FEBS Letters

Dynein-dependent transport of spindle assembly checkpoint proteins off kinetochores toward spindle poles

Patrícia M.A. Silva [a,b], Rita M. Reis [a], Victor M. Bolanos-Garcia [c], Claudia Florindo [b,d], Álvaro A. Tavares [b,d], Hassan Bousbaa [a,e,f,∗]

[a] CESPU, Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, Rua Central de Gandra, 1317, 4585-116 Gandra PRD, Portugal
[b] Centre for Molecular and Structural Biomedicine,CBME/IBB, University of Algarve, Faro 8005-139, Portugal
[c] Faculty of Health and Life Sciences, Department of Biological and Medical Sciences, Oxford Brookes University, Oxford, United Kingdom
[d] Departamento Ciências Biomédicas e Medicina, University of Algarve, Faro 8005-139, Portugal
[e] Centro de Química Medicinal da Universidade do Porto (CEQUIMED-UP), Rua de Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal
[f] Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR/CIMAR), Universidade do Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal

ARTICLE INFO

ABSTRACT

A predominant mechanism of spindle assembly checkpoint (SAC) silencing is dynein-mediated transport of certain kinetochore proteins along microtubules. There are still conflicting data as to which SAC proteins are dynein cargoes. Using two ATP reduction assays, we found that the core SAC proteins Mad1, Mad2, Bub1, BubR1, and Bub3 redistributed from attached kinetochores to spindle poles, in a dynein-dependent manner. This redistribution still occurred in metaphase-arrested cells, at a time when the SAC should be satisfied and silenced. Unexpectedly, we found that a pool of Hec1 and Mis12 also relocalizes to spindle poles, suggesting KMN components as additional dynein cargoes. The potential significance of these results for SAC silencing is discussed.
© 2014 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

Accurate chromosome segregation relies on the activity of the spindle assembly checkpoint (SAC) [1], a surveillance mechanism that monitors the status of kinetochore-microtubule attachment and prevents anaphase onset until all chromosomes are bipolarly attached to the mitotic spindle and aligned at the metaphase plate [2]. Inhibition of anaphase onset is achieved by the SAC through downregulation of the APC/C (anaphase promoting complex/cyclosome). The latter is a E3 ubiquitin ligase required for targeting cyclin B1 and securin for degradation by the 26S proteasome, thereby delaying cell in mitosis and preventing precocious sister-chromatid separation. APC/C inhibition is predominantly achieved

by the MCC, the mitotic checkpoint complex that forms between the SAC proteins Mad2, BubR1 and Bub3, and the cofactor and activator of APC/C, Cdc20 [3]. It is widely believed that unattached or improperly attached kinetochores provide the catalytic platform for the formation of the anaphase inhibitor MCC [1,4]. SAC components also include the evolutionary conserved proteins Mad1, and the kinases Bub1, Mps1, and Aurora B. These proteins localize to kinetochores and participate in the recruitment, activation, or formation of the MCC. Kinetochore levels of these proteins fall upon microtubule attachment [2]. Once all kinetochore-microtubule attachments are efficiently established, SAC must be extinguished in order to allow for mitotic progression, a process known as SAC silencing. Extinguishing SAC requires that assembly of new MCC is prevented and existing inhibitory complexes are disassembled in order to release Cdc20 and, thus, alleviate APC/C inhibition [5–7].

A predominant mechanistic model of SAC silencing postulates the removal of proteins that are required for SAC activation from

∗ Corresponding author at: CESPU, Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, Rua Central de Gandra, 1317, 4585-116 Gandra PRD, Portugal.
E-mail address: hassan.bousbaa@iscsn.cespu.pt (H. Bousbaa).

attached kinetochores via stripping (also known as streaming) [7]. Stripping is mediated by the minus-end directed microtubule motor dynein, which transports certain SAC proteins off kinetochores towards spindle poles along kinetochore-microtubules. Using an ATP depletion assay that maintained dynein activity but prevented release of its cargo from spindle poles, Howell et al. were the first to report that Mad2 and BubR1 (as well as CENP-E, dynein itself and 3F3/2 antigens) are transported to spindle poles, and that inhibition of dynein prevented Mad2 removal from attached kinetochores [8]. Subsequent studies also based on ATP depletion have catalogued several kinetochore components for their ability to be transported by dynein [9]. While it is consensual that Mad1, Mad2, and spindly (which is required for dynein kinetochore binding) are dynein cargoes, there is conflicting data concerning the poleward transport of Bub1 and BubR1 [8,9]. Furthermore, whether all SAC components are transported to spindle poles upon checkpoint silencing is not known yet.

In an attempt to clarify these issues, we used two ATP depletion assays to assess the poleward transport of SAC proteins, focusing the analysis on those that are relevant to the generation of the anaphase inhibitory signal. Through this approach, we confirm the dynein-dependent transport to spindle poles of Mad1 and Mad2; we clarify that Bub1 and BubR1 are indeed dynein cargoes; and propose Bub3 as additional dynein cargo. We also report that the poleward transport of these SAC proteins persists in metaphase-arrested cells, a situation when the cells are in a SAC inactive state with all chromosomes bipolarly attached. Interestingly, we further propose dynein-dependent poleward transport of the KMN components Hec1 and Mis12.

2. Results

We were particularly interested in understanding the behavior of *bona fide* SAC proteins in terms of their dynein-dependent removal from attached kinetochores during SAC silencing. We first examined Mad1 and Mad2 as it has been established that these proteins function as dynein cargoes. After treatment of HeLa cells with sodium azide/2-deoxyglucose (AZ/DOG), we observed a strong accumulation of Mad2 at spindle poles in all prometaphase and metaphase cells analyzed, while Mad2 was undetectable at attached kinetochores (Fig. 1A). Mad1 redistribution from kinetochores to spindle poles mirrored that of Mad2 (Fig. 1B), in accordance with previous reports [8–10]. Treatment with nordihydroguaiaretic acid (NDGA) showed similar results (data no shown).

