

Microtubules do not promote mitotic slippage when the spindle assembly checkpoint cannot be satisfied

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When the spindle assembly checkpoint (SAC) cannot be satisfied, cells exit mitosis via mitotic slippage. In microtubule (MT) poisons, slippage requires cyclin B proteolysis, and it appears to be accelerated in drug concentrations that allow some MT assembly. To determine if MTs accelerate slippage, we followed mitosis in human RPE-1 cells exposed to various spindle poisons. At 37°C, the duration of mitosis in nocodazole, colcemid, or vinblastine concentrations that inhibit MT assembly varied from 20 to 30 h, revealing that

different MT poisons differentially depress the cyclin B destruction rate during slippage. The duration of mitosis in Eg5 inhibitors, which induce monopolar spindles without disrupting MT dynamics, was the same as in cells lacking MTs. Thus, in the presence of numerous unattached kinetochores, MTs do not accelerate slippage. Finally, compared with cells lacking MTs, exit from mitosis is accelerated over a range of spindle poison concentrations that allow MT assembly because the SAC becomes satisfied on abnormal spindles and not because slippage is accelerated.

Introduction

Checkpoint controls delay cell cycle progression in response to conditions that, if uncorrected, generate genetic instability. The spindle assembly checkpoint (SAC) prolongs mitosis until all kinetochores are stably attached to spindle microtubules (MTs; Musacchio and Salmon, 2007). In brief, the SACs “wait-anaphase” signal is generated by several proteins, including Mad1 and Mad2, that are present on unattached but not attached kinetochores. In the presence of unattached or weakly attached kinetochores, these proteins catalyze the formation of an inhibitory complex, which is thought to consist of Mad2, BubR1/Mad3, Bub3, and Cdc20, that prevents anaphase-promoting complexes (APCs) from recognizing and ubiquitinating those proteins required for chromatid cohesion (shugoshin and securin) and the mitotic state (cyclin B). When all kinetochores are under tension, which promotes their saturation with MTs (Waters et al., 1998; Nicklas et al., 2001), the checkpoint is satisfied, production of the inhibitory signal ceases, and APCs are free to target shugoshin, securin, and cyclin B for proteolysis.

As for most checkpoints, satisfaction of the SAC is not required for exiting mitosis: normal (and many cancer) cells that enter mitosis in spindle poisons ultimately exit mitosis and enter the next G1 as tetraploid cells (Rieder and Maiato, 2004). The process by which cells escape mitosis when they cannot satisfy the SAC requires the ubiquitination and proteolysis of cyclin B and is known as mitotic “slippage” (Hunt et al., 1992; Andreassen and Margolis, 1994; Brito and Rieder, 2006). Several factors influence the rate of slippage including the species the cell is from and its genotype. In general, cells from rodents are more resistant to spindle poisons and slip through an active SAC more rapidly than those from primates (Kung et al., 1990). For example, when spindle assembly is inhibited with nocodazole, mouse fibroblasts slip through mitosis in ~4 h (Lanni and Jacks, 1998), whereas human HeLa require ≥20 h (Jordan et al., 1992). The genotype of the cell is important because many transformed cells have a “weakened” SAC, caused by a reduced expression of or mutations in one or more SAC proteins, that translates into an accelerated rate of slippage (Weaver and Cleveland, 2005).

The rate of slippage is also correlated with the concentration of the drug used to poison MT assembly/behavior: as a rule,

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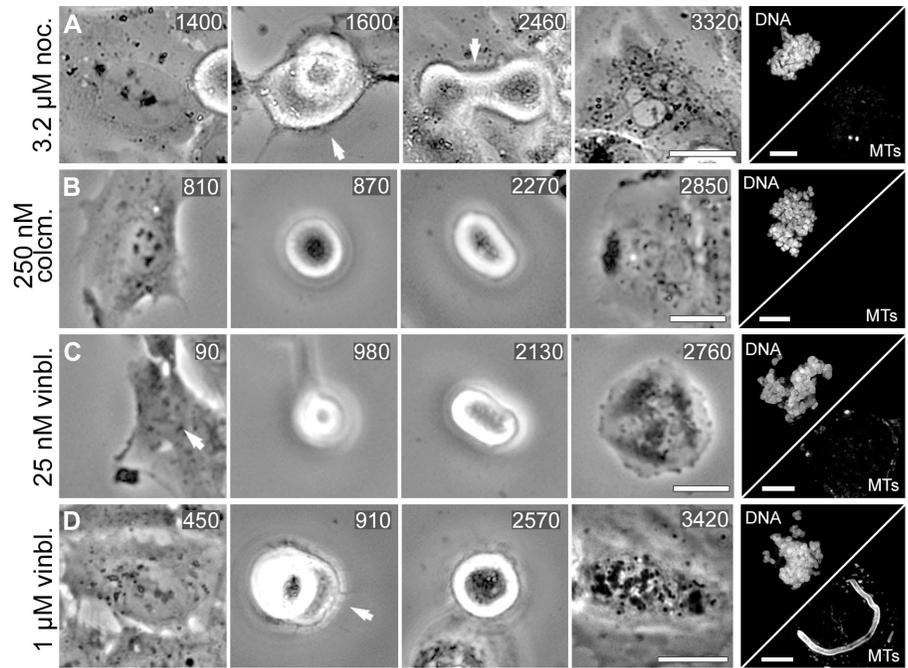
Abbreviations used in this paper: APC, anaphase-promoting complex; MT, microtubule; NEB, nuclear envelope breakdown; SAC, spindle assembly checkpoint.

