

Merotelic kinetochore orientation occurs frequently during early mitosis in mammalian tissue cells and error correction is achieved by two different mechanisms

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Summary

Merotelic kinetochore orientation is an error that occurs when a single kinetochore becomes attached to microtubules from two spindle poles rather than just to one pole. We obtained the first evidence that merotelic kinetochore orientation occurs very frequently during early mitosis in mammalian tissue cells and that two different correction mechanisms are critical for accurate chromosome segregation in cells possessing bipolar spindles and unperturbed chromosomes. Our data show that about 30% of prometaphase PtK1 cells possess one or more merotelically oriented kinetochores. This frequency is increased to over 90% in cells recovering from a nocodazole-induced mitotic block. A delay in establishing spindle bipolarity is responsible for the high frequency of merotelic orientations seen in cells recovering from nocodazole, but not in untreated cells. The frequency of anaphase cells with merotelically oriented lagging chromosomes is 1% in untreated cells and 18% in cells recovering from nocodazole. Prolonging metaphase by 2 hours reduced the frequency of anaphase cells with lagging

chromosomes both for untreated and for nocodazole-treated cells. Surprisingly, anaphase lagging chromosomes represented a very small fraction of merotelic kinetochore orientations present in late metaphase. Our data indicate that two correction mechanisms operate to prevent chromosome mis-segregation due to merotelic kinetochore orientation. The first, a pre-anaphase correction mechanism increases the ratio of kinetochore microtubules attached to the correct versus incorrect pole and might eventually result in kinetochore re-orientation before anaphase onset. The increase in microtubule ratio to opposite poles is the groundwork for a second mechanism, active in anaphase, that promotes the segregation of merotelically oriented chromosomes to the correct pole.

Movies and supplemental data available online

Key words: Merotelic orientation, Lagging chromosomes, Mitotic checkpoint, Chromosome segregation

Introduction

Merotelic kinetochore orientation is an error in kinetochore function and represents a major source of aneuploidy in mammalian tissue cells (Cimini et al., 2002; Cimini et al., 2001). This error occurs during mitosis when a single kinetochore attaches microtubules coming from two spindle poles. Merotelic kinetochore orientation does not inhibit movement of sister chromatids to the spindle equator in prometaphase and it is not detected by the mitotic checkpoint (Cimini et al., 2002; Cimini et al., 2001; Khodjakov et al., 1997; Yu and Dawe, 2000), the cell cycle control mechanism that prevents anaphase onset in the presence of unattached kinetochores or lack of tension between sister kinetochores (Rieder and Salmon, 1998; Shah and Cleveland, 2000). After sister chromatid separation in anaphase, merotelic kinetochore orientation produces a lagging chromosome near the spindle equator with the kinetochore stretched laterally by microtubule connections to opposite spindle poles (Cimini et al., 2001). Lagging chromosomes have been observed in 0.5-5% of

anaphases in human and other mammalian tissue cells in culture (Catalan et al., 2000; Cimini et al., 2002; Cimini et al., 2001; Ford et al., 1988; Izzo et al., 1998; Minissi et al., 1999).

It is likely that merotelic kinetochore orientation is mainly an error inherent to the stochastic nature of the 'search and capture' mechanism of kinetochore microtubule formation that depends on microtubule dynamic instability (Desai and Mitchison, 1997; Inoue and Salmon, 1995; Rieder and Salmon, 1998; Wittmann et al., 2001), although other mechanisms are also possible (Khodjakov et al., 2003). Mammalian kinetochores have multiple microtubule plus end attachment sites, typically 15-25, within the outer plate (McEwen et al., 1997). Attachment to one of these sites stabilizes a plus end and forms a kinetochore microtubule that mechanically links the kinetochore to the site of microtubule nucleation (Rieder and Salmon, 1998). Normally, sister kinetochores face opposite directions so that when one sister captures microtubules from one pole the subsequent chromosome movement toward that pole causes the other sister to face away from the pole. This

mono-orientation prevents both sister kinetochores from capturing microtubule plus ends from the same pole (syntelic orientation) and promotes the correct attachment of sisters to opposite spindle poles (amphitelic orientation, or bi-orientation) (Nicklas, 1997; Rieder and Salmon, 1998).

