2 in human cells is stimulated by Bub1 but appears to be more complex than simply a direct interaction with Bub1.[48,59] In human cells and *Drosophila*, the Rod–ZW10–Zwilch (RZZ) complex has been shown to be required for Mad1 kinetochore localization, but whether this is through direct binding to Mad1 is not clear (**Fig. 4A**).[61,62] The RZZ complex localizes dynein, a minus-end directed microtubule motor, to kinetochores through the adaptor protein spindly. This allows dynein to remove Mad1–Mad2 from kinetochores once they have attached to microtubules (**Fig. 4B**).[63] It is possible that a combined binding interface composed of Bub1 and the RZZ complex mediates the interaction with Mad1 in human cells. Recent work from the Stukenberg laboratory has linked the centromeric protein CENP-I to protection of the Mad1–Mad2 complex from premature stripping from kinetochores that have not established mature microtubule attachments.[64] It will be interesting to understand how CENP-I regulates this and further clarify the interactions of Mad1 with the RZZ complex and Bub1.

Generation of the MCC at Kinetochores

As previously mentioned, the kinetochore localization of the Mad1–Mad2 complex is essential for its activity in the SAC and recent work has shown that SAC strength correlates with the number of Mad2-positive kinetochores.[65,66] Although we do not fully understand the molecular events that lead to MCC generation at kinetochores, the rate-limiting step is the binding of Mad2 to Cdc20. Mad2 exists in at least 2 extreme conformations, open (O-Mad2) and closed (C-Mad2), the latter being able to bind Mad1 and Cdc20. The "template model" for Mad2 activation suggests that the kinetochore localized Mad1–Mad2 complex recruits O-Mad2 to kinetochores through dimerization with C-Mad2 bound to Mad1 and this stimulates the conversion of soluble O-Mad2 into soluble C-Mad2 that can then bind Cdc20 (**Fig. 4A**).[67] These interactions might be directly regulated by Mps1 at kinetochores as Mps1 stimulates O-Mad2 recruitment in human cells and directly phosphorylates Mad2 to allow its incorporation into checkpoint complexes in fission yeast.[68,69] Indeed, O-Mad2 activation has been reconstituted with purified proteins but the low rates measured *in vitro* might suggest that kinetochores provide additional layers of catalysis.[70,71] One clue to this is the recent observation that Mad1 plays a role in the SAC in addition to recruiting C-Mad2, and that this role depends on its C-terminal domain and the RLK motif.[53,58,72] This suggests that Mad1 coordinates the assembly of larger checkpoint complex assemblies at kinetochores and that these are essential for efficient SAC signaling. Indeed, even when Mad1 is artificially tethered to kinetochores there is still a need for Bub1, the cm1 domain of Bub1, and Mps1 to mount an efficient checkpoint.[51,58,72] As BubR1 kinetochore localization is also needed for efficient SAC signaling and BubR1 helps to recruit Cdc20, it is possible that a Mad1–Bub1 interaction precisely coordinates the positioning of all 3 MCC components at kinetochores to allow efficient complex formation (**Fig. 4A**).[40,73] Ultimate testing of this will require biochemical reconstitution and biosensors that can monitor MCC formation spatially and temporally within cells.

MCC Interactions and Inhibition of the APC/C

Binding of Mad2 to Cdc20 stimulates the binding of BubR1 by several mechanisms. First, the crystal structure of the fission yeast MCC reveals that a contact between the Mad2 dimerization surface and BubR1 helps to position the N-terminal KEN box of BubR1 to allow Cdc20 binding.[74] KEN boxes are destruction motifs that are recognized by Cdc20, and the N-terminal BubR1 KEN box is fully conserved and essential for MCC formation and the SAC.[75,76] Second, Mad2 prevents binding of the N-terminal tail of Cdc20 to its own WD40 domain, thus exposing the BubR1 binding site.[77] Despite these important functions of Mad2 in stabilizing the BubR1–Cdc20 interaction, it appears that once this interaction is established Mad2 can leave the MCC.[77] This was elegantly demonstrated by the Cleveland laboratory who showed that controlled removal of Mad2 once the MCC had formed still resulted in robust APC/C inhibition, arguing that the Cdc20–BubR1–Bub3 complex is a potent inhibitor.[77] These *in vivo* experiments were performed in the absence of the $p31^{comet}$ protein that acts to continually remove Mad2 from the MCC, thus under conditions where $p31^{comet}$ is present there would likely be a continual need for Mad2 to counterbalance the continual dissociation of the MCC.

