The spindle assembly checkpoint is satisfied in the absence of interkinetochore tension during mitosis with unreplicated genomes

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The accuracy of chromosome segregation is enhanced by the spindle assembly checkpoint (SAC). The SAC is thought to monitor two distinct events: attachment of kinetochores to microtubules and the stretch of the centromere between the sister kinetochores that arises only when the chromosome becomes properly bioriented. We examined human cells undergoing mitosis with unreplicated genomes (MUG). Kinetochores in these cells are not paired, which implies that the centromere cannot be stretched; however, cells progress through mitosis. A SAC is present during MUG as cells arrest in response to nocodazole, taxol, or monastrol treatments. Mad2 is recruited to unattached MUG kinetochores and released upon their attachment. In contrast, BubR1 remains on attached kinetochores and exhibits a level of phosphorylation consistent with the inability of MUG spindles to establish normal levels of centromere tension. Thus, kinetochore attachment to microtubules is sufficient to satisfy the SAC even in the absence of interkinetochore tension.

Introduction

It has long been established that kinetochores are the source of the signal that prevents anaphase onset before the appropriate time (for review see Musacchio and Salmon, 2007). At the molecular level, the pathway that delays mitotic exit is known as the spindle assembly checkpoint (SAC). The SAC is affected by proteins that are recruited to the kinetochore when the checkpoint is active and released when the SAC becomes satisfied. These proteins inhibit Cdc20, a cofactor of the ubiquitin ligase anaphase-promoting complex (for review see Musacchio and Salmon, 2007). As long as there is a single kinetochore in the cell that is not attached to microtubules, the SAC remains unsatisfied, and initiation of anaphase is inhibited (Rieder et al., 1995). However, it is not clear whether the SAC has the ability to differentiate between proper (amphitelic) and erroneous kinetochore attachments. For example, both kinetochores on a given chromosome can attach to the same spindle pole (syntelic attachment), or a single kinetochore can acquire connections to both spindle poles (merotelic attachment) during intermediate stages of spindle formation. Mitotic exit in the presence of syntelic or merotelic chromosomes would result in the formation of aneuploid progeny and must be prevented. Therefore, it seems reasonable that the SAC should differentiate between proper and erroneous kinetochore attachments to delay mitotic exit until all erroneous attachments are corrected (Li and Nicklas, 1995). However, recent data demonstrate that merotelic attachments are not detected by the SAC (Cimini et al., 2001). Furthermore, under certain conditions, mammalian cells exit from mitosis in the presence of multiple syntelic chromosomes (Lončarek et al., 2007).

A major factor that differentiates between amphitelic and erroneous kinetochore attachments is stretching of the centromere (interkinetochore tension) that occurs only when sister kinetochores attach to opposite spindle poles. Treatments that relieve centromere stretching result in a mitotic delay (Waters et al., 1998; Skoufias et al., 2001). Pulling an improperly attached chromosome away from the spindle pole with a microneedle initiates mitotic exit during meiosis (Li and Nicklas, 1995).
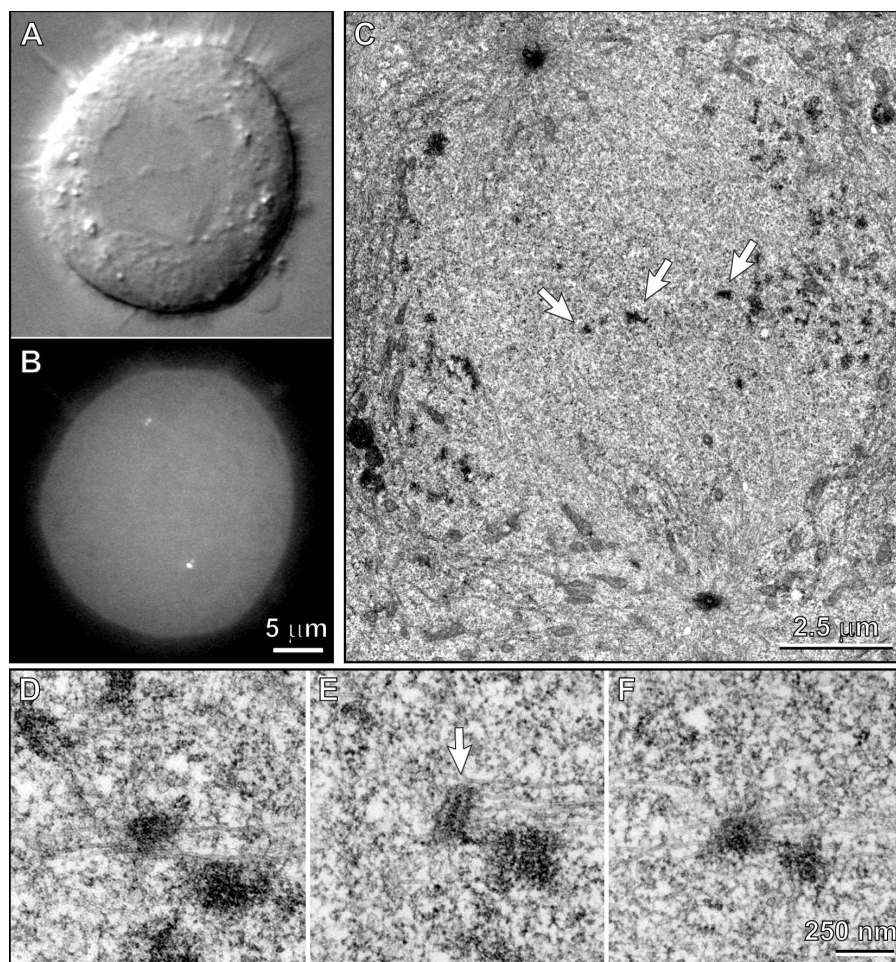
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Abbreviations used in this paper: CENP-A, centromere protein A; HU, hydroxyurea; INCENP, inner centromere protein; MUG, mitosis with unreplicated genomes; SAC, spindle assembly checkpoint.

