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Kinetochore-microtubule interactions "in check" by Bub1, Bub3 and BubR1: The dual task of attaching and signalling

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Review

Kinetochore-microtubule interactions "in check" by Bub1, Bub3 and BubR1

The dual task of attaching and signalling

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The spindle assembly checkpoint (SAC) prevents anaphase onset until all chromosomes accomplish proper bipolar attachments to the mitotic spindle and come under tension, thereby ensuring the fidelity of chromosome segregation. Despite significant advances in our understanding of SAC signalling, a clear link between checkpoint signalling and the molecular mechanisms underlying chromosome attachment to microtubules has not been established so far. However, independent studies from many groups have interestingly found that the bone-a-fide Bub1, BubR1 and Bub3 SAC proteins are themselves required for proper kinetochore-microtubule (K-MT) interactions. Here, we review these findings and discuss the specific contribution of each of these proteins in the regulation of K-MT attachment, taking into consideration their interdependencies for kinetochore localization as well as their relationship with other proteins with a known role in chromosome attachment and congression.

Introduction

The spindle assembly checkpoint (SAC) is a surveillance mechanism that prevents chromosome missegregation in dividing eukaryotic cells by delaying the metaphase-to-anaphase transition until all chromosomes establish proper attachments to spindle microtubules and align at the metaphase plate. Core components of the SAC proteins include the evolutionary conserved Bub1, Bub3, Mad1, Mad2, BubR1 (Mad3 in yeast), Mps1 and Aurora B proteins. Additional proteins that regulate SAC activity in higher eukaryotes include the plus-end directed kinesin motor CENP-E, the minus-end directed motor dynein, dynein-interacting proteins such us dynactin, CLIP170 and LIS1, and the RZZ (ROD-ZW10-ZWILCH) complex (reviewed in refs. 1 and 2). In higher organisms, all these SAC proteins localize to unattached kinetochores during prometaphase, which are specialized protein complexes that assemble on centromeric DNA and provide the site of chromosome binding to spindle microtubules. As long as there are unattached kinetochores or improper attachments unable to produce enough tension between sister chromatids, the SAC pathway will be active, and an active complex containing Mad2, BubR1 and Bub3 (called mitotic checkpoint complex or MCC) will be generated which acts to sequester and inhibit Cdc20, an activator of the E3 ubiquitin ligase called the anaphase promoting complex/cyclosome (APC/C) that is responsible for the metaphase to anaphase transition.[3,4] Once the last chromosome achieves proper bipolar attachment to the mitotic spindle (bi-orientation), the SAC is turned off and the subsequent release of Cdc20 triggers APC/C activation, which in turn ubiquitinates securin and cyclin B targeting them for degradation by the 26S proteasome. Degradation of securin, an inhibitor of the protease separase, leads to cohesin proteolysis and sister chromatid separation, whereas cyclin B degradation induces mitotic exit.

Therefore, by coordinating K-MT interactions and anaphase onset, the SAC reduces the frequency of chromosome missegregation and prevents aneuploidy. How defects in K-MT interactions are monitored and linked to SAC signalling machinery is far from being elucidated. However, recent studies came out with a role of SAC proteins, such as Bub1, BubR1 and Bub3, in the establishment of proper K-MT attachments suggesting that the SAC proteins might be both regulating K-MT interactions and generating the checkpoint signal.[5-9] Nevertheless, given the interdependencies of the Bub proteins for kinetochore localization, together with the complex network of molecular interactions at the kinetochore, questions are raised regarding their specific contribution in the regulation of K-MT attachments which deserve to be addressed.

Regulation of Kinetochore-Microtubule Attachments by BUB1, BUBR1 and BUB3

Small molecule inhibitors have revealed to be powerful tools in the dissection of dynamic cellular processes.[10,11] The cell membrane is permeable to these compounds which, therefore, act quickly, within minutes. Also, their effect is easily reverted by simply washing out the cell media, turning them into ideal tools for switching on/off the function of their targets, allowing observation of the immediate