The online version of this paper contains supplemental material.

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Figure 1. In the absence of spindle MTs, RPE-1 cells spend 20–30 h in mitosis. Images from video records of cells entering and exiting mitosis in 3.2 μ M nocodazole (A), 250 nM colcemid (B), 25 nM vinblastine (C), or 1 μ M vinblastine (D). In each sequence, the second frame defines the start of mitosis, the third the end, and the last depicts the chromosome (DNA) and MT pattern in a mitotic cell from a similarly treated culture fixed and stained for the immunofluorescence localization of MTs. Time from addition of the drug to the medium is in minutes. Arrows note the cell that is followed in subsequent frames. Bars: (IMF images) 5 μ m; (phase images) 20 μ m.



the lower the drug concentration the faster a cell escapes mitosis (Rieder and Maiato, 2004). The reasons for this remain vague. However, work on hamster (Andreassen and Margolis, 1994) and human nontransformed (Brito and Rieder, 2006) and cancer (Jordan et al., 1992) cells reveals that accelerated slippage in low drug concentrations correlates with the formation of some spindle MTs. For example, rat kangaroo cells entering mitosis in 400 nM nocodazole assemble MTs and exit mitosis after \sim 3 h, whereas in 10 μ M nocodazole, MTs fail to form and the cells remain in mitosis for \sim 6 h (Brito and Rieder, 2006). That the formation of some MTs promotes mitotic slippage is also suggested from results that inhibiting aurora B in taxol or monastrol-treated HeLa cells, which contain MTs, rapidly drives them out of mitosis, whereas nocodazole-treated cells lacking MTs remain blocked for hours (Hauf et al., 2003; Ditchfield et al., 2003). It is also consistent with reports that relative to MT poisons, slippage is promoted by MT-stabilizing drugs like taxol (Andreassen et al., 1996; Chen and Horwitz, 2002).

These findings have led to the idea that the duration of mitosis in the presence of MT poisons depends on the number of MTs present in the cell (Weitzel and Vandre, 2000). If this is true, then MTs positively influence the rate that substrates (like cyclin B) are targeted for degradation in the background of an active SAC. This could occur, for example, if MTs facilitate the colocalization of APCs and their substrates. Indeed, because APCs (Acquaviva et al., 2004) and many of their substrates (including cyclin B; Clute and Pines, 1999) are associated with the spindle, inhibiting MT formation will prevent their colocalization. As noted by Melloy and Holloway (2004), although the colocalization of a substrate with APCs may not be required for its ubiquitination, this proximity may accelerate the timing and specificity of the reaction.

The conclusion that the rate cells slip through mitosis in the presence of an active SAC increases with the level of MT assembly (Andreassen and Margolis, 1994) is, however, based on in-

direct data. Because this rate also correlates with drug concentration, it may be influenced more by the drug than by the presence of MTs. Alternatively, drug concentrations that permit the assembly of some MTs may, within a range, allow the SAC to ultimately be satisfied. To evaluate these possibilities, we conducted a series of live cell studies on telomerase immortalized human RPE-1 cells, which have a normal and robust SAC, dividing in the presence of drugs that perturb spindle formation and/or MT assembly.

Results and discussion

We used time-lapse phase-contrast light microscopy to follow fields of RPE-1 cells for 48–72 h at 37°C immediately after treatment with various concentrations of drugs that disrupt MT/spindle assembly differently. We then analyzed these records (e.g., Video 1, available at <http://www.jcb.org/cgi/content/full/jcb.200805072/DC1>) to determine the percentage of dividing cells that slipped through mitosis into the next G1 as well as the duration of mitosis. For this study, we defined the duration of mitosis as that period between the first frame after nuclear envelope breakdown (NEB) and the first frame that includes evidence of cytokinesis and/or the telophase membrane blebbing that precedes cell reflatting.