BubR1 has been shown previously to redistribute to spindle poles in an ATP reduction assay [8]. However, a subsequent study using NDGA treatment reported that BubR1 did not accumulate at spindle poles and therefore ruled out the role of BubR1 as dynein cargo [9]. In an attempt to clarify this crucial aspect of SAC signalling, we treated HeLa cells with AZ/DOG or with NDGA followed by staining of BubR1. In the NDGA assay, BubR1 was observed at kinetochores but not at spindle poles, behaving much like untreated cells (Fig. 1C), thus suggesting that BubR1 is insensitive to NDGA in the experimental conditions here described. In the 30 min AZ/DOG assay, we were unable to detect BubR1 at kinetochores and at spindle poles (Supplementary Fig. 1A). However, in a recovery experiment, 10 min after AZ/DOG washout, BubR1 readily reappeared on kinetochores (Supplementary Fig. 1A). In order to clarify whether BubR1 stability was affected by AZ/DOG, making it inaccessible to the antibodies, we reduced the AZ/DOG incubation time to 10 min. Under such conditions, BubR1 was consistently observed at spindle poles with a clear loss from both unaligned and aligned kinetochores (Fig. 1D), suggesting that BubR1 was affected by ATP reduction at longer incubation periods.

Although definition of how exactly BubR1 is affected by ATP reduction requires further investigation, our results suggest that BubR1 relocalizes to spindle poles upon kinetochore attachment and that conflicting results from previous studies is due to differences in the experimental protocols used by different authors. Such discrepancies extend to the study of Bub1, which has been reported to function as dynein cargo in AZ/DOG but not in NDGA assays [9,11]. Our data shed light into this process as they consistently show that Bub1 leaves the kinetochores toward spindle poles after 30 min incubation with the inhibitors (Fig. 1E) thus supporting the view that Bub1 functions as dynein cargo.

Bub3 is a crucial kinetochore partner of Bub1 and BubR1 that is required for efficient kinetochore-microtubule attachments [12]. Bub3 forms part of the MCC; binds to the recently identified protein BuGZ [13,14] and is required for SAC activation and silencing. Given the multiple roles of Bub3 in SAC signaling, we set out to investigate whether Bub3 is poleward transported by dynein upon kinetochore-microtubule attachment. For this, we used a HeLa cell line stably expressing EGFP-Bub3. Thirty minutes after treatment with AZ/DOG (Fig. 1F) or NDGA (data not shown), we observed EGFP-Bub3 at spindle poles, while a pool was retained at attached kinetochores in all prometaphases and metaphases analyzed (Fig. 1F). The persisting EGFP-Bub3 pool at kinetochores was not due to insufficient inhibition as Mad2 was depleted from kinetochores within the same treated cells (Fig. 1G). Importantly, extension of the incubation time with the inhibitors gave the same results (data not shown). Although the possibility that EGFP-Bub3 competed with the endogenous version cannot be ruled out, our data support the idea of the normal retention of a Bub3 pool at aligned kinetochores until the onset of anaphase [15]. The possibility of our observations being the result of an incomplete enzymatic reaction is remote given the time scale of mitosis in HeLa cells and the fact the assays were performed for up to 45 min. Comparable incubation times for ATP reduction assays have been reported by others [10] thus allowing a fair and meaningful comparison of our experiments with such reports.