Little is known about the initial incidence of merotelic kinetochore orientation and the correction mechanisms that may operate to prevent lagging chromosomes in normal mitotic tissue cells (Ault and Rieder, 1992; Roos, 1973; Roos, 1976). In previous studies merotelic kinetochore orientation in mitotic cells has been studied in fairly abnormal situations, such as impaired sister kinetochore structure (Khodjakov et al., 1997; Wise and Brinkley, 1997; Yu and Dawe, 2000), and multipolar mitoses (Heneen, 1975; Sluder et al., 1997). Studies of normal meiosis in invertebrates have shown that attachment errors, in particular syntelic orientation (both sister kinetochores attached to the same pole), are initially frequent and correction mechanisms exist (Bajer, 1972; Nicklas, 1971; Nicklas, 1997). Recently, correction mechanisms for syntelic kinetochore orientation both in meiosis and mitosis have been identified in budding yeast (Biggins and Murray, 2001; Shannon and Salmon, 2002; Tanaka et al., 2002) and there is evidence that a similar molecular mechanism may function to correct syntelic and merotelic errors during mitosis in higher eucaryotes (Shannon and Salmon, 2002).

A major question is whether merotelic kinetochore orientation in cells with bipolar spindles and unperturbed chromosomes normally occurs in prometaphase at frequencies higher than the frequencies of lagging chromosomes exhibited in anaphase. Another major question is why transient spindle disassembly is such a potent promoter of lagging chromosomes (Cimini et al., 2002; Cimini et al., 2001). Upon spindle reassembly and progression into anaphase the frequency of cells with merotelically oriented lagging chromosomes is substantially increased to 8-30% (Cimini et al., 2002; Cimini et al., 2001; Ladrach and LaFountain, 1986).

To test in mammalian mitosis for the initial incidence and subsequent correction of merotelic kinetochore orientation, we performed several assays using PtK1 cells. To test for the incidence of merotelic attachments in early prometaphase, we induced prometaphase cells to enter anaphase precociously and compared the frequency of lagging chromosomes in these cells and in cells proceeding to anaphase normally. To examine why recovery from spindle depolymerization produces a high incidence of merotelic kinetochore orientation, we analyzed spindle and kinetochore structure in both nocodazole-treated and untreated PtK1 cells. To determine if time before anaphase onset is an important factor in eliminating merotelic kinetochore orientation, we delayed cells in metaphase by a reversible inhibitor of the proteasome. And finally, to see if merotelic orientations are corrected by activities at or after anaphase onset, we used high resolution confocal fluorescence microscopy and 3D image deconvolution to compare the frequency of merotelic kinetochores at late prometaphase/metaphase with the frequency of merotelic orientation in 'delayed' metaphase, and the frequency of lagging chromosomes in anaphase. Finally, we tested whether the ratio of microtubules to opposite poles is an important factor in producing lagging chromosomes by quantification of fiber fluorescence for merotelic kinetochores in normal and delayed metaphases.

Materials and Methods

Cell culture and treatment

PtK1 (American Type Culture Collection; Rockville, MD) and PtK6-23 cells (γ -tubulin-GFP-expressing PtK1 cells; a generous gift from Dr Alexey Khodjakov; Wadsworth Center, Albany, NY) cells were maintained in Ham's F-12 medium (Sigma Chemical Co., St Louis, MO), complemented with 10% fetal bovine serum, antibiotics and antimycotic, and grown at 37°C, in a humidified incubator with 5% CO₂. For microinjection and/or filming, cells were incubated in L-15 medium (Sigma), complemented with 7 mM Hepes, 10% fetal bovine serum, and antibiotics/antimycotic solution. For all experiments, PtK1 cells were grown on sterilized coverslips inside 35 mm Petri dishes. For nocodazole experiments, cells were incubated with 2 μ M nocodazole for 3 hours. At the end of the treatment, cells were either fixed or washed 3 times with medium and incubated in fresh medium at 37°C. Cells were then fixed after the appropriate recovery time required by the experimental protocol. For the MG-132 experiment, untreated cells or cells released from nocodazole for 25 minutes were treated with 5 μ M MG-132 for 2 hours. At the end of the treatment, cells were either fixed for the analysis of merotelic orientations in 'delayed' metaphase, or washed to allow anaphase onset. For the analysis of anaphase lagging chromosomes after MG-132 treatment, cells were washed 3 times for 5 minutes with medium at 37°C and then incubated in fresh medium at 37°C for a further 60 minutes, for a total 75 minutes recovery. Preliminary experiments had shown that most of the cells had accomplished metaphase to anaphase transition within 75 minutes and by that time cells were observed in mid- to late-anaphase.