In the absence of MTs, the rate of mitotic slippage depends on the drug used to prevent spindle assembly

When RPE-1 cells entered mitosis in 3.2 μ M nocodazole, a concentration established empirically to be near the minimum required to completely inhibit spindle MT assembly in these cells, 71% slipped into the next G1 as 4N cells (the remaining 29% died in division), and these survivors spent \sim 19 h on average in mitosis at 37°C (Fig. 1 A, Table I, and Video 2, available at <http://www.jcb.org/cgi/content/full/jcb.200805072/DC1>). Like nocodazole,

Table I. Duration of mitosis in RPE-1 cells exposed to various drugs that perturb spindle formation (37°C)^a

Drug/concentration	MTs	Duration of mitosis ± SD (N) ^b	Approximate hours in mitosis ^c	Survival % ^d
Control	+	18 ± 3 (33)	0.3	100
3.2 μM nocodazole	–	1,140 ± 732 (25)	19	71
250 nM colcemid	–	1,303 ± 362 (24)	22	60
25 nM vinblastine	–	1,784 ± 602 (24) ^e	30	26
1 μM vinblastine	–	1,820 ± 530 (37)	30	21
1 μM maytansine	–	1,558 ± 407 (50)	26	50
500 nM taxol	+	744 ± 399 (117)	12	81
5.0 μM S-Trityl-L-cysteine ^f	+	1,179 ± 310 (160)	20	84
2.5 μM dimethylenastron ^g	+	1,218 ± 424 (241)	20	80
50 nM nocodazole	+	153 ± 58 (200)	3	100
5 nM vinblastine	+	234 ± 140 (180)	4	95
5 nM taxol	+	161 ± 85 (171)	3	99

^aMinutes from first frame after NEB to first frame including the initial signs of cytokinesis and/or telophase membrane blebbing (in cells that exited mitosis).

^bN = total numbers of cells (pooled from ≥2 separate long-term recordings obtained on different days).

^cMean duration of mitosis in hours.

^dPercentage of cells that exited mitosis (versus dying in mitosis).

^eThese data are from a single experiment.

^fAn Eg5 inhibitor (and nonnatural derivative of the α-amino acid cysteine).

^gAn Eg5 inhibitor (a new monastrol analogue).

colcemid binds to the α/β-tubulin heterodimer and modifies MT end dynamics at low concentrations while inducing MT disassembly at high concentrations (Mollinedo and Gajate, 2003). We found that 60% of RPE-1 cells that entered mitosis in 250 nM colcemid, a concentration empirically established to be near the minimum required to completely inhibit spindle MT assembly, slipped into G1 after spending ~22 h in mitosis (Fig. 1 B and Table I).

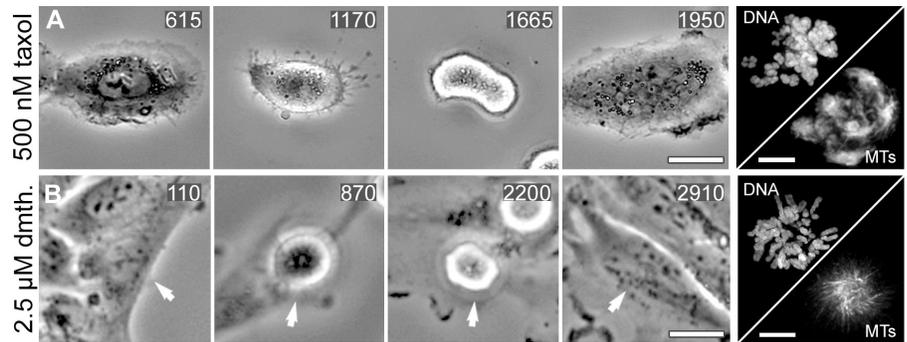
We next asked how vinblastine influences the duration of mitosis. Unlike nocodazole or colcemid, vinblastine prevents MT assembly by binding to the β-tubulin subunit of the α/β-tubulin heterodimer (Rai and Wolff, 1996). This binding induces a conformational change in tubulin that promotes its self-association into aggregates and paracrystals. For this experiment, we used two vinblastine concentrations (Fig. 1, C and D): one that prevents spindle MT assembly (25 nM) and one that induced small aggregates of tubulin and some paracrystals (1 μM). We found that 75–80% of the cells that entered mitosis in both of these concentrations died in mitosis after a prolonged period, and those that survived to enter the next G1 averaged ~30 h in mitosis (Table I). When we repeated this experiment with 1 μM maytansine, which binds to the vinca domain of tubulin without inducing paracrystal formation (Hamel, 1992), we observed the same response: in the absence of MTs, RPE-1 cells remained in mitosis for ~26 h, during which time 50% died (Table I).