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Figure 1. **Spindle and kinetochore morphology of HeLa MUG.** (A and B) Differential interference contrast (A) and centrin1-GFP (B) images of a fixed cell during MUG metaphase. (C–F) This cell was processed for correlative EM. (C) A single lower magnification section through the spindle. Note that chromatin is largely absent from the spindle except for a few small fragments (arrows). (D–F) 70-nm-thick serial sections through a kinetochore. Microtubules attach to the kinetochore end-on as well as laterally (E, arrow).

However, these experiments do not prove that centromere stretching signals directly to the SAC. It has been shown that kinetochore microtubules are not stable in the absence of tension and this instability results in transient reappearance of unattached kinetochores (King and Nicklas, 2000). Thus, the SAC might not directly monitor tension; rather, the intermittent reappearance of unattached kinetochores caused by low stability of erroneous microtubule attachments is what delays mitotic exit (Nicklas et al., 2001). Therefore, the role of centromere stretching in checkpoint signaling is a matter of ongoing debate (Pinsky and Biggins, 2005).

To directly address whether the SAC can be satisfied in the absence of stretched centromeres, we examined human cells undergoing mitosis with unreplicated genomes (MUG; Brinkley et al., 1988). During MUG, kinetochores separate from the bulk of chromatin and are unpaired so that interkinetochore tension cannot arise. Here, we provide evidence that the SAC is nevertheless satisfied in MUG cells.

Results and discussion

Spindle morphology and kinetochore behavior in human MUG

Normally, mitosis does not commence before DNA replication because of cell cycle checkpoints. However, this regulation can

be overcome when DNA replication is inhibited with hydroxyurea (HU), and the DNA damage checkpoint is overridden by caffeine (Schlegel and Pardee, 1986). Under these conditions, cells initiate mitosis with unreplicated chromosomes. Although these cells contain normal centrosomes and form bipolar spindles, kinetochores are not duplicated.