Microinjection and time-lapse microscopy

Coverslips with untreated cells or with nocodazole-released cells were mounted into modified Rose chambers (Rieder and Hard, 1990) without the top coverslip, the chambers were filled with Hepes-buffered L-15 medium, and a thin layer of mineral oil was poured on the medium to avoid evaporation. Stage temperature was maintained at ~35°C using an air curtain incubator (Nevtek, Model ASI 400, Burnsville, VA). Microinjection with GST-Mad1F10 and Mad2 Δ C was performed as previously described (Canman et al., 2002a). Control prometaphase cells were microinjected with either 2 mg/ml (needle concentration) purified GST in HEK buffer (20 mM Hepes, 100 mM KCl, and 1 mM DTT, pH 7.7 with KOH), HEK buffer alone, 2 mg/ml GST-Mad1F10, or 7 mg/ml Mad2 Δ C in HEK buffer. Nocodazole-released cells were either time-lapsed uninjected or were microinjected with either GST-Mad1F10 or Mad2 Δ C after about 15 minutes recovery from the mitotic block. Phase contrast time-lapse images were acquired every 30 seconds using a MetaView image acquisition system (Universal Imaging Corp., Downingtown, PA). γ -tubulin-GFP-expressing PtK1 cells (PtK6-23) were time-lapsed by phase contrast and fluorescence microscopy using a Nikon TE300 inverted microscope equipped with a 100 \times (1.4 NA) Plan Apo phase 3 objective, a Chroma Hy-Q FITC filter set, and an Orca II CCD camera (Hamamatsu Photonics, Bridgewater, NJ). The microscope was controlled by MetaMorph imaging software (Universal Imaging). Phase contrast and fluorescence images were acquired almost simultaneously every 2 minutes, using a 4 \times 4 binning.

Immunostaining with CREST, anti- α -tubulin, anti- γ -tubulin and anti-Mad2 antibodies

For CREST staining and combined CREST and α -tubulin immunostaining, cells were fixed and processed as previously described (Cimini et al., 2001). For combined Mad2/ γ -tubulin and Mad2/ α -tubulin immunostaining the same procedure was used, except for the fixation, which was performed in a freshly prepared 4% formaldehyde solution at room temperature. We used the following

primary antibodies: CREST SH serum (a generous gift from Dr B. R. Brinkley, Baylor College of Medicine, TX), diluted 1:600; mouse anti- α -tubulin antibody (Sigma), diluted 1:600; rabbit anti-Mad2 antibody (Waters et al., 1998), diluted 1:100; mouse anti γ -tubulin antibody (Sigma), diluted 1:5000. The following secondary antibodies were used in various combinations in different experiments: Rhodamine Red-X anti-human (Jackson ImmunoResearch, West Grove, PA), diluted 1:200; Alexa 488 anti-mouse antibody (Molecular Probes, Eugene, OR), diluted 1:1000; Alexa 488 anti-rabbit antibody (Molecular Probes), diluted 1:1000; Rhodamine Red-X anti-mouse (Jackson ImmunoResearch), diluted 1:200.