Interestingly, we observed that upon AZ/DOG or NDGA treatment, Mad1, Mad2, BubR1, Bub1, and Bub3 accumulate at spindle poles from prometaphase through anaphase (Fig. 1), suggesting that their poleward transport may occur even in cells with the checkpoint satisfied. This was particularly clear in the case of Mad2 in metaphase when this protein was absent from attached kinetochores [16]. To assess if this could be the case of other SAC proteins, we treated cells with the proteasome inhibitor MG132 to arrest cells in metaphase before performing the ATP reduction assay. In these conditions, all SAC proteins were still able to accumulate at spindle poles (Fig. 2A). We took advantage of the HeLa cell line stably expressing EGFP-Bub3 to perform live-cell imaging experiments in order to monitor spindle pole relocalization of Bub3 upon ATP reduction. In control cells, the behavior of EGFP-Bub3 was consistent with the cellular distribution pattern described for endogenous Bub3 throughout mitosis (Fig. 2B and Supplementary Movie 1) [12]. We noted, however, that mitotic progression stopped almost instantaneously upon addition of AZ/DOG, indicating that it is affected by ATP reduction. This aspect was not appreciated in previous works that used ATP reduction assays, which precludes any comparison with such reports. We therefore monitored mitotic progression by time-lapse microscopy, and once cells reached the desired mitotic phase AZ/DOG was added to the medium and cells immediately filmed. Under these conditions, EGFP-Bub3 was observed to immediately accumulate to spindle poles with a pool retained at kinetochores (Fig. 2B), confirming the results described above. Moreover, while no accumulation of EGFP-Bub3 at spindle poles was apparent in MG132 metaphase-arrested cells during the 30 min of filming, addition of AZ/DOG to these MG132-pretreated cells resulted in
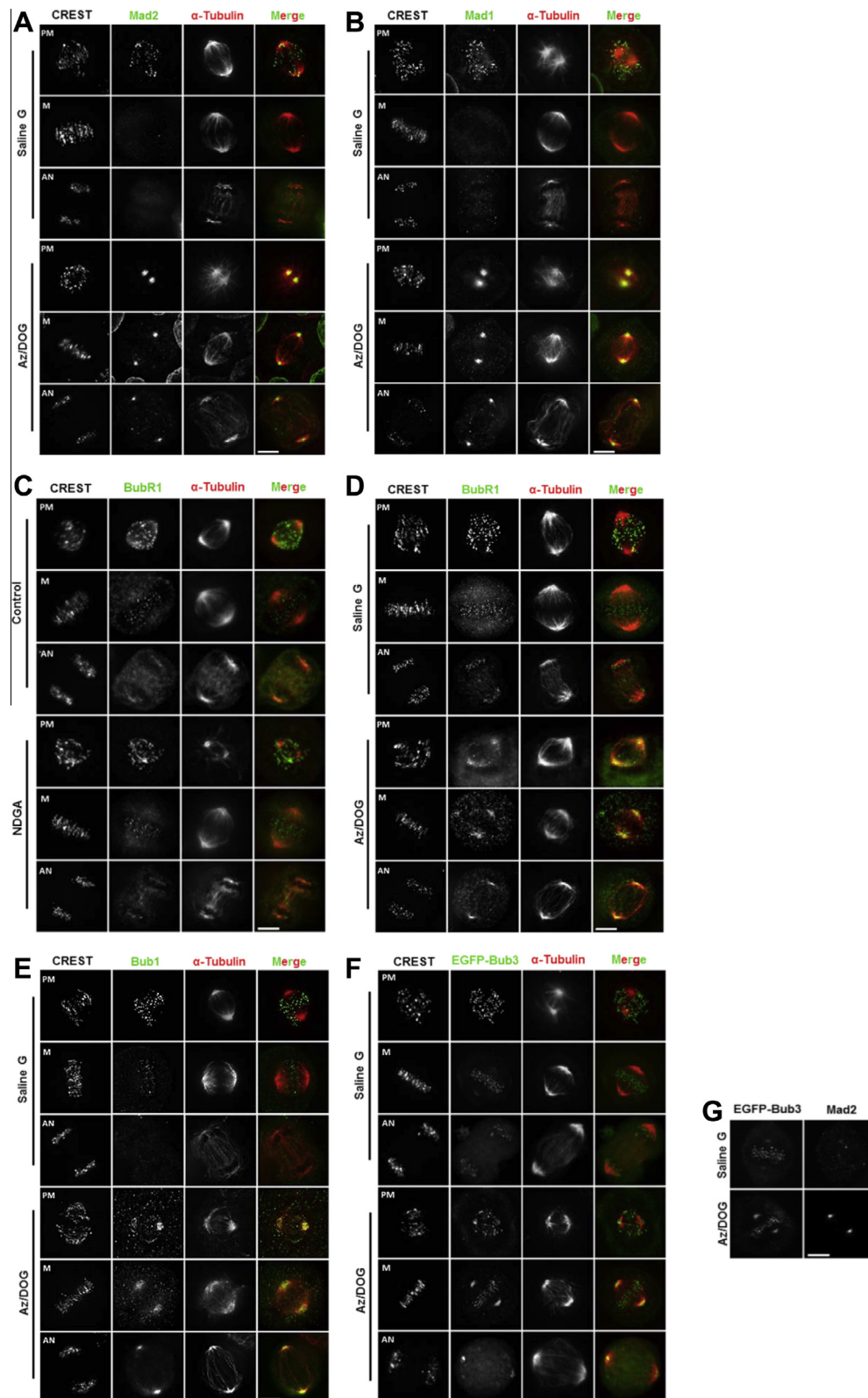
Fig. 1. SAC proteins accumulate at spindle poles upon ATP reduction. (A) ATP reduction was performed by 30 min incubation either with isotonic salt solution with glucose (Saline G) or with sodium azide/2-deoxyglucose (AZ/DOG) [8]. Cells were stained with anti-Mad2, CREST as kinetochore marker, and anti-tubulin to localize spindle pole position. (B) Cells were treated as in (A) except that they were stained for Mad1. (C) ATP reduction was performed by 30 min incubation with nordihydroguaiaretic acid (NDGA) and cells were stained for BubR1, CREST, and tubulin. (D) Cells were treated as in (A) except that they were incubated for 10 min only with AZ/DOG and stained for BubR1. (E) Cells were treated as in (A) except that they were stained for Bub1. (F) Cells were treated as in (A) except that a HeLa line stably expressing EGFP-Bub3 was used to visualize Bub3 spindle pole relocalization, with a persistent pool at kinetochores. (G) A cell treated as in (A) showing that the persistence of the EGFP-Bub3 kinetochore pool is not due to insufficient inhibition as Mad2 was completely removed from kinetochores in the same cell. PM = Prometaphase; M = Metaphase; AN = Anaphase; Bars, 5 μm.
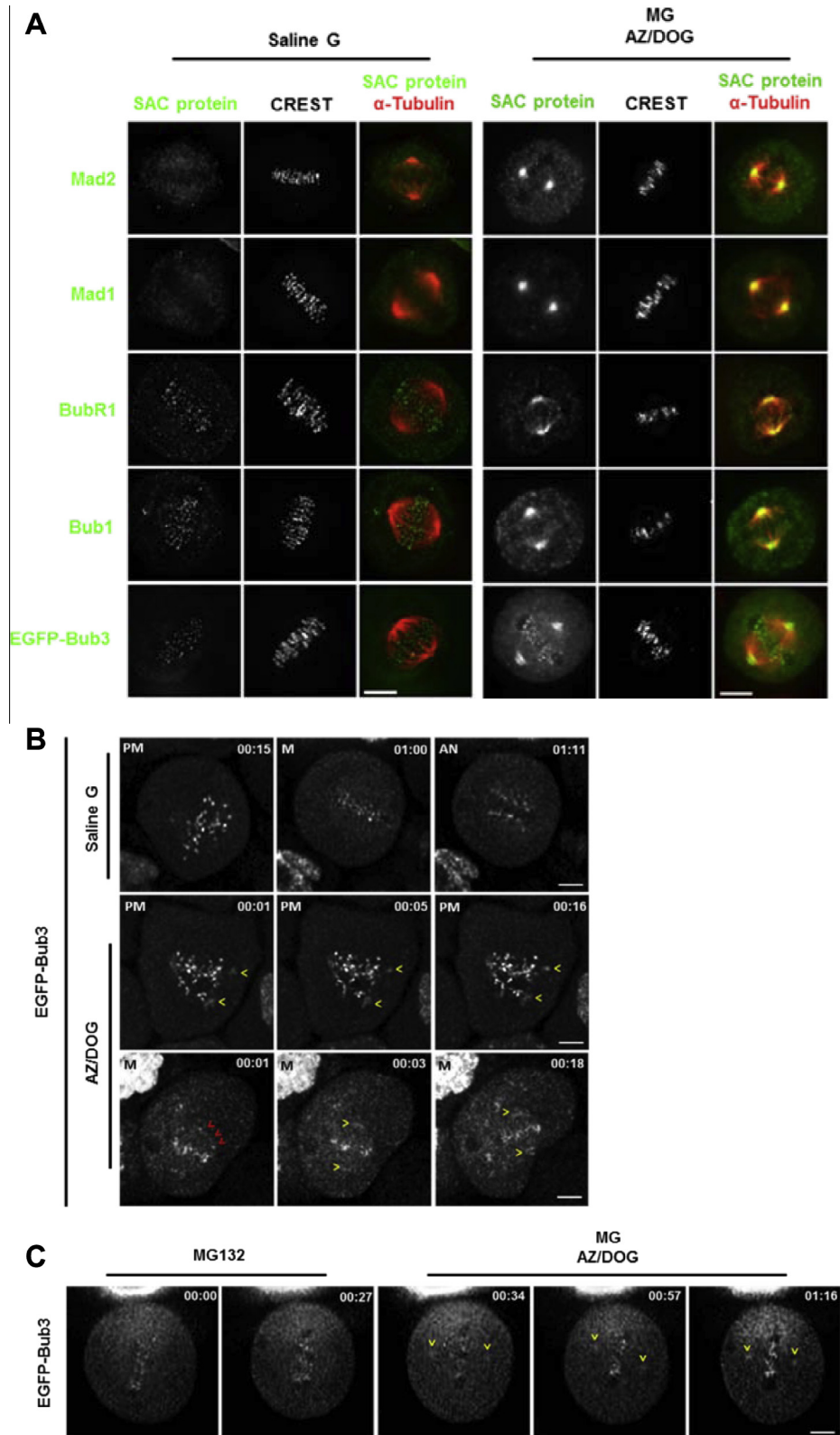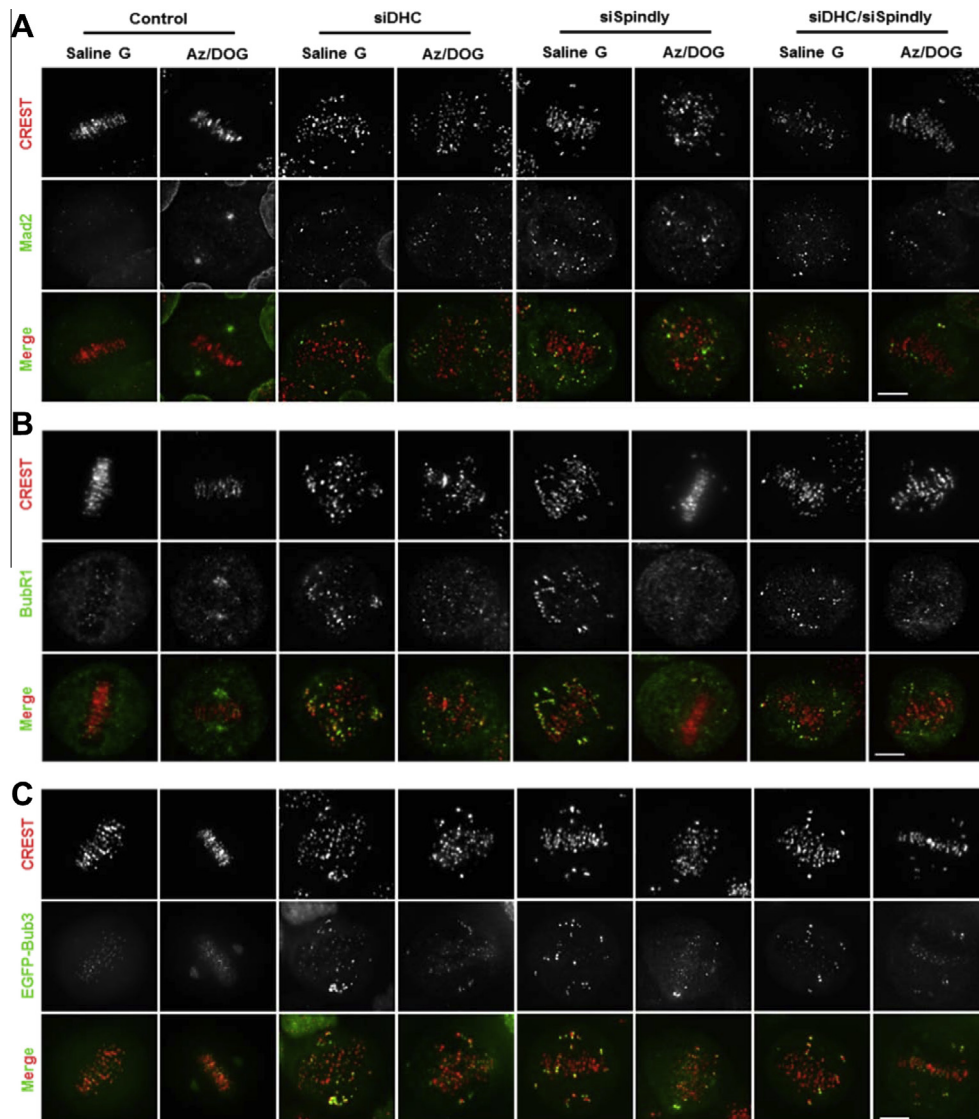
Fig. 2. Poleward transport of SAC proteins still occurs at metaphase. (A) HeLa cells were pre-treated with MG132 for 1 h, subjected to ATP reduction assay, and stained for Mad2, Mad1, BubR1, or Bub1. For Bub3, the EGFP-Bub3 expressing HeLa line was used. CREST was used to localize kinetochore position, and anti-tubulin to localize spindle pole position. (B) Selected 1 μm Z-stacks collected every minute in time-lapse experiments using HeLa cells stably expressing EGFP-Bub3. Control saline G cells (top) show intense EGFP-Bub3 staining on prometaphase (PM) kinetochores and reduced staining on metaphase (M) and anaphase (AN) kinetochores. EGFP-Bub3 relocalizes to spindle poles (yellow arrowheads) almost instantaneously upon AZ/DOG treatment in prometaphase (middle) and metaphase (bottom). The red arrows indicate EGFP-Bub3 foci streaming towards the pole. (C) HeLa cells stably expressing EGFP-Bub3 were treated with MG132 and filmed for 30 min, then AZ/DOG was added and the cells were further filmed for 60 min. EGFP-Bub3 relocalized to spindle poles (yellow arrowheads) at the start of filming. Time is shown in hours:minutes. Bar, 5 μm.