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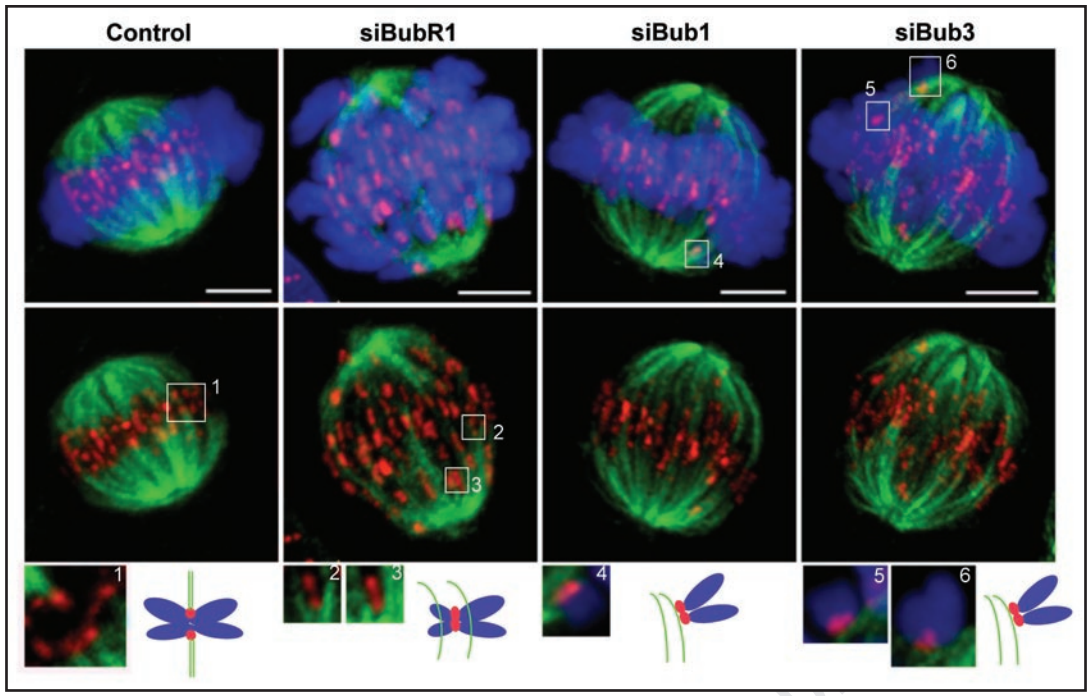

Figure 1. Misalignment defects caused by BubR1, Bub1 and Bub3 repressions. Control, BubR1, Bub1 and Bub3 siRNA-depleted cells were incubated with MG132, and stained for CREST, tubulin and DNA. Maximal intensity projections of the entire cell Z-stack are shown. Insets are projections of 4–8 Z-stacks for individual K-MT attachments of misaligned chromosomes. Note the lateral attachments (insets 4–6) of Bub3- and Bub1-depleted misaligned chromosomes. As a comparison, end-on attached kinetochores of correctly aligned sister chromatids are shown (inset 1), as well as detached BubR1-depleted chromosomes (insets 2 and 3). Bars, 5 μm.

effects and live imaging. Combined with RNAi-based knockdown experiments, small molecule inhibitors have been very helpful in elucidating some functional aspects of SAC proteins. In particular, the small molecule MG132, a powerful inhibitor of the proteasome, has been used to uncouple the function of SAC proteins in mitotic progression timing from their eventual role in chromosome bi-orientation and congression.[11]

Ditchfield et al.[5] were the first to report such a dual function for a checkpoint protein, namely for BubR1. They found BubR1 RNAi cultures to often exhibit abnormal prometaphases with chromosomes aligned along the length of the spindle rather than at the metaphase plate (Fig. 1), and anaphases with lagging chromosomes. As depletion of checkpoint proteins leads to premature mitotic exit before chromosomes get properly attached to the spindle and aligned, they treated cells with MG132 in order to prevent anaphase onset and, thereby, to assess the effects of BubR1 repression on chromosome alignment independently of its effects on mitotic checkpoint activity. They found BubR1 to be required for chromosome alignment as misalignment persisted after MG132 treatment. RNAi-mediated Bub1 repression was also shown to affect chromosome alignment, however differently from BubR1 depletion, as most chromosomes did align on the metaphase plate with only one or a few lying outside the plate (Fig. 1).[12] Furthermore, these unaligned chromosomes could eventually align if anaphase was prevented with MG132. This suggested Bub1 to be required for chromosome congression.