MUG was originally observed in hamster cells (BHK, CHO, and V79-8; Schlegel and Pardee, 1986; Brinkley et al., 1988) and subsequently in HeLa cells overexpressing cyclin A (Balczon, 2001). Serendipitously, we observed spontaneous MUG in a strain of HeLa cells that has been used in several recent studies on centrioles and mitosis (Piel et al., 2000; La Terra et al., 2005; Thery et al., 2007). We find that many cells in this strain begin MUG ~40 h after the addition of 2 mM HU. MUG is characterized by the assembly of a robust bipolar spindle (Fig. 1, A and B). Uncondensed chromatin remains granular in appearance and is largely excluded from the spindle (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200801038/DC1). In some cells, variable degrees of chromatin condensation are observed (Fig. S1 B). Increased condensation may arise as a result of partially replicated chromosomes in cells that were in S phase at the time of HU treatment or those that somehow progress through the block imposed by HU. Such cells were not considered to be in true MUG for the purpose of this study and were disregarded.
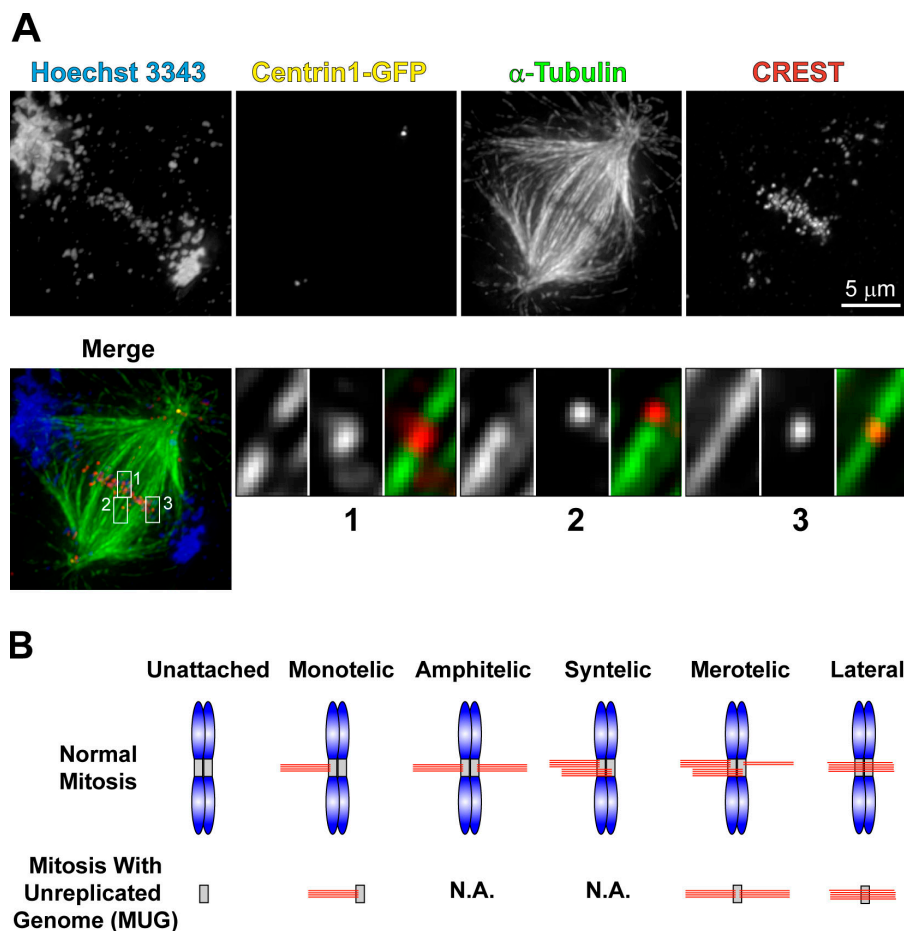
A

Hoechst 3343   Centrin1-GFP   α-Tubulin   CREST

5 μm

Merge

1   2   3

Figure 2. **Geometries of kinetochore attachment in MUG.** (A) Cells undergoing MUG were fixed and stained for chromatin, microtubules, and kinetochores. Images are maximum intensity projections of deconvolved z slices. Insets (single z slices) illustrate the types of microtubule–kinetochore attachments achievable in MUG: (1) merotelic, (2) monotelic, and (3) lateral. (B) Comparison of kinetochore attachments to spindle poles in normal mitosis versus MUG. Amphitelic or syntelic arrangements are not applicable (N.A.) for unpaired kinetochores.

B

Unattached   Monotelic   Amphitelic   Syntelic   Merotelic   Lateral

Normal Mitosis

Mitosis With Unreplicated Genome (MUG)   N.A.   N.A.

Serial-section EM of HeLa MUG reveals that kinetochores are, in fact, unpaired (Fig. 1, D–F). Kinetochore morphology is normal with typical trilaminar appearance. Many kinetochores associate with small pieces of chromatin that likely represent kinetochore-bound centromeric DNA. Except for these pieces, chromatin is virtually absent within the spindle (Fig. 1 C). Most kinetochores align along the spindle equator (Fig. 2 A) as reported for hamster cells (Brinkley et al., 1988). Microtubules from both spindle poles interact with kinetochores through end-on binding and lateral association (Fig. 1, D–F; and Fig. 2 A). Thus, the nature of kinetochore–microtubule interactions is preserved relative to normal mitosis. However, proper amphitelic attachment of two sister kinetochores to opposite spindle poles cannot be achieved in MUG (Fig. 2 B). MUG kinetochores are either monotelic (connected to only one pole), merotelic (connected to both spindle poles simultaneously), or laterally bound to spindle microtubules (Fig. 2).

The process by which unpaired MUG kinetochores align at the spindle equator is unknown because previous work examined only fixed samples (Brinkley et al., 1988; Wise and Brinkley, 1997). To follow kinetochore dynamics in individual cells, we constitutively expressed the innerplate component centromere protein A (CENP-A) fused to GFP (Fig. 3). Time-lapse recordings revealed that upon nuclear envelope breakdown, most kinetochores rapidly become aligned at the metaphase plate (Fig. 3 A). However, individual kinetochores frequently

escape toward one of the spindle poles and then return to the equator throughout metaphase (Fig. 3 B and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200801038/DC1). As a result, anaphase is initiated in the presence of unaligned kinetochores that subsequently move poleward. Moreover, fixed preparations of cells in MUG metaphase always display kinetochores near spindle poles (Fig. 2 A). Thus, although most kinetochores in MUG achieve biorientation (most likely via establishment of merotelic attachments), this biorientation is not as robust as in a normal amphitelic arrangement. The presence of scattered kinetochores at anaphase onset indicates that cells initiate mitotic exit in the presence of both merotelic and monotelic attachments.