From these direct data, we conclude that in the absence of MTs, the rate that RPE-1 cells slip through an active SAC can differ depending on the drug. Compared with the duration of mitosis in concentrations of nocodazole (19 h) or colcemid (22 h) that inhibit spindle MT assembly, which was the same for both drugs (P = 0.3335), mitosis was significantly prolonged in vinblastine (30 h; P = 0.0016). This implies that in the background of an active SAC, different MT poisons directly or indirectly differentially depress either the rate that cyclin B is ubiquitinated during slippage or the rate that ubiquitin-tagged cyclin B is destroyed by the proteasome.

When the SAC cannot be satisfied, the rate of slippage is not accelerated by the presence of numerous dynamic spindle MTs
At this point, we can conclude that in the absence of MTs, the duration of mitosis in RPE-1 cells varies from ~20 to 30 h depending on the MT poison. We next asked if the mere presence of MTs accelerates mitotic slippage. In our first study on this question, we treated RPE-1 cells entering mitosis with the MT “stabilizing” drug taxol at a concentration (500 nM) that suppresses both MT plus- and minus-end dynamics to the same extent in vitro (Derry et al., 1995). Under this condition, RPE-1 cells formed spindles consisting of several (5–7) dense astral MT arrays linked by their associated chromosomes (Fig. 2 A). We found that, on average, these cells remained in mitosis for ~12 h before reflattening into multinucleated G1 cells (Fig. 2 A), and 81% survived mitosis (Table I). A statistical analysis reveals that compared with nocodazole or colcemid-treated RPE-1 cells lacking MTs, mitotic slippage was accelerated in the presence of 500 nM taxol (P = 0.0002).

This finding is consistent with the conclusions of others based on less direct data (Andreassen and Margolis, 1994) and implies that the rate of mitotic slippage is accelerated by MTs. However, unlike in the absence of MTs, at any time almost all kinetochores in taxol-treated cells are stably attached to MTs and are not Mad2 positive (Waters et al., 1998). This raises the possibility that relative to cells lacking MTs, taxol-treated RPE-1 cells spend less time in mitosis because the SAC ultimately becomes satisfied and not because slippage is accelerated. To eliminate this concern, we treated RPE-1 cultures with either 5 μM S-trityl-L-cysteine (DeBonis et al., 2004) or 2.5 μM dimethylenastron (Gartner et al., 2005), two new small molecule inhibitors of the kinesin-like Eg5 motor protein that are, respectively, 40 and 100 times more potent than monastrol (a moderate allosteric Eg5 inhibitor that is used at 100–200-μM

Figure 2. When RPE-1 cells cannot satisfy the SAC, the formation of spindle MTs does not accelerate mitotic slippage. (A and B) Selected images from video recordings of cells entering and exiting mitosis in the presence of 500 nM taxol (A), which stabilizes spindle MTs, or 2.5 μ M of the Eg5 inhibitor dimethylnastron (B). In each sequence the second frame defines the start of mitosis, the third the end, and the last depicts the chromosome (DNA) and MT pattern in a mitotic cell from a similarly treated culture fixed and stained for the immunofluorescence localization of MTs. Time from addition of drug to the medium is in minutes. See text for details. Arrows note the cell that is followed in subsequent frames. Bars: (IMF images) 5 μ m; (phase images) 20 μ m.



concentrations). Unlike the response to 500 nM taxol, inhibiting Eg5 leads to the formation of monopolar spindles containing near normal numbers of dynamic MTs and numerous persistently monooriented chromosomes with Mad2-positive kinetochores (Kapoor et al., 2000). We found that 81–84% of RPE-1 cells entering mitosis in dimethylnastron (Fig. 2 B and Video 3, available at <http://www.jcb.org/cgi/content/full/jcb.200805072/DC1>) or S-trityl-L-cystine (not depicted) survived to enter the next G1 after a mean of \sim 20 h in mitosis (Table I). Statistical analyses revealed no significant difference between the duration of the monopolar mitosis induced by dimethylnastron (or S-trityl-L-cystine) and the duration of mitosis in colcemid- ($P = 0.3486$) or nocodazole ($P = 0.4201$)-treated cells lacking MTs. From these data, we conclude that when the SAC cannot be satisfied, the presence of relatively normal numbers of dynamic spindle MTs does not accelerate mitotic slippage.