Fluorescence microscopy and image acquisition

Wide-field epifluorescence was used for analysis of anaphase lagging chromosomes by CREST staining. For each slide all the anaphase cells were analyzed and recorded as normal or as showing one or multiple lagging chromosomes. The data shown in the paper are the sum of three independent experiments. For Mad2/ γ -tubulin and Mad2/ α -tubulin immunostained cells z-series stacks of images at 0.2 μ m intervals through each cell analyzed were collected. The images were obtained by projecting into a single image the maximal brightness at each pixel location. The microscope used was a Nikon TE300 inverted microscope equipped with a 100 \times (1.4 NA) Plan Apo phase 3 objective, Chroma filters, and an Orca II CCD camera (Hamamatsu Photonics). To analyze merotelic kinetochore orientation in late prometaphase and metaphase we acquired z-series stacks of confocal images at 0.2 μ m intervals through each cell analyzed and processed the image stacks to obtain 3D projections after digital image deconvolution using a Delta Vision image processing workstation (Applied Precision; Seattle, WA). To improve the accuracy of the analysis, two different people performed the analysis blindly and independently. Images were recorded with a Nikon TE300 inverted microscope equipped with epifluorescence illumination by a Yokogawa CS10 spinning disk confocal attachment (Perkin Elmer Life Sciences Wallac; Gaithersburg, MD), containing filters and filter wheels for illumination at 488 nm or 568 nm from a 60 mW argon/krypton laser, and equipped with a 100X/1.4 NA plan-apochromatic phase contrast objective lens. Digital images were obtained with an Orca ER cooled CCD camera (Hamamatsu Photonics). Image acquisition, microscope shutters and z-axis focus were all controlled by MetaMorph (Universal Imaging) software on PC computers. Adobe Photoshop software was used to process all digital images.

Fluorescence measurements

Fluorescence intensity measurements of the two microtubule bundles attached to a merotelic kinetochore were performed on deconvolved images by measuring the fluorescence intensity of the microtubule bundle, as detected by α -tubulin immunostaining, and subtracting the average background intensity. The fluorescence intensity of the microtubule bundle was determined in a 5 \times 5 pixel square region on the microtubule side of the microtubule-kinetochore interface. The background fluorescence intensity was measured in two 5 \times 5 pixel regions on both sides of the same microtubule bundle, and then these values were averaged. The measurements were made using the data inspector tool of the Deltavision's Softworx Deconvolution package (Applied Precision), which is run on a Silicon Graphics platform.

Online supplemental material

QuickTime movies and a supplemental table (Table S1) summarizing both data 'per cell' and 'per kinetochore' in different experimental conditions are also available online (<http://JCS.biologists.org/supplemental/>).

Results

Merotelic kinetochore orientation occurs frequently in early prometaphase

To investigate the incidence of merotelic kinetochore orientation early in mitosis, PtK1 prometaphase cells were induced to enter anaphase precociously and the frequency of chromosomes lagging behind at the spindle equator at anaphase was evaluated. To induce precocious anaphase we used two different inhibitors of the mitotic checkpoint, GST-Mad1F10 and Mad2 Δ C, recently developed by Canman and coworkers (Canman et al., 2002a). Cells were microinjected in prometaphase, when most of the chromosomes were not aligned at the metaphase plate. Usually, by the time anaphase began, about 10-15 minutes after microinjection, most of the chromosomes had congressed to the spindle equator. However, occasionally mono-oriented chromosomes were present at the time of anaphase onset. These mono-oriented chromosomes were positioned close to the pole they were attached to and they remained close to that pole throughout anaphase. Thus, such mono-oriented chromosomes did not interfere with our analysis of lagging chromosomes, which are typically localized at the cell equator.

When PtK1 prometaphase cells were microinjected with either GST-Mad1F10 (11 cells microinjected) or with Mad2 Δ C (11 cells microinjected), they entered anaphase precociously and 27.3% (for GST-Mad1F10) and 36.4% (for Mad2 Δ C) of the cells showed lagging chromosomes at anaphase (Fig. 1B, Fig. 2). As a control, we microinjected 10 PtK1 prometaphase cells with GST-containing buffer and 10 PtK1 prometaphase cells with the Mad2 Δ C dilution buffer. None of the 20 microinjected cells entered anaphase precociously and none showed anaphase lagging chromosomes (Fig. 1A, Fig. 2), as expected for normal untreated PtK1 cells, where the frequency of anaphase lagging chromosomes is about 1% (Cimini et al., 2001). To test if lagging chromosomes were somehow induced by the injected proteins, we injected 19 metaphase PtK1 cells with Mad2 Δ C and found that none of them displayed lagging chromosomes at anaphase.