immediate EGFP-Bub3 relocalization to the poles (Fig. 2C and Supplementary Movie 2).

We then inhibited dynein in order to establish if poleward transport of the aforementioned SAC proteins was dependent on this motor protein. Inhibition of dynein was achieved either by direct RNAi depletion or by preventing its kinetochore localization through RNAi depletion of spindly, a protein indispensable for the kinetochore localization of dynein (Supplementary Fig. 1B). Our results show that dynein inhibition abolished spindle pole relocalization of Mad1, Mad2, BubR1, Bub1, and Bub3 following AZ/DOG treatment (Fig. 3). Also, we found that dynein-inhibited cells, without or with AZ/DOG treatment, did not retain SAC proteins on kinetochores of aligned chromosomes. Instead, these proteins accumulated only on misaligned kinetochores (Fig. 3). The same results were obtained in cells co-depleted of dynein and spindly in order to maximize dynein depletion from kinetochores, therefore ruling out the possibility that residual dynein delocalised SAC proteins from kinetochores (Fig. 3). These results support the idea of the existence of a dynein-independent mechanism of protein stripping from attached kinetochores during SAC silencing [10,17,18]. Moreover, we noted that Cdc20 was not subjected to poleward transport (data not shown) which is in good agreement with similar, previous observations [9].