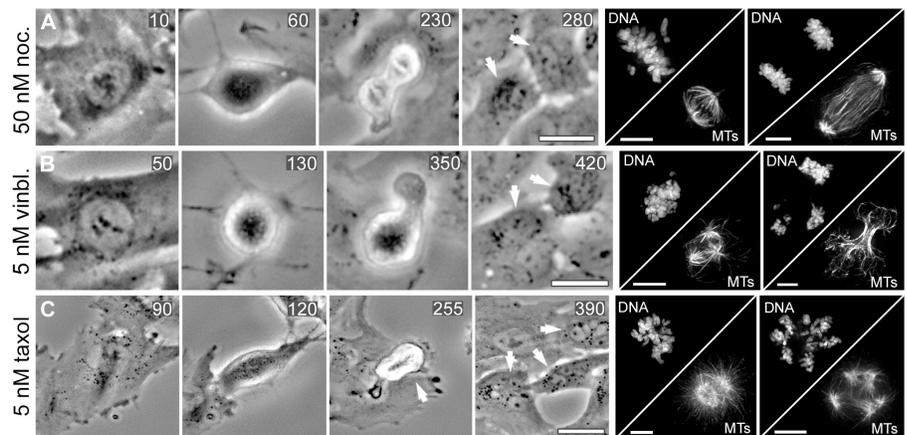
Because Eg5 inhibitors do not retard spindle formation or delay anaphase onset when applied after centrosome separation (Kapoor et al., 2000), they do not deleteriously affect cyclin B ubiquitination or proteolysis. However, when cells enter mitosis in the absence of functional Eg5, the SAC cannot be satisfied (at any time \sim 50% of the kinetochores are unattached), and we found that, on average, \sim 20 h is required for the cyclin B concentration to drop below that needed to sustain the mitotic state. If we use this 20-h figure as the normal “background” rated in RPE-1 cells for cyclin B ubiquitination or destruction in the presence of an active SAC,

then vinblastine (even at 25 nM) significantly depresses these rates ($P < 10^{-8}$), whereas 3.2 μ M nocodazole or 250 nM colcemid have little effect ($P = 0.4201$ and $P = 0.3486$, respectively; Table I).

Slippage is not accelerated in low concentrations of MT poisons. Rather, satisfaction of the SAC is delayed on abnormal spindles

Analyses of fixed-cell populations reveal that there is a narrow range of concentrations in which MT poisons allow cells to escape mitosis more rapidly in the presence of MTs than they would via slippage in the absence of MTs. For example, when the percentage of HeLa cells in mitosis after 18–20 h is plotted against increasing nocodazole concentrations, a rapid rise is seen from \sim 2 to 90% between the 10–100-nM concentration range (Jordan et al., 1992; In HeLa these ranges for vinblastine and taxol are, respectively, 0.5–10 nM [Jordan et al., 1991] and 1–20 nM [Jordan et al., 1993]). This rapid and progressive rise in the mitotic index means that the mean duration of the mitotic “arrest” increases with increasing nocodazole concentrations that, at the structural level, correlate with progressively more abnormal spindles. Then, when the concentration reaches the upper range limit, the percentage of cells in mitosis reaches its maximum (90–100%), even as the drug concentration is increased further. At this point, HeLa are blocked in mitosis for at least 18–20 h, whether they enter mitosis in concentrations of nocodazole that allow the formation of highly distorted spindles

Figure 3. In low concentrations of MT poisons or stabilizers, RPE-1 cells form aberrant spindles that segregate chromosomes into two or more daughter cells. Each row depicts a cell entering (second frame) and exiting (third frame) mitosis in either 50 nM nocodazole (A), 5 nM vinblastine (B), or 5 nM taxol (C). The split-screen fluorescence images at the end of each row depict the distribution of chromosomes (DNA) and MTs in cells fixed during spindle assembly (fifth frame) and telophase/cytokinesis (sixth). Time from addition of drug to the medium is in minutes. Arrows note the cell of interest. Bars: (IMF images) 5 μ m; (phase images) 20 μ m.