The SAC is active and robust during MUG

Duration of MUG in HeLa cells is more variable and on average longer than normal mitosis in the same HeLa cell line (Table I), which suggests that spindle assembly and satisfaction of the SAC in cells undergoing MUG is often impeded. This is expected considering the lower stability of mono- and merotelic microtubule attachments.

As for MUG in hamster cells (Brinkley et al., 1988), MUG in HeLa is arrested when microtubules are depolymerized with nocodazole. This arrest is robust, and many cells die without exiting mitosis. Cells that survive nocodazole treatment eventually escape MUG (Fig. 4 A and Table I) in a manner similar to
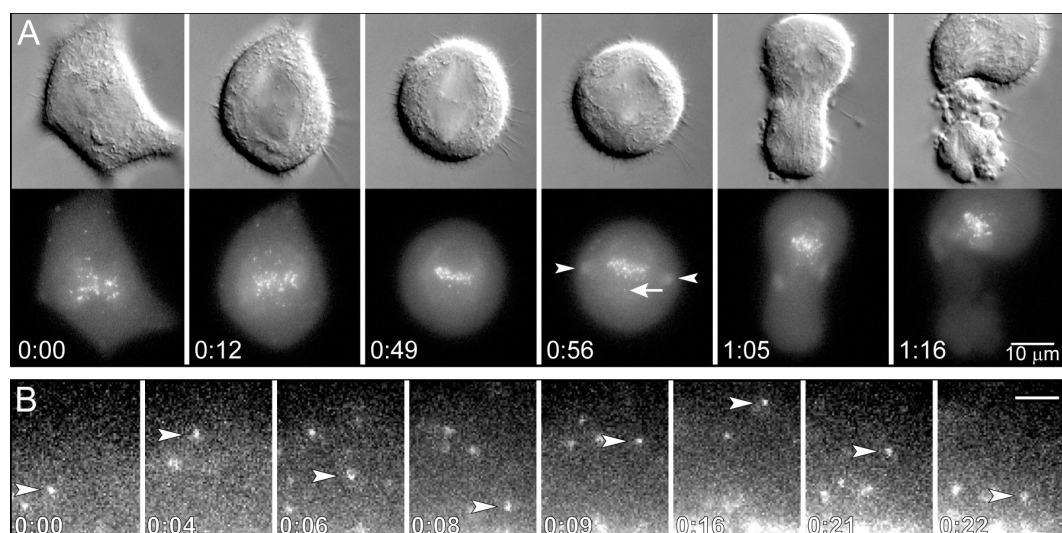
Figure 3. **Behavior of unpaired kinetochores.** (A) MUG in HeLa cells stably expressing CENP-A–GFP followed by combinational differential interference contrast (top) and three-dimensional fluorescence microscopy (bottom). Kinetochores are extremely dynamic, exhibiting rapid poleward and away from pole movements with a mean velocity of 1.8 ± 0.8 μm/min. Anaphase in this cell (56 s) is marked by cessation of kinetochore dynamics, spindle shortening (compare 49 s with 56 s), and outward movement of chromatin (arrowheads). Not all kinetochores congress before anaphase (arrow). (B) Tracking of a single kinetochore (arrowheads) in the cell shown in A. This as well as many other kinetochores exhibits rapid back and forth movement toward and away from the spindle equator. Time is given in hours/minutes. Bar, 2.5 μm.

normal mitosis. When nocodazole is washed out from arrested cells during MUG, a spindle rapidly assembles, and cells exit mitosis in 1–1.5 h (Fig. 4 B).

When treated with taxol, HeLa MUG is delayed for >4 h. Similarly, MUG cells treated with monastrol, a kinesin-5 inhibitor, remain in mitosis for ∼4.5 h. The increase in MUG duration in the presence of nocodazole, taxol, or monastrol is statistically significant (P < 0.01 in the two-tailed Student's *t* test). Thus, both taxol and monastrol also delay satisfaction of the SAC in MUG. These delays are shorter in MUG than in normal mitoses. However, the observation that MUG is extended from 1.5 h to 4–10 h in taxol, monastrol, or nocodazole demonstrates that exit from MUG is normally dependent in satisfaction of the SAC.