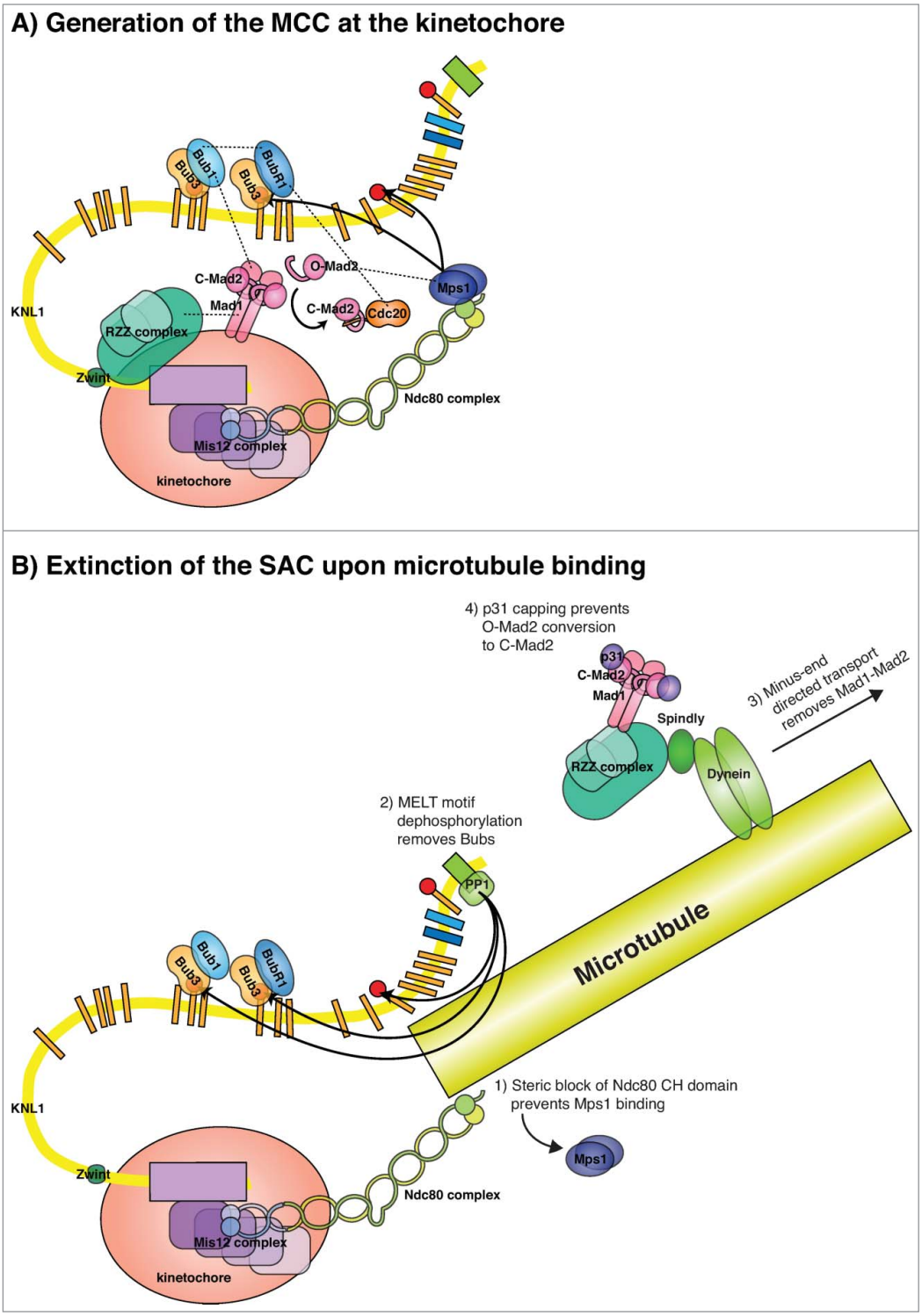

Figure 4. Generation of the MCC at kinetochores and silencing of this signal. (**A**) A schematic of the KMN network and the interaction of checkpoint proteins with this. Interactions between the RZZ complex and Bub1 could facilitate the localization of Mad1–Mad2 in human cells and an interaction between Cdc20 and BubR1 could position Cdc20 close to MCC components. The proper positioning of all MCC components, potentially scaffolded by a Mad1–Bub1 interaction, catalyzes the loading of soluble O-Mad2 onto Cdc20, locking Mad2 in its closed conformation (C-Mad2), and BubR1 subsequently binds. Kinetochore localized Mps1 might also directly regulate Mad2. (**B**) Several mechanisms might contribute to the removal of SAC proteins from the kinetochores once they have bound microtubules including (1) blocking of the Mps1 binding site on Ndc80, (2) dephosphorylation of the MELT motifs by PP1, (3) removal of the Mad1–Mad2 complex by dynein-mediated transport, and (4) capping of C-Mad2 by p31.

The MCC exists both in a free state and stably bound to the APC/C, and stable interaction of the MCC with the APC/C is important for a functional SAC.[5,78] BubR1 and the IR motif of Cdc20 contribute to stable MCC binding to the APC/C whereas Mad2 opposes this as the Mad2 binding site of Cdc20 is also required for APC/C interaction and activation.