We next tested for the incidence of merotelic orientation in prometaphase of cells recovering from a nocodazole-induced mitotic block because of the higher incidence of anaphase lagging chromosomes compared to controls (Cimini et al., 2001; Cimini et al., 1999). Cells were released from the mitotic block for about 15 minutes to allow spindle reassembly and then microinjected with either GST-Mad1F10 (13 cells) or Mad2 Δ C (11 cells) to induce precocious anaphase. The frequencies of cells showing lagging chromosomes in anaphase were 92.3% and 90.9% for GST-Mad1F10 and Mad2 Δ C, respectively, indicating a very high incidence of merotelic kinetochore orientation (Fig. 1D, Fig. 2; Movie 1: <http://JCS.biologists.org/supplemental/>). As a control, we measured by live cell imaging the frequency of lagging chromosomes in 10 PtK1 cells recovering from nocodazole and progressing normally into anaphase. This frequency was 20% (Fig. 1C, Fig. 2), similar to the 16-18% obtained by fixed time-point assays (Cimini et al., 2001).

Merotelic kinetochore orientation is responsible for the induction of lagging chromosomes in cells progressing normally into anaphase (Cimini et al., 2001). To confirm the

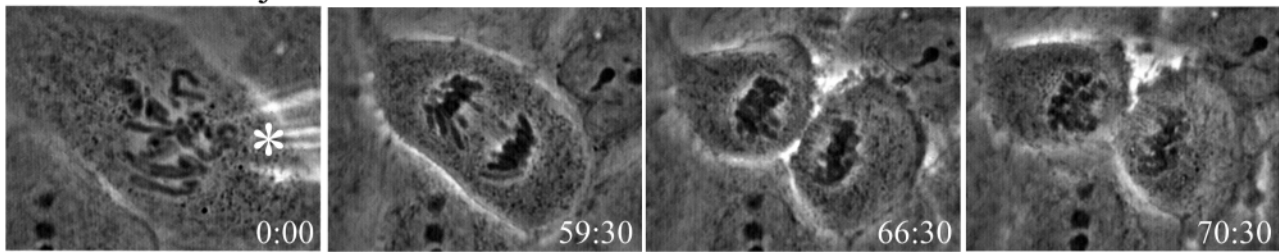
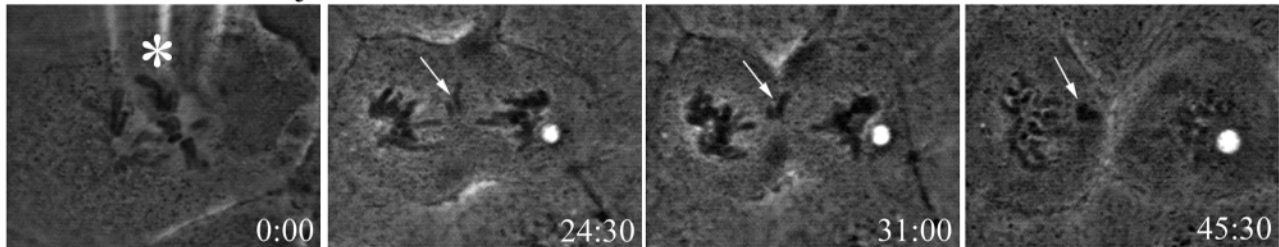
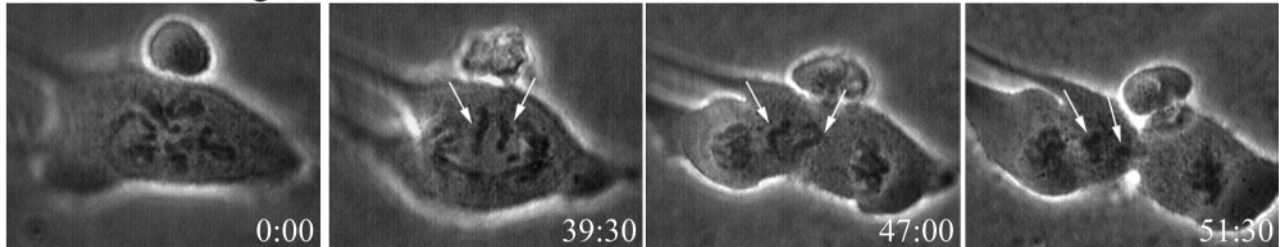
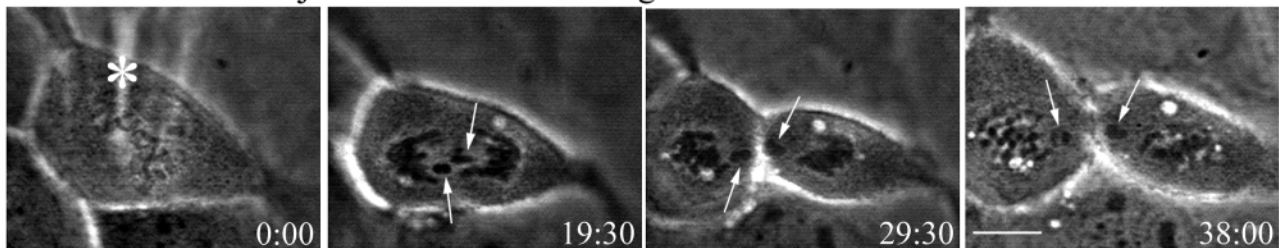
A. Buffer microinjection in control cell**B. Mad2 Δ C microinjection in control cell****C. Cell recovering from a nocodazole-induced mitotic block****D. Mad2 Δ C microinjection in cell recovering from a nocodazole-induced mitotic block**