The mechanism of mitotic arrest in cells treated with taxol or monastrol is not completely understood. Both drugs clearly decrease centromere stretching, which can be directly monitored by the SAC. Alternatively, lack of centromere tension results in destabilization of kinetochore attachments. It has been demonstrated that both taxol- or monastrol-treated cells consistently contain several Mad2-positive (unattached) kinetochores

(Waters et al., 1998; Kapoor et al., 2000; Khodjakov et al., 2003; Lončarek et al., 2007). Destabilization of the tensionless kinetochore fibers is mediated by the activity of aurora B, a centromere-associated kinase (Hauf et al., 2003). Aurora B is targeted to the centromere through its association with the chromosomal passenger complex inner centromere protein (INCENP). The complex activates aurora B in the absence of centromere tension, normally allowing cells to differentiate between amphitelic attachments that should remain stable and erroneous attachments that need to be destabilized (Lens and Medema, 2003). We find that during metaphase of MUG, INCENP associates with small pieces of chromatin that are aligned at the spindle equator along with kinetochore fragments (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200801038/DC1). After the completion of MUG, INCENP is restricted to the midbody (Fig. S2 B), a behavior similar to normal mitosis. This suggests that the mechanisms used to sense and signal tension are preserved in MUG despite the unpaired organization of kinetochores. Furthermore, we find that hesperadin, a cell-permeable inhibitor of aurora B, overrides mitotic arrest in response to taxol and monastrol in MUG (Table I).

Table I. **Duration of control mitosis versus MUG**

Treatment	Control	MUG
Untreated[a]	0.7 ± 0.1 (n = 47)	1.6 ± 1.1 (n = 43)
5 μM nocodazole[b]	24.3 ± 9.1 (n = 31)	10.1 ± 5.6 (n = 34)
200 μM monastrol[b]	15.8 ± 5.9 (n = 35)	4.5 ± 2.5 (n = 35)
200 μM monastrol + 100 nM hesperadin[b]	ND	1.8 ± 1.6 (n = 34)
500 nM taxol[b]	20.5 ± 5.3 (n = 50)	4.6 ± 3.9 (n = 43)
500 nM taxol + 100 nM hesperadin[b]	ND	2.6 ± 1.2 (n = 32)

Time is given in hours. Error is reported as SD.

[a]Defined as nuclear envelope breakdown to maximum furrow ingression.

[b]Defined as nuclear envelope breakdown to first cortical telophase activity (blebbing, furrowing, etc.).
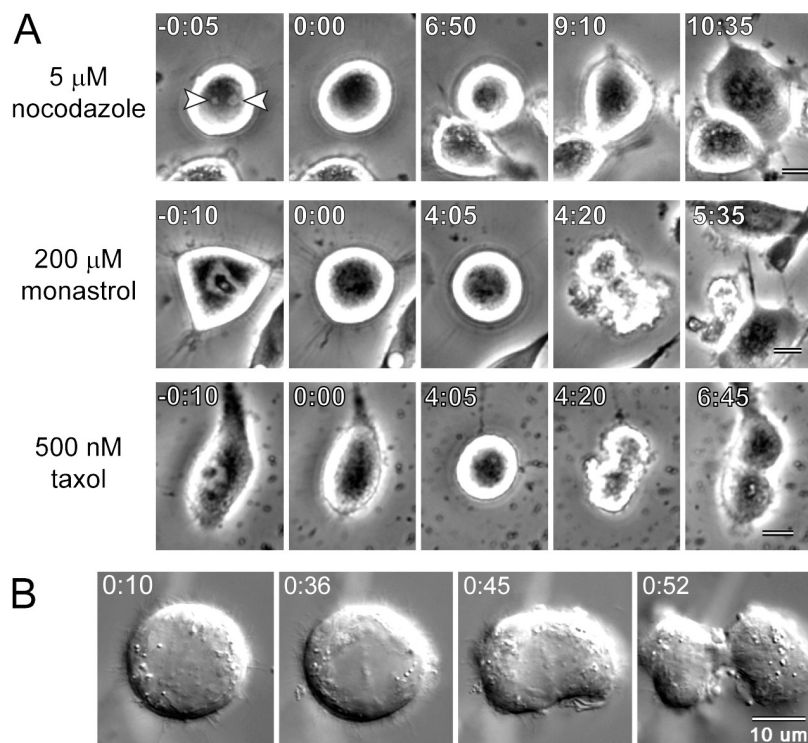
A

5 μM
nocodazole

200 μM
monastrol

500 nM
taxol

B

10 um

Figure 4. **Evidence of a robust SAC during MUG.** (A) HeLa cells undergoing MUG were treated with the indicated drugs and monitored by time-lapse microscopy. The disappearance of the nucleolus (arrowheads) indicates entry into MUG at t = 0. In the presence of nocodazole, there is a prolonged checkpoint arrest followed by escape that is marked by formation of micronuclei and respreading. Exit from MUG is also delayed when cells are treated with monastrol or taxol. Bar, 10 μm. (B) A cell in MUG after washout (t = 0) of 1.5 μM nocodazole. A bipolar spindle forms, and kinetochores congress to the equator (36 s). Soon after, the cell exits MUG, demonstrating satisfaction of the SAC. Time is given in hours/minutes.