[78-80] The exact details of MCC interaction with the APC/C are still unclear but with recent advancements in APC/C and MCC structures and their docking into the cryo-EM map of the APC/C-MCC complex we now have a good understanding of how the MCC inhibits the APC/C.[5,7,74] One surprising observation is that MCC binding actually induces the active conformation of the APC/C; however, a number of mechanisms prevent APC/C–MCC activity. Within the MCC, Cdc20 is displaced from its normal position so that it no longer forms a combined binding site for D-boxes and therefore cannot bind substrates.[5,7] The second KEN-box of BubR1 further prevents substrate binding by an unknown mechanism.[40] In addition, the MCC contacts the catalytic APC2–APC11 module, potentially preventing interaction with the E2-ubiquitin complex and providing further inhibition.[7] Given the strong inhibition of the APC/C by the MCC, it is puzzling why apo-APC/C also exists in cells and it will be important to determine whether distinct forms of the APC/C exist and whether only some of these have to be inhibited.

SAC Silencing

The rapid activation of APC/C–Cdc20 in response to attachment of the last kinetochore suggests that Cdc20 is quickly liberated from inhibition. This is because MCC disassembly is constantly occurring even during an active checkpoint, and as soon as MCC production ceases free Cdc20 accumulates.[81,82] The generation of active Cdc20 consists of at least 2 steps, namely killing of the kinetochore signal and disassembly of existing MCC complexes.