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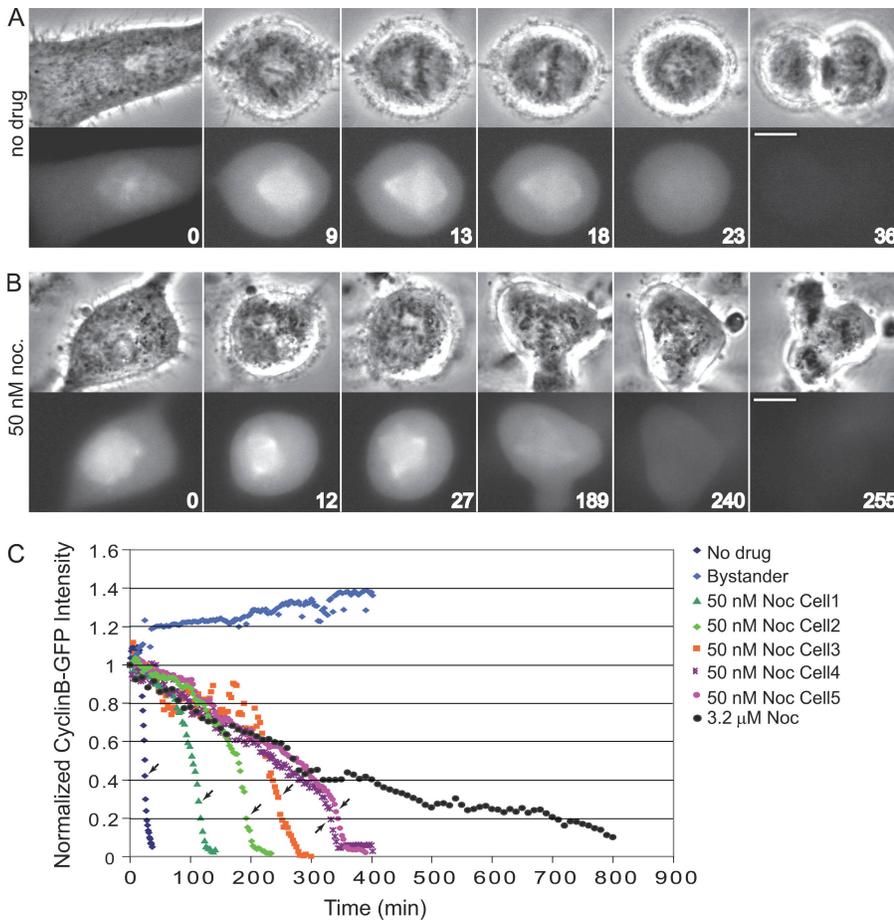


Figure 4. Exit from mitosis in the presence of low concentrations of spindle MT poisons occurs from SAC satisfaction and not slippage.

(A and B) Selected phase-contrast (top) and fluorescence (bottom) frames from video recordings of cyclin B/GFP-expressing RPE-1 cells as they enter and exit mitosis after no treatment (A) or in the presence of 50 nM nocodazole (B). Numbers define the time in minutes from NEB. (C) GFP intensity versus time plots reveal that the fluorescence intensity of control cells (e.g., no drug = cell shown in A) begins to rapidly decay at metaphase (18 min) after the SAC is satisfied and, once initiated, the chromatids disjoin ~ 5 min later (23 min; arrow in C). A similar behavior is seen in cells treated with 50 nM nocodazole, except that the rapid decay of fluorescence intensity is delayed for a variable period during the prolonged mitosis (cell in B = cell 3). The cyclin B/GFP intensity in cells that enter and exit mitosis in 3.2 μ M nocodazole exhibits a slow steady decline, with a slope reflecting the duration of mitosis, until the cell exits mitosis. Bars, 10 μ m.

(e.g., 330 nM) or in concentrations (>1 μ M) that completely inhibit MT assembly (Jordan et al., 1992).

This direct relationship between the drug concentration and the duration of the ensuing mitotic block has been interpreted to mean that the formation of some spindle MTs accelerates mitotic slippage (Introduction). However, this is not consistent with our finding that when the SAC cannot be satisfied, mitotic slippage is not accelerated by the mere presence of MTs. An alternative, but yet to be tested, interpretation of these data is that for MT poisons, a range of concentrations exists in which satisfaction of the SAC is retarded but not prevented. In other words, within this range MT dynamics are not completely inhibited, which ultimately allows all kinetochores to become saturated with MTs regardless of how the spindle is structured.