Together, these data reveal that HeLa MUG is controlled by a SAC that responds to the factors known to prevent satisfaction of the SAC during normal mitosis. The fact that mitotic exit in MUG treated with nocodazole, taxol, or monastrol is significantly delayed implies that under normal conditions the SAC in these cells becomes satisfied.

Behavior of Mad2 and BubR1 during MUG

Mad2 and BubR1 are two major components of the SAC that are found on unattached kinetochores during prometaphase and disappear from the properly attached kinetochores before anaphase onset. It is established that Mad2 is removed from kinetochores solely as the result of microtubule attachment (Waters et al., 1998). BubR1 associates with both unattached and attached kinetochores when the centromere is relaxed, but its amount is drastically reduced when sister kinetochores are pulled apart (Hoffman et al., 2001; Skoufias et al., 2001). Furthermore, proper amphitelic attachments result in loss of Plk1-dependent BubR1 phosphorylation (Elowe et al., 2007; Wong and Fang, 2007). Because our data suggest that the SAC can be satisfied in the absence of interkinetochore tension, we followed the behavior of Mad2 and BubR1 during MUG.

When microtubules are depolymerized by nocodazole during MUG, kinetochores stain positively for Mad2. MUG spindles that are fully assembled show no prominent Mad2 staining on kinetochores (Fig. 5 A). Quantification of fluorescence intensity (Hoffman et al., 2001) demonstrated a threefold reduction of kinetochore-bound Mad2 in the presence of microtubules ($27,876 \pm 1,587$ [$n = 50$] vs. $8,825 \pm 875$ [$n = 57$]), supporting the idea that Mad2-based attachment signaling to the SAC is functional during MUG. In sharp contrast, aligned and unaligned kinetochores contained similar amounts of BubR1 in MUG

(Fig. 5 B and Table II). Interestingly, BubR1 levels at kinetochores remained unchanged during MUG anaphase (Fig. 5, B and C; and Table II), demonstrating that removal of BubR1 from kinetochores is not required for mitotic exit in this system.

Treatments that relieve interkinetochore tension have been shown to increase the amount of BubR1 phosphorylation on S676 at properly aligned kinetochores (Elowe et al., 2007). We reasoned that if MUG kinetochores are not under tension, the amount of phospho-BubR1 should not change in response to treatments that decrease centromere stretching. Indeed, there was no statistically significant difference ($P > 0.05$) between the amount of phospho-S676 at kinetochores in untreated ($11,666 \pm 1,302$ [$n = 30$]) versus taxol-treated ($10,852 \pm 1,126$ [$n = 30$]) MUG (Fig. S3 C, available at http://www.jcb.org/cgi/content/full/jcb.200801038/DC1).