Subsequent studies, again using the proteasome inhibitor, further elucidated the nature of K-MT interactions defects caused by BubR1 and Bub1 RNAi-depletions.[6,7] High resolution immunofluorescence microscopy was used to show that kinetochores of BubR1-depleted cells retain high levels of Mad2 and p150 (a subunit of the dynactin complex), proteins known to delocalize from the kinetochores upon microtubule binding. Interestingly, the BubR1-depletion phenotype was found to depend on Aurora kinase B activity, which acts to destabilize tension-defective attachments.[6,13-15] Inhibition of Aurora B activity, either by the small molecule inhibitors, hesperadin and AKI-1,[5,16] or by RNAi, restored attachment and suppressed the misalignment phenotype in BubR1-depleted cells. Therefore, it was proposed that BubR1 links stabilization of K-MT attachments to SAC signalling. Consistently, a parallel study came out with a dual function of Bub1 in SAC and chromosome congression. Bub1 depletion was found to cause an accumulation of misaligned chromosomes in which kinetochores failed to achieve end-on binding (Fig. 1).[7] Inhibition of Aurora kinase B activity in Bub1-RNAi cultures resulted in an additive misalignment phenotype suggesting, therefore, that Bub1 and Aurora B regulate chromosome attachment via separate pathways.

Bub1 and BubR1 form cell cycle constitutive complexes with Bub3 and these proteins were reported to be interdependent for kinetochore localization.[17,18] This led us to address whether Bub3 is also required for the establishment of proper K-MT interactions, and whether each Bub protein has a specific role in K-MT interactions. We found Bub3 RNAi to induce chromosome misalignment (Fig. 1),[9] and through a comparative analysis we showed that: (i) Bub3 and Bub1 RNAi induce similar misalignment phenotypes which are different from the BubR1 RNAi phenotype; and (ii) Bub3 is required for the establishment of K-MT end-on attachments, as previously suggested for Bub1 while BubR1 regulates K-MT stability. It is noteworthy that the Bub RNAi-repressions do not largely affect kinetochore assembly as judged by CENP-E and NDC80/Hec1 immunostainings, among others; and they are specific as RNAi

of another bonafide SAC protein, Mad2, does not affect chromosome alignment, suggesting that Mad2 is exclusively implicated in the regulation of mitotic timing.[19,20] However, the complex nature of K-MT interactions together with protein interdependencies for kinetochore localization turns out difficult to assign a specific role to each Bub protein in the regulation of K-MT attachments. In the next sections, we will focus on this issue and then speculate on the linkage between the microtubule-binding machinery at kinetochores and the Bub proteins.

Assigning Specific Roles to BUB1, BUBR1 and BUB3 in Kinetochore-Microtubule Attachments

Kinetochore localization of SAC proteins seems to be hierarchical, implying that the recruitment of some depends on the prior recruitment of others.[21,22] It is generally believed that Bub1, BubR1 and Bub3 function in a complex and that they are interdependent for kinetochore localization. However, independent studies have reported contradictory findings concerning kinetochore localization interdependencies amongst SAC proteins. For example, some groups found Bub1 to be required for kinetochore localization of BubR1, Bub3 and CENP-E,[9,12] while others described it not to be the case.[7,19] Similarly, different results were reported regarding the requirement of Bub3 for kinetochore localization of Bub1 and BubR1.[9,18,19] In one comprehensive study of kinetochore assembly, Lin et al. carefully quantified the degree of siRNA depletion of specific proteins as well as analyzed kinetochore localization interdependencies using high quality antibodies.[22] This study placed Bub1 at the nexus of several kinetochore assembly pathways and showed that Bub1 is required for BubR1 but not for CENP-E kinetochore localization. Whether discrepancies are due to the fact that assembly of kinetochore proteins depends on multiple rather linear branches, or reflect variations in cell type, repression levels and/or off-target RNAi effects, is unclear. Therefore, kinetochore localization interdependencies between SAC proteins should be taken into account carefully when studying their functions. Although the assignment of a clear-cut role to a SAC protein in K-MT interaction implies it not to be required for kinetochore localization of other SAC proteins, one cannot exclude the above mentioned factors of variability as well as the possibility that kinases (like Bub1 and BubR1) might act independently of their kinetochore localization. One approach to overcome the difficulty of function assignment face to interdependent protein localizations is to examine double repression phenotypes and determine whether distinct aspects of K-MT interaction, such as achievement of end-on attachment, attachment stability and chromosome congression, might be differently affected by the SAC proteins (see below).