An unexpected result from our analyses concerned the protein Hec1, a subunit of the outer kinetochore KMN network that constitutes the core site for microtubule attachment. In the present study we first intended to use Hec1 as kinetochore marker instead of CREST. Interestingly, we noted that 30 min after AZ/DOG treatment, Hec1 accumulated at spindle poles and a pool remained at attached kinetochores, in a manner that mirrored Bub3 localization (Fig. 4A). The localization of Hec1 to spindle poles was prevented upon dynein inhibition (Fig. 4A), thus suggesting that Hec1 function as an additional dynein cargo [9,18]. Similar results were obtained for the other component of the KMN network, Mis12, except that its spindle pole localization was not observed at prometaphase but only when a metaphase plate became evident (Fig. 4B).

Fig. 3. Poleward transport of SAC proteins is suppressed upon dynein depletion. Cells were depleted of dynein (siDHC), spindly (siSpindly), or both (siDHC/siSpindly), subjected to ATP reduction assay, and stained for (A) Mad2, (B) BubR1 and (C) Bub3 (analyzed using the EGFP-Bub3 expressing cell line). Note that in all cases, SAC proteins accumulate on unaligned but not on aligned kinetochores. Mad1 behaves identically to Mad2 (not shown); and Bub1 behaves identically to Bub3 (not shown). Bars, 5 μm.
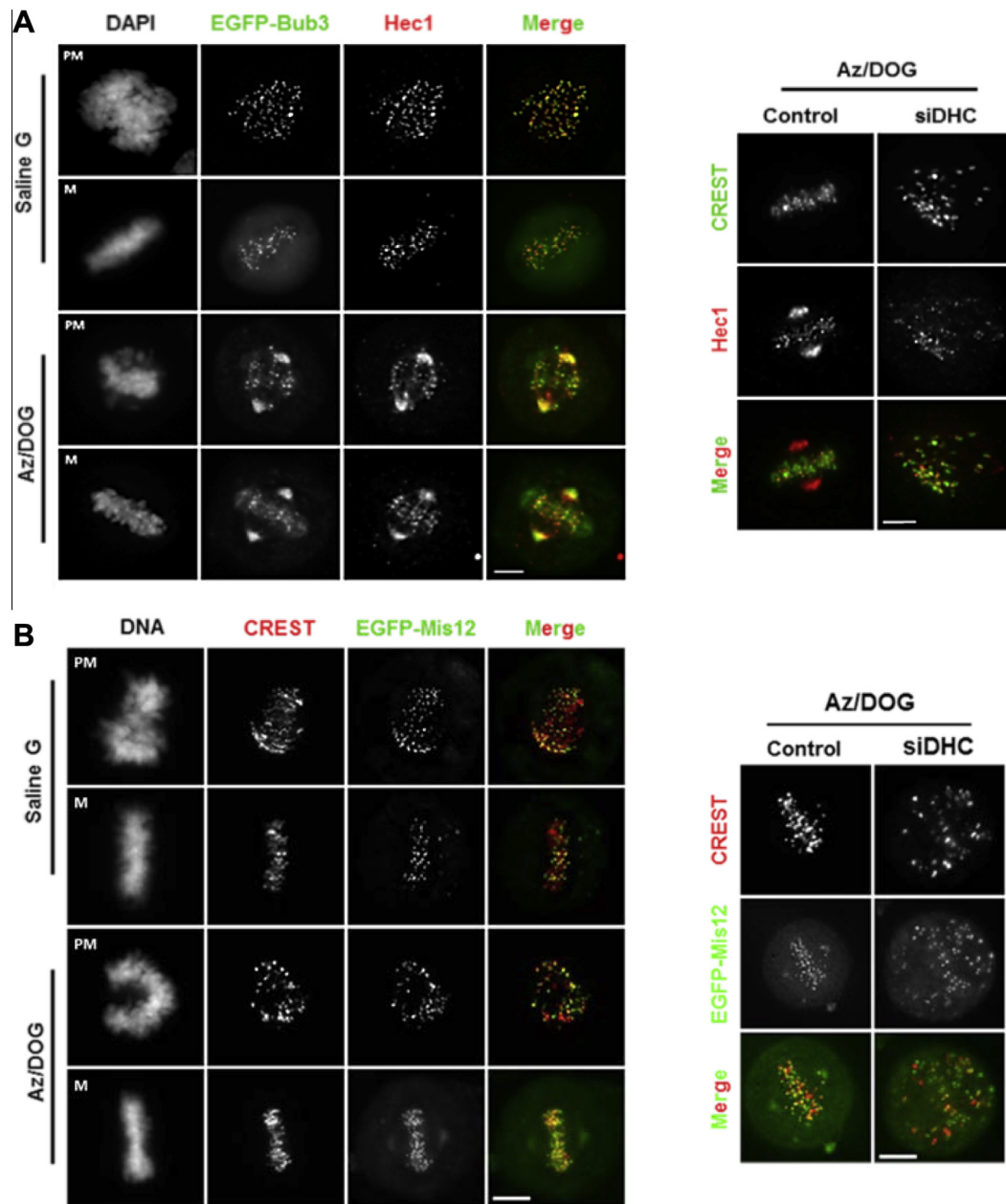
Fig. 4. KMN components are dynein-dependent cargoes. (A) EGFP-Bub3 expressing HeLa cells were subjected to ATP reduction assay and stained for Hec1. (Left) Upon the assay, Hec1 redistributes to spindle poles with a pool left on kinetochores, mirroring the behavior of Bub3. (Right) Dynein depleted cells (siDHC) were subjected to ATP reduction assay and stained for Hec1 and CREST. Note the absence of Hec1 at spindle poles in cells depleted of dynein. (B) HeLa cells were transiently transfected with pBABEblast YFP-Mis12 construct and subjected to ATP reduction assay. (Left) Upon the assay, Mis12 redistributed to spindle poles with a pool left on kinetochores. (Right) YFP-Mis12 expressing cells were depleted of dynein (siDHC) and subjected to ATP reduction; CREST was used as kinetochore marker. Note the absence of Mis12 at spindle poles in cells depleted of dynein PM = Prometaphase; M = Metaphase; Bars, 5 μm.