Fig. 1. (A-D) Effect of microinjection of dominant negative mitotic checkpoint proteins on the presence of lagging chromosomes in anaphase in PtK1 cells under different experimental conditions. Arrows point at lagging chromosomes in anaphase. In microinjected cells, the tip of the needle is indicated by an asterisk. Scale bar: 10 μ m. A QuickTime movie (Movie 1) of the cell shown in D is available at <http://jcs.biologists.org/supplemental/>

merotelic orientation of lagging chromosomes in cells induced to enter anaphase precociously, some of the cells microinjected with GST-Mad1F10 were followed by time-lapse microscopy until mid-anaphase and then fixed and immunostained for kinetochores and microtubules. The immunostaining showed that lagging chromosomes had merotelically oriented kinetochores that were stretched laterally between bundles of microtubules from opposite spindle poles (Fig. 3).

A delay in achieving spindle bipolarity promotes very high frequencies of merotelic orientations in nocodazole-recovering cells

When mitotic spindle assembly is inhibited by nocodazole

treatment, centrosomes appear close to each other (Cassimeris et al., 1990) and kinetochores expand into curved crescents around the centromeres (Cimini et al., 2001; Hoffman et al., 2001). It has been proposed that the enlargement of kinetochores into a crescent shape around their centromeres might promote merotelic kinetochore orientation (Cimini et al., 2001). However, a delay in establishing spindle bipolarity during recovery from nocodazole might also contribute to the very high frequency of merotelic kinetochore orientation seen during recovery from nocodazole treatment.

To test whether and how these two mechanisms contribute to the incidence of merotelic kinetochore orientation, we fixed cells after a 3 hour nocodazole treatment and after 1, 5 and 15

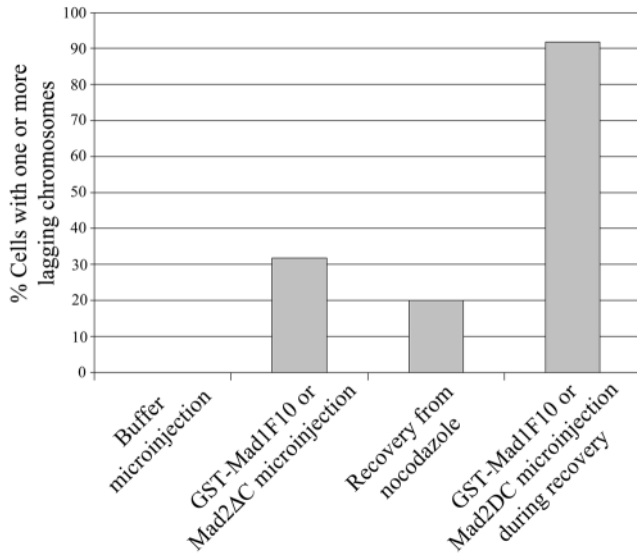


Fig. 2. The data obtained in microinjection experiments (shown in Fig. 1) are summarized. Results obtained by microinjection of GST-Mad1F10 and Mad2ΔC were pooled.

minutes recovery from the nocodazole-induced mitotic block. These cells were immunostained either for γ -tubulin (centrosomes) and Mad2 (kinetochore outer domain), or for α -tubulin (microtubules) and Mad2. As expected (Cimini et al., 2001; Hoffman et al., 2001), Mad2 accumulated at kinetochores in cells arrested in mitosis by a 3 hour nocodazole treatment and some of the kinetochores showed a clear crescent structure (Fig. 4A, Fig. 5A'). Furthermore, the centrosomes, as seen by γ -tubulin staining, were unseparated (Fig. 4A).