In our recent work, RNAi-mediated double depletion experiments shed light upon the specific requirement of each Bub protein for K-MT attachments.[9] Remarkably, chromosome alignment defects in the Bub3/Bub1 double repression are worst than in Bub3 and Bub1 single knockdowns. Such an additive phenotype is expected for proteins with parallel function and explains why depletion of either one individually is not enough to create a dramatic effect. Furthermore, Bub1/BubR1 and Bub3/BubR1 double repressions exhibit similar misalignment phenotypes, not significantly different from that in BubR1 single repression, but less severe than that in Bub1/Bub3 depletion. Again this is consistent with parallel functions converging on the regulation of a specific K-MT event, with Bub3 still operating in the absence of Bub1 and vice-versa. However, considering that even small amounts of proteins could still account for significant function on K-MT interaction, the possibility that Bub3 and Bub1 may act cooperatively should not be excluded, as in this case co-repression would also produce an additive effect on misalignment compared to single repressions. Assuming the caveat that siRNAs are inefficient, the Bub3/Bub1 phenotype could be due to full inhibition of Bub1 activity achieved by the co-repression of Bub3, initially described as a potential co-factor of Bub1.[23,24] Nevertheless, this seems unlikely as the misalignment phenotype of previously reported complete Bub1 RNAi was not as severe as that of Bub3/Bub1 RNAi.[7] Finally, it is noteworthy that for all SAC protein repression data reported so far, a significant fraction of chromosomes do align, suggesting that parallel pathways do exist for the formation of K-MT attachments.

BUB1/BUB3 and BUBR1 Regulate Distinct Aspects of Prometaphase Kinetochore-Microtubule Interactions

Kinetochores bind MTs laterally in a transient fashion, and stably by insertion of plus-ends (reviewed in refs. 25 and 26). These pathways might exist to carry out distinct tasks during different stages of mitosis, and likely depend on distinct molecular mechanisms where different SAC proteins could be participating. This might also be related to the fact that different SAC proteins monitor distinct aspects of K-MT binding (attachment versus tension).[27]

Mechanisms for kinetochore transport are poorly understood. In yeast, two mechanisms were recently shown to be involved in MT-dependent poleward kinetochore transport: (i) Sliding along the MT lateral surface, which is driven by Kar3 (a kinesin-14 family member), and by dynein in metazoans; (ii) End-on pulling, i.e., kinetochore tethering at MT-distal ends and poleward pulling as MTs shrink, which is partly hindered by Kar3 and requires the Dam1 complex for its progression, for which possible functional counterparts of vertebrate kinetochores have been suggested but remain elusive.[28-30]

After kinetochores are transported to the vicinity of a spindle pole in prometaphase, they move towards the spindle equator to form the metaphase plate (congression). Congression often occurs after sister chromatid bi-orientation, regulated mostly by the kinase activities of Aurora B, Plk1 and Mps1.[13,31,32] But in metazoan cells, kinetochore-bound CENP-E facilitates this process before bi-orientation occurs, by transporting mono-oriented chromosomes away from the pole along MTs that are attached to other already bi-oriented and congressed kinetochores.[33] Yet another alternative strategy exists in which the unattached kinetochore in the mono-oriented chromosome can develop K-fibers by centrosome-independent mechanisms that subsequently capture astral MTs and are transported to the distal spindle pole.[34] Considering the above mechanisms of kinetochore movement during prometaphase, which of them could be regulated by Bub1, Bub3 and BubR1 functions?