Silencing the kinetochore signal requires the removal of the checkpoint proteins from the kinetochore. As discussed, one mechanism is the dynein-mediated removal of Mad1–Mad2, but the Mad1–Mad2 complex can also be removed from kinetochores in the absence of kinetochore–microtubule interactions. In yeast there is no RZZ complex and Mad1–Mad2 removal is coupled with Bub1 removal.[43] The Mad1–Mad2 complex is also inhibited by "capping" of C-Mad2 by p31^{comet}, which prevents binding of O-Mad2 to the complex once it is removed from kinetochores.[83] In addition, protein phosphatase 1 (PP1) is required for SAC silencing, and this activity requires its interaction with kinetochores. KNL1 contains a conserved PP1 binding site close to the region of KNL1 that interacts with microtubules, and binding of PP1 to KNL1 contributes to SAC silencing.[35,46,84-87] An obvious target of KNL1-bound PP1 is the phosphorylated MELT motifs and indeed increased kinetochore levels of Bub1 and BubR1 are observed when the PP1 binding site is removed.[46] A model for PP1-mediated SAC silencing is thus dephosphorylation of MELT motifs to remove Bub1 and BubR1 from kinetochores (**Fig. 4B**). The binding of PP1 to KNL1 appears to be tightly regulated so that PP1 only strongly accumulates at kinetochores in metaphase.[85] The PP1 binding site on KNL1 contains a phosphorylation site for Aurora B, which when phosphorylated prevents PP1 binding.[85] Thus, PP1 and Aurora B antagonize each other on the outer kinetochore and when microtubules bind the balance tips toward PP1 binding and SAC silencing. Furthermore, as microtubule binding might prevent Mps1–Ndc80 interactions this would further favor removal of Bub1 and BubR1 from KNL1. In humans and *C. elegans*, preventing PP1 binding to KNL1 has a mild effect on SAC silencing, which might suggest that additional phosphatases play an important role in these organisms.[46,87]

MCC and APC/C–MCC are stable complexes and their disassembly is an active process. The exact mechanism of disassembly, or indeed whether free MCC and APC/C-bound MCC disassemble by the same mechanism, is not clear. At least 2 distinct pathways have been suggested to remove Mad2 from Cdc20: a p31^{comet}-catalyzed mechanism and APC/C-mediated ubiquitination of Cdc20. p31^{comet} binds specifically to C-Mad2 at the same dimerization surface as O-Mad2 and BubR1, therefore p31^{comet} could facilitate MCC disassembly by preventing Mad2–BubR1 interactions within the MCC and destabilizing the entire complex.[74,82,88] To facilitate efficient C-Mad2 removal p31^{comet} might collaborate with the AAA-ATPase TRIP13.[89,90] Why this then leads to selective removal of Mad2 and not BubR1 from the MCC is unclear, but as discussed above it appears that once the BubR1–Cdc20 interaction is established Mad2 is no longer needed. An interesting observation is that p31^{comet} acts very inefficiently on APC/C-bound MCC, suggesting that the entire MCC might have to dissociate from the APC/C before p31^{comet} can act on it.[82] A second proposed mechanism of MCC dissociation, which would be specific for APC/C bound MCC, is ubiquitination of Cdc20 by the APC/C, a process regulated by APC15.[79,91-93] APC15 is a small APC/C subunit close to the region of APC8 that likely engages the C-box of co-activators, and removal of APC15 leads to elevated levels of Cdc20 and MCC on the APC/C. Similar observations have been obtained with the budding yeast homolog Mnd2.[7,79,91,92] Although different studies on APC15 and Mnd2 agree on a role in SAC silencing, they differ over whether this involves Cdc20 ubiquitination. One cautionary note is that since APC15 RNAi or Mnd2 deletion results in elevated total levels of Cdc20 and thus MCC, it is difficult to compare these to the wild-type situation. It might be that the cell has to reduce MCC levels below a certain threshold before anaphase is allowed and so even similar rates of MCC disassembly would result in slower mitotic exit if the initial MCC levels were higher. It will be important to clarify whether there is a threshold level of MCC that prevents anaphase and how this relates to free Cdc20 levels.

Concluding Remarks

Since the discovery of the SAC and its tight link to kinetochore status we now have a very detailed picture of the molecular events leading to APC/C inhibition. However, the more we understand, the more we realize how complex the system is and how much there still is to learn. Given the complexity of the kinetochore and the fact that that it might scaffold large checkpoint complex assemblies in a unique manner, it will be a challenge to study checkpoint protein interactions using traditional biochemical approaches. It might necessary to design novel quantitative tools to look at interactions *in vivo* before we can fully understand this checkpoint.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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