Surprisingly, 1 minute after nocodazole washout most of the Mad2 staining had disappeared, even though centrosomes had not separated, yet (Fig. 4B). Most of the cells showed clear centrosome separation 5 minutes after release from the mitotic block (Fig. 4C). At this recovery time, however, not all the chromosomes were aligned at the metaphase plate and some Mad2 was still present at kinetochores. Well separated centrosomes and chromosomes aligned at the metaphase plate were observed after 15 minutes recovery from the mitotic block (Fig. 4D).

When cells recovering from the mitotic block were analyzed by α -tubulin immunostaining, multiple sources of microtubules surrounding the chromosomes (typically about 4–8 microtubule organizing centers) were observed after 1 minute recovery (Fig. 5B). After 5 minutes recovery the number of α -tubulin-positive microtubule organizing centers ranged between 2 and 5, with most of the cells showing 3 or 4 (Fig. 5C). At 15 minutes recovery, many cells (50%) showed 2 major spindle poles and a clearly bi-polar spindle (Fig. 5D), but a minor fraction (35%) of the population still showed 3 spindle poles. However, by anaphase, most of the cells reached bi-polarity and only about 4% of the anaphases were tri- or tetrapolar. This frequency was not significantly higher than the 3% occurrence of multipolar anaphases observed in control PtK1 cells. These data suggest that the high incidence of merotelic kinetochore orientation observed during recovery from a nocodazole-induced mitotic block is primarily produced by single kinetochores becoming attached to microtubules coming from microtubule organizing centers that are initially close together and later separate into opposite spindle poles. The enlargement of kinetochores into crescents might favor their interaction with more than one microtubule organizing center.