High-resolution confocal imaging suggests that misaligned chromosomes in Bub1 and Bub3 RNAi-depleted cells exhibit distinct MT-binding defects compared to those in BubR1 RNAi. Whereas the former frequently show MTs running past the kinetochore pairs, suggesting side-on binding to the walls of MTs, the latter are mostly detached.[6-9] Also, the misaligned chromosomes in Bub1 and Bub3

RNAi-depleted cells are not centrophilic (tightly associated with the centrosomes) as those typically found in CENP-E RNAi-depleted cells.[35] Inter-kinetochore distances, measured in all the defective K-MT attachments analysed, further informed that while Bub3- and Bub1-depleted misaligned chromosomes often exhibit intermediate levels of tension, those in CENP-E and BubR1-depleted cells have low levels of tension. Interestingly, in BubR1-depleted cells, misaligned chromosomes despite detached have kinetochore pairs parallel to the spindle axis, with inter-kinetochore distances slightly higher than those produced by nocodazole treatment, which is in agreement with the fact that BubR1 depletion destabilizes K-MT attachments rather than impairing MT binding.[6] In Bub3- or Bub1-depleted cells the prevalence of side-on attachments, rarely found in control cells due to their transient nature, indicates impairment of poleward movement and/or switching to end-on attachment.[9]

Further supporting the idea that Bub1/Bub3 and BubR1 act in different regulatory pathways of K-MT interactions, Aurora B kinase inhibition was shown to restore K-MT attachments in BubR1- but not Bub3- and Bub1-depleted cells.[6,7,9] Aurora kinase inhibition has a suppressing effect in BubR1 phenotype as it helps to stabilize the K-MT attachments, but in contrast, it has an additive effect on the misalignment phenotype of Bub3 and Bub1 RNAi. This suggests that the defective attachments in Bub3- and Bub1-depleted cells are detected by active Aurora B, which acts to destabilize them, thereby creating the possibility for new correct attachments to be established.

In summary, Bub1 and Bub3 seem to regulate the switching from lateral to end-on attachment, while BubR1 is required for stabilization of K-MT attachments (Fig. 1).

Linking the BUB Proteins to the Microtubule-Binding Machinery at Kinetochores

Understanding how kinetochores coordinate the formation of K-MT and SAC downregulation is crucial to future progress. Kinetochore protein complexes have five major functions: (i) kinetochore specification; (ii) kinetochore assembly; (iii) microtubule binding; (iv) monitoring and (v) K-MT attachment regulation (reviewed in refs. 1 and 2). The Bubs are K-MT attachment monitoring proteins that do not seem to affect kinetochore specification or assembly. Although still elusive, it is tempting to foresee a link between the Bub proteins and the MT-binding proteins at kinetochores on the basis of the available data (Fig. 2).

In higher eukaryotes, kinetochore proteins with MT-binding function include proteins that form the core attachment site (Ndc80/HEC1 complex); proteins that control microtubule dynamics (CLASP, CLIP170, APC/EB1 and MAP215); and proteins that promote kinetochore motility (dynein and CENP-E). Therefore, these proteins are attractive targets of the SAC proteins. No direct association between the Bub proteins and the Ndc80 complex components has been reported so far. Ndc80 complex is required for proper kinetochore recruitment of Mps1 but does not affect the kinetochore localization of Bub1 and BubR1.[36] However, as Mps1 is required for kinetochore recruitment of BubR1, Bub1 and Bub3, an indirect link between Ndc80 and Bubs can not to be excluded.[37]

Regarding proteins that control microtubule dynamics, the MT plus-end associated protein APC (Adenomatous Polyposis Coli) has been shown to form a complex with the mitotic checkpoint proteins Bub1 and Bub3 and to be a substrate for Bub1/BubR1 kinases in vitro.[38] Both APC and its binding partner EB1 have been implicated in chromosome behaviour in mammalian cells.[39,40] More recent findings clearly support a model in which BubR1 kinase activity directly regulates APC function for the positioning of chromosomes at the metaphase plate and the establishment of stable K-MT attachments (Fig. 2).[41] Furthermore, APC has been found to associate with MCAK at kinetochores in Xenopus egg extracts.[42] MCAK is involved in the depolymerization of improperly attached MTs (merotelic and syntelic attachments) to ensure accurate chromosome segregation,[43] being its MT depolymerising activity reduced upon Aurora B phosphorylation in vitro.[44,45] Therefore, it is possible that BubR1, APC/EB1, Aurora B and MCAK cooperate to ensure proper K-MT attachment.