As a first step toward evaluating this idea, we followed mitosis in RPE-1 cells exposed to concentrations of nocodazole (50 nM), vinblastine (5 nM), or taxol (5 nM) that lie near the mid-range supporting accelerated exit from mitosis in HeLa. We found that in 50 nM nocodazole, RPE-1 cells averaged ~ 3 h in mitosis (vs. 18 min in untreated controls), 100% survived (Table I), and 90% divided into two or three cells (Fig. 3 A and Video 4, available at <http://www.jcb.org/cgi/content/full/jcb.200805072/DC1>). In 5 nM vinblastine, RPE-1 cells averaged ~ 4 h in mitosis, and $>80\%$ ultimately divided into two or more daughter cells (Fig. 3 B). Finally, as for HeLa (e.g., Ikui et al., 2005), RPE-1 cells that entered division in 5 nM taxol averaged ~ 3 h in mitosis (Table I),

99% survived, and $>90\%$ divided into two or more daughter cells (Fig. 3 C). Together, these results confirm that, compared with drug concentrations that prevent MT assembly, exit from mitosis is accelerated in concentrations that allow some MT assembly.

As expected, spindle structure in mitotic RPE-1 cells fixed after 12–16 h in 50 nM nocodazole, 5 nM vinblastine, or 5 nM taxol varied considerably, from diminished bipolar to distorted nonpolar and multipolar spindles (Fig. 3). However, the SAC is satisfied once all kinetochores become stably attached to spindle MTs regardless of whether the spindle is structurally normal (Loncarek et al., 2007; Yang et al., 2008). In this regard, and consistent with our live cell observations, a minor fraction of the mitotic cells in these fixed cultures contained anaphase or telophase spindles (Fig. 3). Yet because some of the APC/Cdc20 substrates responsible for chromatid cohesion are also destroyed during slippage, including shugoshin (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200805072/DC1>), these anaphase and telophase cells could have been generated via accelerated slippage and not from satisfaction of the SAC.

To evaluate this possibility, we used immunofluorescence microscopy to determine if Mad2 or BubR1 are present on any anaphase kinetochores in cells treated with 50 nM nocodazole or 5 nM taxol. However, because of the large number of kinetochores (92), high background staining, and the fact that only one signaling kinetochore prevents SAC satisfaction, this study proved inconclusive.

In our final study, we examined the kinetics of cyclin B/GFP degradation in RPE-1 cells treated with 50 nM nocodazole. If it takes several hours to satisfy the SAC in 50 nM nocodazole, then the cyclin B/GFP levels should slowly decline because of background degradation. But then, as occurs when the SAC is satisfied during a normal mitosis, it should suddenly drop precipitously just before chromatid separation. In contrast, if the cells are undergoing accelerated slippage, then cyclin B/GFP levels should show a slow steady continuous decline until they exit mitosis. As reported previously for HeLa cells (Clute and Pines, 1999), satisfaction of the SAC control RPE-1 cells transiently expressing cyclin B/GFP was followed by a sudden steep drop in cyclin B/GFP fluorescence intensity at metaphase, after which the chromatids disjoined 5–7 min later (Fig. 4 A and Video 5, available at <http://www.jcb.org/cgi/content/full/jcb.200805072/DC1>). When we repeated this study in the presence of 50 nM nocodazole, we consistently obtained a similar result: in all five cells followed from NEB through cytokinesis, the cyclin B/GFP fluorescence intensity showed a similar slow progressive decay after NEB until a variable point, ~5–7 min before chromatid disjunction/anaphase onset, when it suddenly exhibited a sharp and continuous decline (Fig. 4 B, Video 6). This behavior was also seen in five other cells in which observations were initiated after NEB. In contrast, the cyclin B/GFP fluorescence intensity in cells entering mitosis in 3.2 μ M nocodazole exhibited a relatively steady decline, with a slope reflecting the duration of mitosis, until the cells finally exited mitosis 10–30 h later (Fig. 4 C; Brito and Rieder, 2006). From these experiments, we can conclude that compared with cells lacking MTs, cells spend less time in mitosis when exposed to low concentrations of spindle poisons because they ultimately satisfy the SAC and not because slippage is accelerated.

In summary, the rate of mitotic slippage is defined by the rate cyclin B is ubiquitinated and destroyed in the background of an active SAC. This rate is influenced by the organism the cells are from and their genotype. In this paper, we show that mitotic slippage in human cells with a normal robust SAC requires ~20 h under conditions that do not influence the background level of cyclin B destruction and also that some drugs (notably vinblastine) considerably delay slippage. This delay could reflect a retardation in the background rate that cyclin B is ubiquitinated, either via the APC or some other E3 ubiquitin ligase (e.g., NIPA; Bassermann et al., 2005), or its rate of destruction by proteolysis. Contrary to conclusions from indirect data, our live cell work reveals that the presence of numerous dynamic spindle MTs does not influence the rate of slippage. Instead, for drugs that perturb MT assembly a range of concentrations exists in which, rather than being arrested, mitosis is simply prolonged until all of the kinetochores become stably attached to an abnormal spindle (i.e., until the SAC is satisfied). Above this concentration the SAC cannot be satisfied, even in the presence of some MTs, before the cell exits mitosis via slippage. Our finding that cells satisfy the SAC in low concentrations of MT poisons may be clinically relevant. It implies that drugs like taxol do not work in situ by arresting cells in mitosis and also that during therapy, such drugs can induce high numbers of genetically abnormal cells.