Our data are not consistent with the idea that stretching of the centromere between sister kinetochores (interkinetochore tension) is monitored by the SAC. Cells undergoing MUG possess a SAC, as evident from their responses to all standard treatments known to affect satisfaction of the SAC during normal mitosis. Although the duration of mitotic arrests in MUG is shorter than in normal mitosis, the fact that MUG is significantly prolonged in nocodazole, taxol, or monastrol reveals that untreated MUG cells manage to satisfy the SAC in the absence of centromere stretching. These data are consistent with several previous observations that mammalian cells exit mitosis in the presence of merotelic and syntelic attachments that lower interkinetochore tension (Kline-Smith et al., 2004; Ganem et al., 2005; Lončarek et al., 2007). Furthermore, our results are consistent with the demonstration that monotelic chromosomes are not detected by the SAC if the sister (unattached) kinetochore is destroyed (Rieder et al., 1995). However, our conclusions do not imply that interkinetochore tension is irrelevant for mitotic
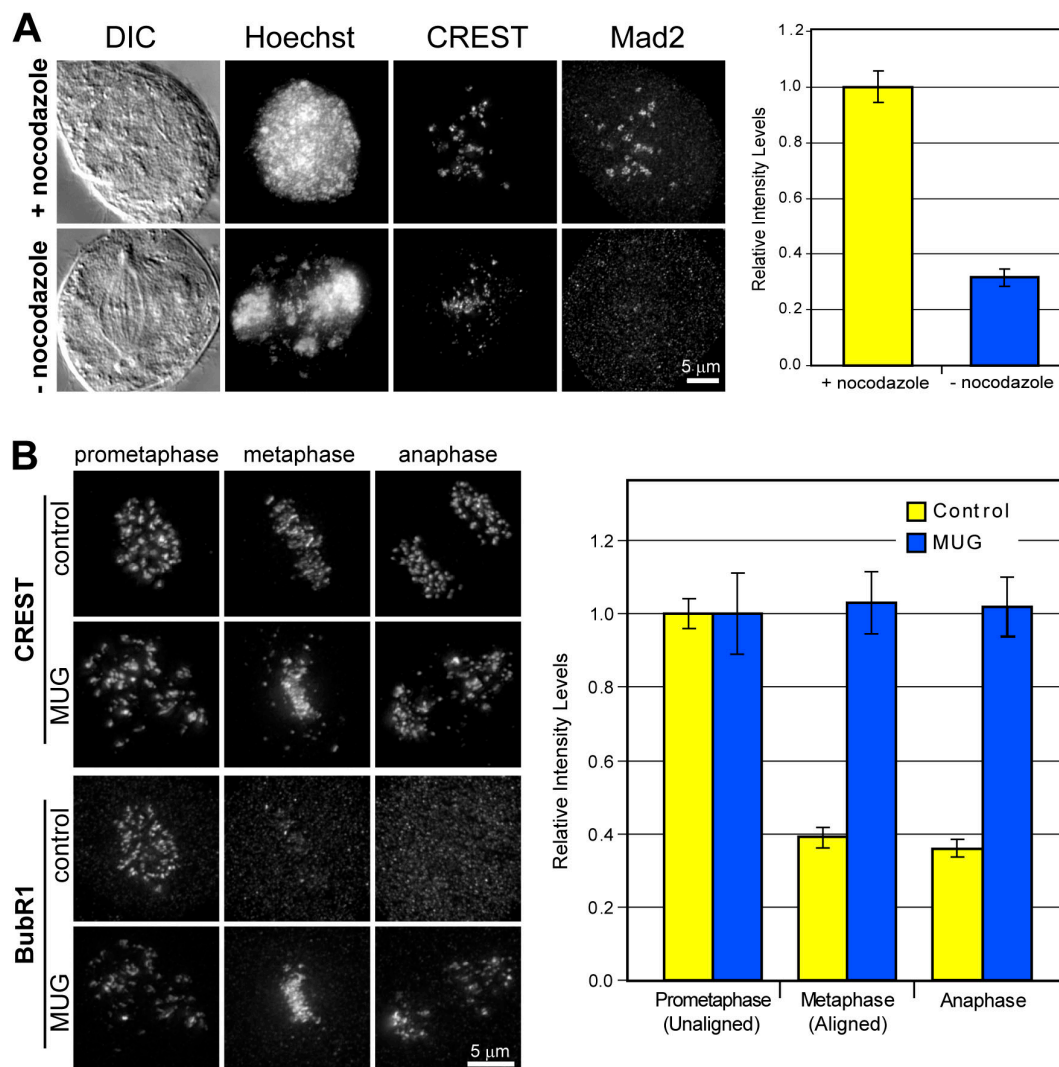
Figure 5. **Behavior of Mad2 and BubR1 during MUG.** (A) Cells in MUG were treated with nocodazole to generate unattached kinetochores or were left untreated. Differential interference contrast and fluorescence images of cells stained for DNA, kinetochores, and Mad2. The histogram presents the integrated intensity of Mad2 normalized to nocodazole-treated cells. (B) The distribution of BubR1 during normal mitosis and MUG. The histogram presents the integrated intensity of BubR1 on control and MUG kinetochores normalized to prometaphase. Error bars indicate SEM.

mechanisms such as correction of erroneous kinetochore attachments. Also, we cannot rule out that attachment to highly dynamic microtubules induces intrakinetochore deformations that may play a role in the SAC.

Erroneous kinetochore attachments are inevitable during spindle formation, and it is imperative that they are resolved before the cell exits mitosis. This goal can be achieved either by delaying mitotic exit in the presence of erroneously attached chromosomes or by using a speedy correction mechanism that makes the delay unnecessary. Our data suggest that mammalian cells rely on the latter approach. Furthermore, the absence of tension is indirectly manifested through aurora B–mediated destabilization of kinetochore microtubules on erroneously attached kinetochores. Repetitive reappearance of unattached kinetochores is responsible for the stringent mitotic arrest in cells with monopolar spindles, where ∼70% of chromosomes are syntelic at any given time (Kapoor et al., 2000; Khodjakov et al., 2003). It is interesting that during correction of syntelic attachments, only one of the two sister kinetochores detaches from microtubules (Lampson et al., 2004; Kapoor et al., 2006). This can explain why unpaired kinetochores in MUG ultimately attain at least a quasistable attachment and satisfy the checkpoint.