3. Discussion

Unattached kinetochores activate and accumulate critical SAC components such as Mad1, Mad2, Bub1, BubR1, and Bub3. Binding of Mad2 to Mad1 to the kinetochore induces significant conformational changes that promote Mad2 binding to Cdc20, which in turn makes Cdc20 unable to activate the APC/C [19]. Additionally, the Mad2-Cdc20 complex associates with Bub3 and BubR1 to form the MCC, the protein assembly that acts as a pseudosubstrate to inhibit the APC/C [20–22]. Bub1 is required to recruit all the above SAC components to the kinetochore and contributes to APC/C inhibition through Cdc20 phosphorylation [7,23].

We used two previously described ATP reduction assays [8,9] which prevent release of dynein and its cargoes from spindle poles, in order to identify core SAC proteins that are transported off kinetochores toward spindle poles. We found that Mad1, Mad2, Bub1, BubR1, and Bub3 accumulated at spindle poles following treatment with the inhibitors. This poleward transport was dynein-dependent as it was abolished upon dynein inhibition. The result confirmed that Mad1 and Mad2 function as dynein cargoes, a feature shown in earlier reports [8–10]. We argue that the discrepancy between previous studies concerning BubR1 and Bub1 function as dynein cargoes is due to differences in the experimental approaches, a concern that has been expressed by others [8,9].

We found that BubR1 was the only SAC protein which was insensitive to NDGA but not to AZ/DOG as co-staining of NDGA-treated cells showed BubR1 only at kinetochores while Bub3 was relocalized to spindle poles (Supplementary Fig. 1A). We have also determined that Bub3 is a new dynein cargo that is poleward transported upon kinetochore attachment, suggesting a role in SAC silencing. Such role seems consistent with Bub3 direct involvement in the MCC complex whose assembly must be prevented to relieve inhibition exerted on the APC/C thus allowing mitotic progression. Interestingly, the SAC proteins were not retained at aligned kinetochores upon dynein depletion, supporting the existence of a dynein-independent mechanism for SAC silencing as previously suggested [10,17,18]. Also, it should be noted that spindle pole accumulation of SAC proteins reported in the present study depends on the specific treatment we used to inhibit dynein cargo release at the poles; such poleward transport needs, however, to be confirmed under physiological conditions upon chromosome attachment.

We found that ATP depletion assays performed on cells arrested at metaphase after pretreatment with MG132 still resulted in a poleward transport of all the SAC proteins reported in this study, in a manner that mirrored that of ATP depletion assays without prior MG132 treatment. This poleward relocalization was dynein-dependent as it was abolished by dynein inhibition. These finding suggests that metaphase cells with complete chromosome alignment are still competent to generate a SAC signal. This is consistent with the recently reported ability to reestablish SAC activity after initial silencing [24], and with the persistence of SAC activity observed in MG132-treated mitotic cells and in proteasome deficient cells [25,26]. As MG132-treated cells can be arrested in metaphase with kinetochores that develop normal tension [27], it was assumed that the kinetochores in these cells would be depleted of Mad2, a condition that should inactivate the SAC [9]. Although the absence of Mad2 poleward stripping in metaphase-arrested cells, in a previously reported ATP reduction assay, seems to support this assumption [8,9], Zeng et al. showed that cells treated with the proteasome inhibitor MG132 were unable to sustain a prolonged metaphase arrest after depletion of Mad2, suggesting that MG132-mediated metaphase arrest may depend on continued inhibition of APC/C by the SAC [28]. The latter notion would explain why SAC components could be detected at spindle poles of metaphase-arrested cells in our ATP depletion assays. Intriguingly, it is unclear how SAC proteins appeared at spindle poles in a stage when their levels are lowered since all kinetochores are attached and under tension, particularly in the case of Mad2, which leaves the kinetochore upon attachment [16].

Interestingly, and contrary to what has been reported [9,18], we observed that a pool of Hec1, a subunit of the outer kinetochore KMN network that constitutes the core site for microtubule attachment [29], accumulated at spindle pole in a dynein-dependent manner. Hec1 is one of four proteins of the outer kinetochore Ndc80 complex that together with the KNL-1 protein and the Mis-12 complex form the conserved KMN (KNL-1/Mis-12/Ndc80) network that constitutes the core microtubule binding site at the kinetochore [29]. The observed dynein-dependent relocalization of Hec1 to spindle poles is consistent with early studies where Hec1 was reported to localize at the centrosome [30,31]. As Mis-12 exhibited similar behaviors, it is tempting to speculate that KMN streaming from kinetochores would partially disassemble the docking platform that recruits SAC proteins, therefore contributing to SAC silencing by preventing further assembly of anaphase inhibitors. At the same time, a significant pool of KMN components must be retained at kinetochores in order to ensure efficient attachment and chromosome transport at anaphase onset. This is consistent with the decrease in Hec1 kinetochore levels following an ATP reduction assay [32], and with the recently reported disassembly of all KMN components at the onset of anaphase [33]. We have previously shown that Bub3 is required for efficient kinetochore-microtubule attachment [12,34]. Accordingly, the pool of Bub3 retained at the kinetochores, as observed in the present study, might serve to sustain attachment during metaphase and anaphase.

In summary, this report presents evidence of additional SAC components to be subjected to dynein-mediated transport to the spindle pole. We also show that poleward transport still occurs in metaphase-arrested cells consistent with the reported SAC reactivation ability at metaphase [24], and that a pool of Hec1 and Mis12, may function as additional dynein cargoes, thus providing new insights into the contribution of the KMN network to SAC silencing.

4. Materials and methods

4.1. Cell culture, drug treatment, and RNAi

HeLa cells were cultured in DMEM (PAA Laboratories, E15-883), supplemented with 10% FBS (GIBCO, 10500-064) and 1% antibiotic/antimycotic (GIBCO, 12605-028) and maintained at 37 °C, in a 5% CO_2 humidified atmosphere. EGFP-Bub3 stably-expressing HeLa cell line was generated by inserting the hBub3 cDNA in frame into pIC113 vector [35]. Clones expressing near endogenous levels of EGFP-Bub3 were selected and maintained in culture under 400 μg/ml of G418 (Sigma, A1720-1G). MG132 (Sigma, 2211-5MG) was used at 10 μM for 60 min; nocodazole (Sigma, M1404-2MG) was used at 1 μM for 60 min; nordihydroguaiaretic acid (Biomol, BML-EI101-0001) was used at 30 μM for 30 min as described [9]; The ATP inhibition assay was performed as described [8].