Materials and methods

Stock cultures of RPE-1 cells were maintained at 37°C in a humidified 5% CO₂ environment in DME supplemented with 10% FBS. Before each experiment, cells were subcultured onto 25 mm² glass coverslips and grown as in the previous sentence for at least 16 h.

Before imaging, drugs were added to the medium at the concentrations noted in the text. The drugs used in this study were purchased either from Sigma-Aldrich (colcemid, taxol, and vinblastine) or EMD (nocodazole and Eg5 Inhibitor III dimethylenastron). Maytansine was provided by the National Cancer Institute (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnostics).

For filming, coverslip cultures were assembled into Rose chambers (Khodjakov and Rieder, 2006) containing phenol-free L-15 medium supplemented with 10% FBS. Recordings were made at 37°C on microscopes housed inside custom-built temperature-controlled incubators. Time-lapse images were captured every 2 (control)–10 (experimental) min for 48–72 h, with a low magnification (10 \times 0.3NA) PlanFluor phase-contrast objective lens mounted on a microscope (Diaphot; Nikon) equipped with a video camera (Spot Insight QE) and fast UniBlitz shutters (Vincent Associates). This hardware was driven by Image-Pro Plus 5.1.020 (Media Cybernetics, Inc.). Image sequences were compiled with ImageJ 1.35c (National Institutes of Health), and the contrast was adjusted in Photoshop CS2 (Adobe).

Indirect immunofluorescence staining for spindle MTs was conducted as previously detailed (Brilo and Rieder, 2006). After staining, select cells were imaged as a Z-series with a microscope (IX70; Olympus) equipped with a camera (CM350; Photometrics). The image stacks were then deblurred using the SoftWoRx 2.5 deconvolution algorithm (Applied Precision, LLC) and presented as maximal intensity projections.

For cyclin B/GFP studies, RPE-1 cells were cultured and transfected as described previously (Brilo and Rieder, 2006). 15 h after transfection, cells on coverslips were mounted in Rose chambers and cyclin B/GFP-expressing prophase cells were located and followed through mitosis. Near simultaneous phase-contrast and GFP fluorescence images were acquired every 1 min for control (no drug) and 3 min for nocodazole-treated cells. Images were obtained using a 40 \times 0.75 NA lens on a microscope (TE2000E; Nikon) equipped with a shuttered camera (Orca ER; Hamamatsu Photonics) as well as a narrow band pass excitation filter for minimizing GFP photobleaching. Integrated fluorescence intensities were measured by using ImageJ via the method described by Hoffman et al. (2001). All intensities were normalized as the ratio to that in the first (zero) time-point image. Surrounding interphase cells expressing cyclin B/GFP were used as bystander controls. Data processing and graph plotting were conducted in Excel (Microsoft). The cyclin B/GFP construct was provided by J. Pines (University of Cambridge, Cambridge, UK). The Sgo1 antibody was obtained from Y. Watanabe (University of Tokyo, Tokyo, Japan).

Online supplemental material

Fig. S1 shows that indirect immunofluorescence studies reveal that shugoshin (Sgo1) is destroyed as RPE-1 cells slip through mitosis in the presence of nocodazole or vinblastine. Video 1 shows a low-magnification phase-contrast sequence of cultured RPE-1 cells entering and exiting mitosis over a period of 3.5 h. Video 2 is similar to video 1, except the culture was treated with 3.2 μ M nocodazole and the framing rate was once every 15 min for 50 h. Video 3 is similar to video 1, except the culture was treated with 250 nM dimethylenastron and the framing rate was once every 10 min. Video 4 is the same as video 1, except the culture was treated with 50 nM nocodazole and the framing rate was once every 10 min. Video 5 shows near-simultaneous phase-contrast (left) and epifluorescence (right) sequences of a cyclin B/GFP-expressing RPE-1 cell entering and completing mitosis. Video 6 shows near simultaneous phase-contrast (left) and epifluorescence (right hand) sequences of a cyclin B/GFP-expressing RPE-1 cell as it enters and exits mitosis in the presence of 50 nM nocodazole. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200805072/DC1>.

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