Regarding proteins that promote kinetochore motility, CENP-E and dynein molecular motors are appealing candidates for a link with the Bub proteins. The kinase activity of BubR1 was shown to be controlled by CENP-E and downregulated upon CENP-E binding to microtubules,[46] providing an example of coordination between microtubule binding and SAC signalling. However, CENP-E does not seem to account, at least not exclusively, for the chromosome misalignment phenotype in Bub depleted cells since it still localizes at the kinetochores in those cells.[6-9] Moreover, Bub1 haplo-insufficient mouse embryonic fibroblasts (MEFs), which exhibit normal CENP-E recruitment to kinetochores, display similar rates of congression failure as Bub1 hypomorphic MEFs in which CENP-E target is perturbed.[47] In addition, whereas misaligned chromosomes in CENP-E-depleted cells are typically centrophylic and align if cells are blocked in metaphase with MG132, misaligned chromosomes in Bub-RNAi exhibit a K-MT attachment defect which positions them between the pole and the equatorial plate and that persists after proteasome inhibition.[9] These results clearly suggest that Bub depletion compromises other mechanism of K-MT attachment rather than congression. Recently, Bub3 was found to be a specific binding partner of cytoplasmic dynein light chain DYNLT3.[48] Dynein selective depletion from kinetochores, achieved by either mutating or depleting the ZW10 protein required to target dynein to kinetochores, leads to chromosome misalignment and tension reduction defects very similar to those caused by Bub1 and Bub3 RNAi.[29,49] Importantly, ZW10 depletion does not affect congression of bi-oriented chromosomes. Thus, one possible model is that, acting through dynein, Bub3/Bub1 may promote the generation of a poleward pulling force that facilitates proper bipolar attachment, even though for Bub1 an interaction with dynein stills needs to be tested (Fig. 2). As a consequence, many aspects of K-MT interactions would be affected in Bub3- and perhaps Bub1-depleted cells such as stable monotelic attachments, which come out from the pulling forces that drive rapid movement of kinetochore along transiently laterally-associated MTs towards the pole; orientation of a mono-oriented chromosome, which depends on chromosome oscillation powered by the pulling force and the polar ejection force; and correction of syntelic attachments, which depends on the movement to the attaching pole. Interestingly, and related to this latter aspect of K-MT interaction, Bub1 has been shown to be required for the kinetochore localization of MCAK.[22] It will be important to determine if the dynein-dependent steps are compromised in Bub3-as well as in Bub1-depleted cells.