Materials and methods

Cell culture and drug treatments
HeLa cells were cultured in DME supplemented with 10% FBS and penicillin/streptomycin at 37°C in an atmosphere of 5% CO_2. Cells stably transfected with centrin1-GFP were provided by M. Bornens (Institut Curie, Paris, France). Lentiviral transfection (Rubinson et al., 2003) was used to stably integrate a human CENP-A sequence fused to GFP (Sullivan et al., 1994). After transfection, GFP-positive cells were enriched by FACS, and individual clones from the enriched population were screened by fluorescence microscopy for suitable levels of expression.

For induction of MUG, mitotic cells were shaken off and plated immediately with 2 mM HU (Sigma-Aldrich) for at least 40 h. Shake off provides a synchronous population of cells that results in a more homogeneous response to HU arrest. Mitotic arrests were induced by 1.5 or 5 µM nocodazole (EMD). For Mad2 localization in the absence of microtubules,

Table II. **Integrated BubR1 fluorescence during control mitosis and MUG**

	Control	MUG
Prometaphase (unaligned)		
n	59	59
Mean	33,777 ± 1,412	17,283 ± 1899
Ratio to prometaphase	1	1
Metaphase (aligned)		
n	52	55
Mean	13,262 ± 883	17,793 ± 1,441
Ratio to prometaphase	0.39	1.03
Anaphase		
n	50	57
Mean	12,140 ± 771	17,559 ± 1,407
Ratio to prometaphase	0.36	1.02

Error is reported as SEM.

nocodazole was added for 20–30 min before fixation. Hesperadin and monastrol (provided by T. Kapoor, The Rockefeller University, New York, NY) were used at concentrations of 100 nM and 200 µM, respectively.

Microscopy and immunostaining
Cells for correlative EM were fixed and processed for serial sectioning as previously described (Rieder and Cassels, 1999). Serial 70-nm sections were examined on an electron microscope (model 910; Carl Zeiss, Inc.) at 80 kV.

Multimode live cell time-lapse sequences were recorded on a custom-modified microscope (TE-2000E; Nikon) with a Plan Apo 100× 1.4 NA oil immersion objective. Images were captured using either iXon 897 (Andor) or CoolSnap HQ (Photometrics) charge-coupled device cameras. Cells on the microscope stage were maintained at 37°C using custom-built environmental chambers. The system was driven by IPLab software (version 4.0; BD Biosciences).

Images for deconvolution were collected on a DeltaVision system (Applied Precision, LLC) with a 100× UPlan Apo 1.35 NA oil immersion objective (Olympus). Stacks were deconvolved using SoftWoRx software (version 2.5; Applied Precision, LLC). For fixed preparations, z series were obtained with 0.2-µm steps. During live cell imaging, the z interval was increased to 1 µm to limit phototoxicity.

For CREST, Mad2, and BubR1 staining of kinetochores, cells were rinsed twice with warm PBS, fixed in 3.5% paraformaldehyde for 10 min, and extracted for 20 min with 0.2% Triton X-100. The distribution of INCENP was determined by fixation in −20°C methanol for 5 min. BubR1 phosphorylation at S676 was assessed using an antibody generated against a synthetic phosphopeptide and fixation as described previously (Elowe et al., 2007).

Intensities of BubR1, phospho-S676, and Mad2 on kinetochores were measured as described previously (Hoffman et al., 2001). In brief, pixel intensities were integrated in a small, 9 × 9–pixel window centered on a kinetochore (F_S). Background fluorescence was assessed by integrating pixels of a larger, 13 × 13 square (F_l). These values were used to calculate background (F_B) with the following equation: $F_B = (F_L − F_S)(81/88)$, which takes into consideration the smaller area of the 9 × 9 square. The integrated intensity (I) of a given kinetochore is $I = F_S − F_B$. MUG kinetochores sometimes form large aggregates that were excluded from quantitative analyses because they were larger than a 9 × 9– pixel area. Large aggregates were found in all cells during MUG prometaphase, metaphase, and anaphase, so the exclusion did not selectively affect measurements. Results of kinetochore intensity measurements are presented in the text as mean ± SEM.

Primary antibodies used in this study include polyclonal anti-Mad2 (provided by T. Kapoor), polyclonal anti-BubR1 (provided by T. Yen, Fox Chase Cancer Center, Philadelphia, PA), polyclonal anti-INCENP (Sigma-Aldrich), human CREST-SH serum (provided by B. Brinkley, Baylor College of Medicine, Houston, TX), and anti-phospho-S676 BubR1 (provided by E. Nigg, Max Planck Institute of Biochemistry, Munich, Germany).

Online supplemental material
Figs. S1 and S2 present fluorescence characterization of chromatin condensation and INCENP distribution during MUG. Fig. S3 illustrates the behavior of phosho-BubR1 in response to taxol treatment. Video 1 illustrates kinetochore movements during MUG. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801038/DC1.

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