Dynein knockdown was performed using validated siRNA oligoduplexes against Dynein Heavy Chain [36] (Santa Cruz Biotechnology, sc-43738). Spindly knockdown was performed using validated siRNAs sequence [10] that was synthetized by (Sigma). Cells were transfected as previously described [12], except that INTERFERin siRNA Transfection Reagent (PolyPlus, 409-10) was used. For immunoblotting, cells were collected by centrifugation, lysed, and resuspended on SDS-sample buffer containing protease inhibitors (Sigma cocktail, P2850-1ML), and loaded onto a 7.5% SDS–PAGE and western blots were processed and analyzed as described [12].

4.2. Immunofluorescence

Cells were fixed for 12 min in freshly prepared 2% paraformaldehyde (Sigma, 158127-500G) in phosphate-buffered saline (PBS), permeabilized with 0.5% Triton X-100 in PBS for 7 min, rinsed in PBS and blocked with 10% FBS in PBST (PBS plus 0.05% Tween-20) for 30 min. Cells were then incubated for 1 h with primary antibodies diluted in PBST with 5% FBS. After three washes in PBST for 5 min each, cells were incubated with fluorescently-labeled secondary antibodies for 1 h, and then washed three times 5 min in PBST. DNA was stained with 2 μg/ml DAPI (Sigma, D9542-5MG) diluted in Vectashield mounting medium (Vector, H-1000). Images were acquired on a Spinning Disc AxioObserver Z.1 SD confocal microscope (Carl Zeiss, Germany), coupled to an AxioCam MR3, and with a Plan Apochromatic 63×/NA 1.4 objective. Z-stacks were acquired at 0.5 μm intervals, deconvolved using AxioVision Release 4.8.2 SPC software, and projected using ImageJ software (http://rsbweb.nih.gov/ij/).

4.3. Antibodies

Primary antibodies used were: mouse anti-Mad2L1 (1:200, Sigma, WH0004085M1-100UG); mouse anti-Bub1 (1:400, Abcam, ab54893); mouse anti-BubR1 (1:1000, BD Biosciences, 612503); rabbit anti-Cdc20 (1:1000, Sigma, C5497); rabbit anti-Mad1 (1:100, gift from Dr. Kuan-The, NIH, USA); human anti-CREST (1:4500, gift from Dr. Bronze-da-Rocha, IBMC, Portugal); mouse anti-Hec1 (1:600, Abcam, ab3613); rabbit anti-α-Tubulin (1:100, Sigma, T5168); mouse anti-dynein intermediate chain (1:200, Sigma, D5167); mouse anti-spindly (1:100, Sigma, WH0054908M1-100UG); rabbit anti-spindly (1:3000, gift from Dr. R. Gassmann, IBMC, Portugal). Alexa Fluor 488- and 568-conjugated secondary antibodies were used at 1:1500, and 647-conjugated secondary antibody which was used at 1:2500 (Molecular Probes). Horseradish peroxidase (HRP)-conjugated secondary antibodies was used at 1:1500 (Vector).

4.4. Live-cell imaging

HeLa cells stably expressing EGFP-Bub3 were seeded on glass bottom dish (Bioptechs) and synchronized in S phase using a double thymidine (2.5 mM) block of 24 h, with a 12 h interval between the blocks. Cells were filmed 8–10 h after the final release as they entered mitosis, on a Delta Vision microscope, using a $100\times$/NA 1.4 Plan Apo objective, and an EMCCD camera (Cascade2_1 k) with gain 75. Time-lapse images were acquired every minute (7 stacks at 1 μm intervals).

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.07.011.

References

[1] Musacchio, A. and Salmon, E.D. (2007) The spindle-assembly checkpoint in space and time. Nat. Rev. Mol. Cell Biol. 8, 379–393.

[2] Foley, E.A. and Kapoor, T.M. (2013) Microtubule attachment and spindle assembly checkpoint signalling at the kinetochore. Nat. Rev. Mol. Cell Biol. 14, 25–37.

[3] Sudakin, V., Chan, G.K. and Yen, T.J. (2001) Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. J. Cell Biol. 154, 925–936.

[4] Kops, G.J. and Shah, J.V. (2012) Connecting up and clearing out: how kinetochore attachment silences the spindle assembly checkpoint. Chromosoma 121, 509–525.

[5] Howell, B.J., Hoffman, D.B., Fang, G., Murray, A.W. and Salmon, E.D. (2000) Visualization of Mad2 dynamics at kinetochores, along spindle fibers, and at spindle poles in living cells. J. Cell Biol. 150, 1233–1250.

[6] Howell, B.J., Moree, B., Farrar, E.M., Stewart, S., Fang, G. and Salmon, E.D. (2004) Spindle checkpoint protein dynamics at kinetochores in living cells. Curr. Biol. 14, 953–964.

[7] Lara-Gonzalez, P., Westhorpe, F.G. and Taylor, S.S. (2012) The spindle assembly checkpoint. Curr. Biol. 22, R966–980.

[8] Howell, B.J., McEwen, B.F., Canman, J.C., Hoffman, D.B., Farrar, E.M., Rieder, C.L. and Salmon, E.D. (2001) Cytoplasmic dynein/dynactin drives kinetochore protein transport to the spindle poles and has a role in mitotic spindle checkpoint inactivation. J. Cell Biol. 155, 1159–1172.

[9] Famulski, J.K., Vos, L.J., Rattner, J.B. and Chan, G.K. (2011) Dynein/Dynactin-mediated transport of kinetochore components off kinetochores and onto spindle poles induced by nordihydroguaiaretic acid. PLoS One 6, e16494.

[10] Gassmann, R., Holland, A.J., Varma, D., Wan, X., Civril, F., Cleveland, D.W., Oegema, K., Salmon, E.D. and Desai, A. (2010) Removal of Spindly from microtubule-attached kinetochores controls spindle checkpoint silencing in human cells. Genes Dev. 24, 957–971.

[11] Yan, X., Li, F., Liang, Y., Shen, Y., Zhao, X., Huang, Q. and Zhu, X. (2003) Human Nudel and NudE as regulators of cytoplasmic dynein in poleward protein transport along the mitotic spindle. Mol. Cell. Biol. 23, 1239–1250.

[12] Logarinho, E., Resende, T., Torres, C. and Bousbaa, H. (2008) The human spindle assembly checkpoint protein Bub3 is required for the establishment of efficient kinetochore-microtubule attachments. Mol. Biol. Cell 19, 1798–1813.