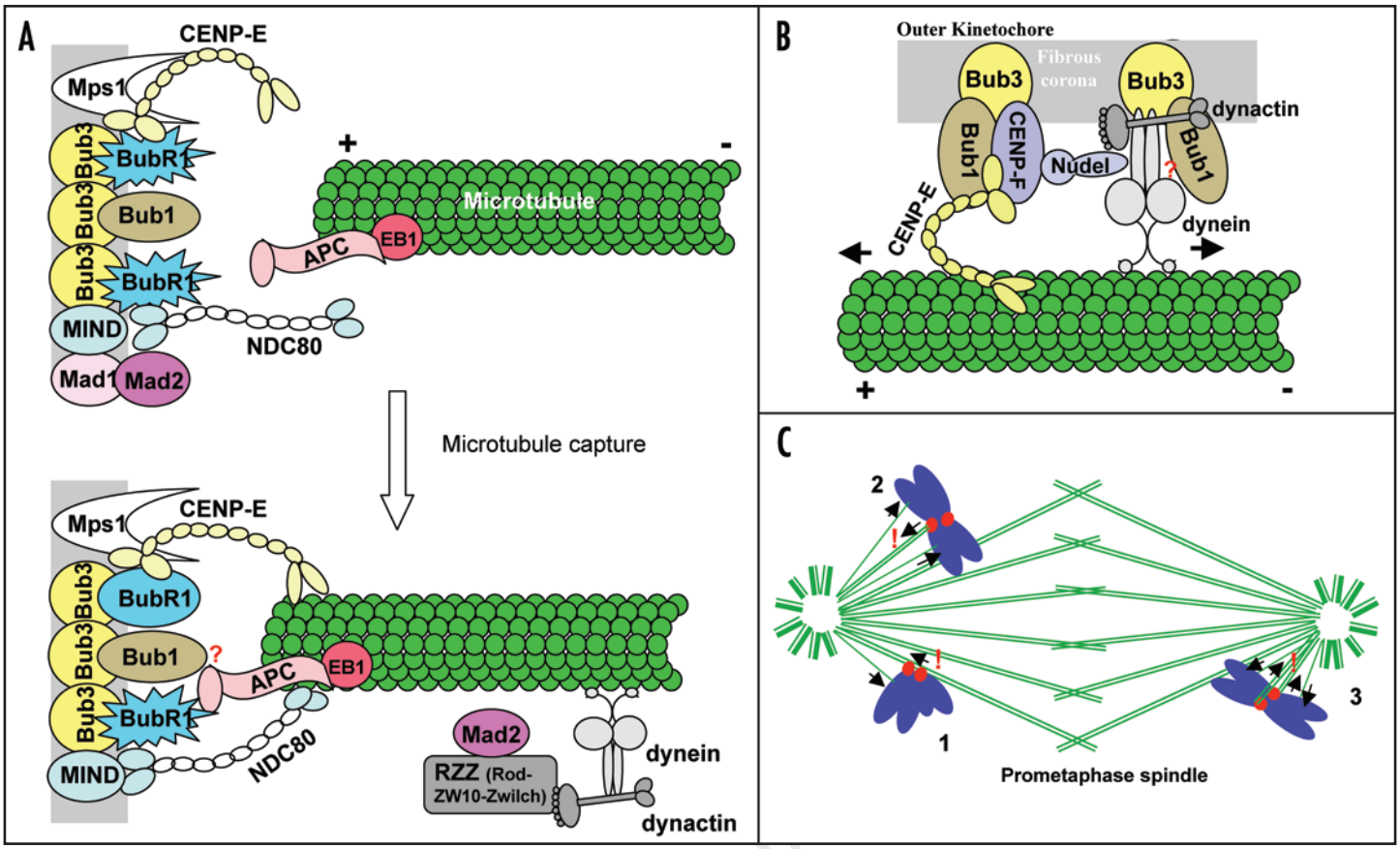

Figure 2. Models for BubR1 and Bub1/Bub3 functions on the establishment of K-MT attachments. (A) Model for BubR1 action. BubR1 is recruited into unattached kinetochores and the microtubule-associated proteins EB1-APC bind to the plus ends of microtubules. Once the initial capture of microtubules by kinetochores takes place, the interaction between BubR1 and APC/EB1 stabilizes K-MT attachment, which possibly requires APC phosphorylation by BubR1 (which should happen before inactivation of BubR1 kinase activity upon CENP-E binding to microtubules). Bub1 also interacts and phosphorylates APC but its function on stabilization of K-MT attachments remains undetermined. (B) Model for Bub3 and Bub1 action. Bub3 interacts directly with dynein light chain. Dynein translocates laterally associated kinetochores to the vicinity of spindle poles thereby regulating the switching to end-on attachments. Interaction between Bub1 and dynein remains unknown. Bub1 also interacts with CENP-F and CENP-E to form a complex which drives plus end directed motility. (C) Compromised chromosome movements in cells lacking Bub3/dynein activity (as adapted from Liang et al.,).[54] Lack of poleward movement reduces the efficiency of lateral to end-on attachment transition (1). Lack of chromosome oscillation leads to misorientation and impairment of MT capture by the unattached chromosome (2). Chromosomes with syntelic attachments do not move to the spindle pole (3). See text for details.

Conclusion

Understanding how chromosome-microtubule interactions are transduced into signals that can be integrated by the SAC molecular pathway has remained a challenge since the discovery of this mitotic surveillance mechanism. The data here reviewed stress that the connection may rely on the dual function of checkpoint proteins. This way these proteins are at the right place at the right time, establishing an interface between target molecules involved in diverse aspects of chromosome-to-spindle binding and the checkpoint signalling and attachment-correction mechanisms. Interestingly, this dual function is also found for proteins of the DNA damage checkpoint that participate in both signalling and DNA repair.[50] As a consequence, cells with decreased levels of SAC proteins not only will experience defective K-MT attachments, but will also be unable to sense the errors, being thereby highly prone to genetic instability.[51] Furthermore, and importantly in this review context, spindle checkpoint defects have been associated with resistance to spindle damaging agents such as taxanes and vinca alkaloids,[52,53] a fact that might be related to the molecular mechanisms here described for the checkpoint proteins, turning them of significant clinical impact.

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