[13] Toledo, C.M., Herman, J.A., Olsen, J.B., Ding, Y., Corrin, P., Girard, E.J., Olson, J.M., Emili, A., Deluca, J.G. and Paddison, P.J. (2014) BuGZ is required for Bub3 stability, Bub1 kinetochore function, and chromosome alignment. Dev. Cell 28, 282–294.

[14] Jiang, H., He, X., Wang, S., Jia, J., Wan, Y., Wang, Y., Zeng, R., Yates 3rd, J., Zhu, X. and Zheng, Y. (2014) A microtubule-associated zinc finger protein, BuGZ, regulates mitotic chromosome alignment by ensuring Bub3 stability and kinetochore targeting. Dev. Cell 28, 268–281.

[15] Martinez-Exposito, M.J., Kaplan, K.B., Copeland, J. and Sorger, P.K. (1999) Retention of the BUB3 checkpoint protein on lagging chromosomes. Proc. Natl. Acad. Sci. USA 96, 8493–8498.

[16] Waters, J.C., Chen, R.H., Murray, A.W. and Salmon, E.D. (1998) Localization of Mad2 to kinetochores depends on microtubule attachment, not tension. J. Cell Biol. 141, 1181–1191.

[17] Barisic, M., Sohm, B., Mikolcevic, P., Wandke, C., Rauch, V., Ringer, T., Hess, M., Bonn, G. and Geley, S. (2010) Spindly/CCDC99 is required for efficient chromosome congression and mitotic checkpoint regulation. Mol. Biol. Cell 21, 1968–1981.

[18] Chan, Y.W., Fava, L.L., Uldschmid, A., Schmitz, M.H., Gerlich, D.W., Nigg, E.A. and Santamaria, A. (2009) Mitotic control of kinetochore-associated dynein and spindle orientation by human Spindly. J. Cell Biol. 185, 859–874.

[19] De Antoni, A., Pearson, C.G., Cimini, D., Canman, J.C., Sala, V., Nezi, L., Mapelli, M., Sironi, L., Faretta, M., Salmon, E.D., et al. (2005) The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. Curr. Biol. 15, 214–225.

[20] Lara-Gonzalez, P., Scott, M.I., Diez, M., Sen, O. and Taylor, S.S. (2011) BubR1 blocks substrate recruitment to the APC/C in a KEN-box-dependent manner. J. Cell Sci. 124, 4332–4345.

[21] Buschhorn, B.A., Petzold, G., Galova, M., Dube, P., Kraft, C., Herzog, F., Stark, H. and Peters, J.M. (2011) Substrate binding on the APC/C occurs between the coactivator Cdh1 and the processivity factor Doc1. Nat. Struct. Mol. Biol. 18, 6–13.

[22] Han, J.S., Holland, A.J., Fachinetti, D., Kulukian, A., Cetin, B. and Cleveland, D.W. (2013) Catalytic assembly of the mitotic checkpoint inhibitor BubR1-Cdc20 by a Mad2-induced functional switch in Cdc20. Mol. Cell 51, 92–104.

[23] Tang, Z., Shu, H., Oncel, D., Chen, S. and Yu, H. (2004) Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint. Mol. Cell 16, 387–397.

[24] Kuijt, T.E., Omerzu, M., Saurin, A.T. and Kops, G.J. (2014) Conditional targeting of MAD1 to kinetochores is sufficient to reactivate the spindle assembly checkpoint in metaphase. Chromosoma.

[25] Ohi, M.D., Feoktistova, A., Ren, L., Yip, C., Cheng, Y., Chen, J.S., Yoon, H.J., Wall, J.S., Huang, Z., Penczek, P.A., et al. (2007) Structural organization of the anaphase-promoting complex bound to the mitotic activator Slp1. Mol. Cell 28, 871–885.

[26] Visconti, R., Palazzo, L. and Grieco, D. (2010) Requirement for proteolysis in spindle assembly checkpoint silencing. Cell Cycle 9, 564–569.

[27] Famulski, J.K. and Chan, G.K. (2007) Aurora B kinase-dependent recruitment of hZW10 and hROD to tensionless kinetochores. Curr. Biol. 17, 2143–2149.

[28] Zeng, X., Sigoillot, F., Gaur, S., Choi, S., Pfaff, K.L., Oh, D.C., Hathaway, N., Dimova, N., Cuny, G.D. and King, R.W. (2010) Pharmacologic inhibition of the anaphase-promoting complex induces a spindle checkpoint-dependent mitotic arrest in the absence of spindle damage. Cancer Cell 18, 382–395.

[29] Cheeseman, I.M., Chappie, J.S., Wilson-Kubalek, E.M. and Desai, A. (2006) The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. Cell 127, 983–997.

[30] Diaz-Rodriguez, E., Sotillo, R., Schvartzman, J.M. and Benezra, R. (2008) Hec1 overexpression hyperactivates the mitotic checkpoint and induces tumor formation in vivo. Proc. Natl. Acad. Sci. USA 105, 16719–16724.

[31] Lin, Y.T., Chen, Y., Wu, G. and Lee, W.H. (2006) Hec1 sequentially recruits Zwint-1 and ZW10 to kinetochores for faithful chromosome segregation and spindle checkpoint control. Oncogene 25, 6901–6914.

[32] DeLuca, J.G., Dong, Y., Hergert, P., Strauss, J., Hickey, J.M., Salmon, E.D. and McEwen, B.F. (2005) Hec1 and nuf2 are core components of the kinetochore outer plate essential for organizing microtubule attachment sites. Mol. Biol. Cell 16, 519–531.

[33] Gascoigne, K.E. and Cheeseman, I.M. (2013) CDK-dependent phosphorylation and nuclear exclusion coordinately control kinetochore assembly state. J. Cell Biol. 201, 23–32.

[34] Logarinho, E. and Bousbaa, H. (2008) Kinetochore-microtubule interactions "in check" by Bub1, Bub3 and BubR1: the dual task of attaching and signalling. Cell Cycle 7, 1763–1768.

[35] Cheeseman, I.M. and Desai, A. (2005) A combined approach for the localization and tandem affinity purification of protein complexes from metazoans. Sci. STKE 2005, pl1.

[36] Nakano, H., Funasaka, T., Hashizume, C. and Wong, R.W. (2010) Nucleoporin translocated promoter region (Tpr) associates with dynein complex, preventing chromosome lagging formation during mitosis. J. Biol. Chem. 285, 10841–10849.