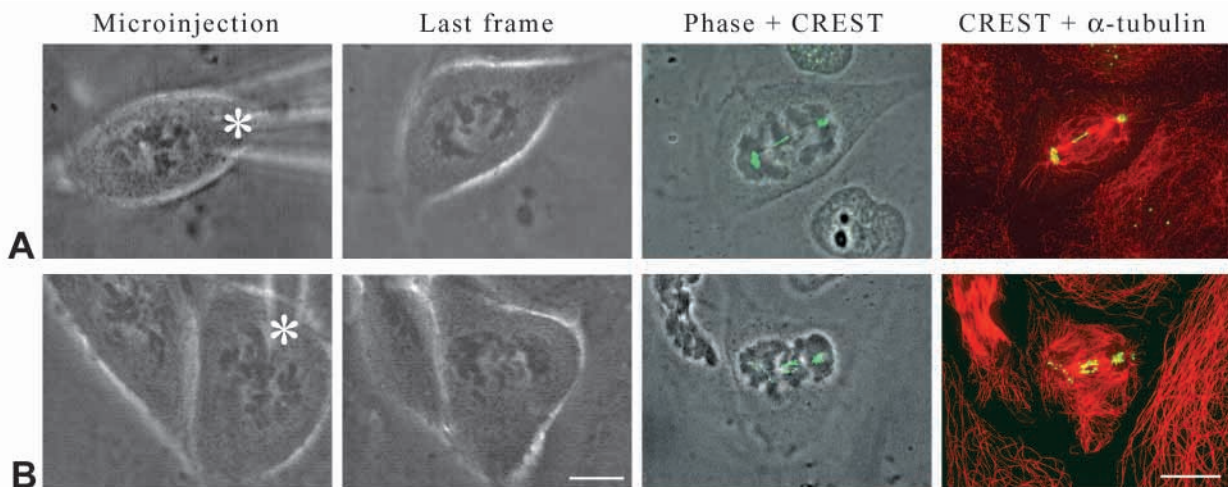


Fig. 3. Merotelic orientation of lagging chromosomes after microinjection of Mad1F10. PtK1 cells recovering from a nocodazole-induced mitotic block were microinjected with GST-Mad1F10 to inactivate the mitotic checkpoint and to induce precocious anaphase. Cells were followed by time-lapse microscopy and fixed in mid-anaphase if they possessed one or more lagging chromosomes. Cells were then immunostained for CREST and α -tubulin. After immunostaining, cells were re-localized to verify if lagging chromosomes were merotelically oriented. Two cells showing lagging chromosomes after microinjection of GST-Mad1F10 are shown. The frame showing microinjection, the last frame photographed by time-lapse microscopy before fixation, the phase contrast/CREST overlay, and the CREST/ α -tubulin overlays are shown in the four columns. (A) Microinjected cell showing one merotelically oriented lagging chromosome at anaphase. (B) Microinjected cell showing multiple merotelically oriented lagging chromosomes. Asterisks indicate the tip of the needle at the moment of microinjection. Scale bars: 10 μ m.

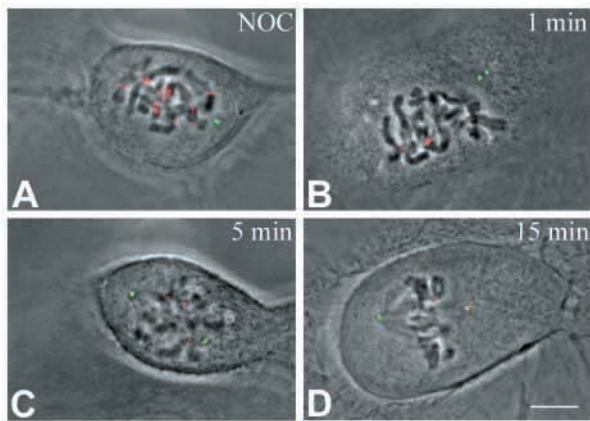


Fig. 4. PtK1 cells immunostained for Mad2 (red) and γ -tubulin (green). The overlay of phase contrast images, Mad2, and γ -tubulin staining is shown. (A) Cell arrested in mitosis by a 3-hour nocodazole treatment. (B-D) Cells fixed after 1, 5 and 15 minutes recovery from the nocodazole-induced mitotic arrest. Scale bars: 10 μ m.

Merotelic orientations in untreated cells are not caused by a delay in establishing spindle bipolarity

To test in untreated cells whether and how often centrosomes fail to separate to the opposite sides of the nucleus before nuclear envelope breakdown and spindle assembly, we used γ -tubulin-GFP-expressing PtK1 cells (PtK6-23; a generous gift from Dr Alexey Khodjakov, Wadsworth Center, Albany, NY) to follow spindle pole separation in living cells. As detected by CREST staining on fixed cells, only 1% of the anaphases (3/300) in PtK6-23 cells showed lagging chromosomes, indicating that the expression of the chimeric protein alone does not induce an increase in anaphase lagging chromosomes. PtK6-23 cells were recorded by time-lapse phase contrast and fluorescence microscopy from prophase to telophase. One out of 30 cells analyzed showed a lagging chromosome in anaphase. This frequency is not significantly different from the 1% frequency of anaphase lagging chromosomes in fixed PtK6-23 cells (χ^2 test, $P=0.811$). However, in all cells analyzed, including the one showing a lagging chromosome in anaphase, centrosomes were well separated, 29 out of 30 to opposite sides of the nucleus, when nuclear envelope breakdown occurred (see Movies 2 and 3: <http://JCS.biologists.org/supplemental/>). These data indicate that defects in centrosome separation are not responsible for the high incidence of merotelic kinetochore orientation in normal prometaphase.

We then tested whether ectopic microtubule nucleation centers are present in early prometaphase of untreated cells, as seen by α -tubulin staining in nocodazole-recovering cells. Almost all the cells showed normal bipolar spindles with two well separated spindle poles, and no ectopic microtubule nucleation centers were observed in cells fixed immediately after nuclear envelope breakdown (Fig. 6A,B) or later in prometaphase (Fig. 6C). A small number of cells (2%) showed tripolar or tetrapolar spindles, but this represents a multipolar subpopulation always observed in cultured PtK1 cells, at any mitotic stage.

In control PtK1 cells immediately after nuclear envelope breakdown, there was great variability seen between different chromosomes in the orientation of the centromere axis through sister kinetochores relative to the inter-polar spindle axis (Fig.

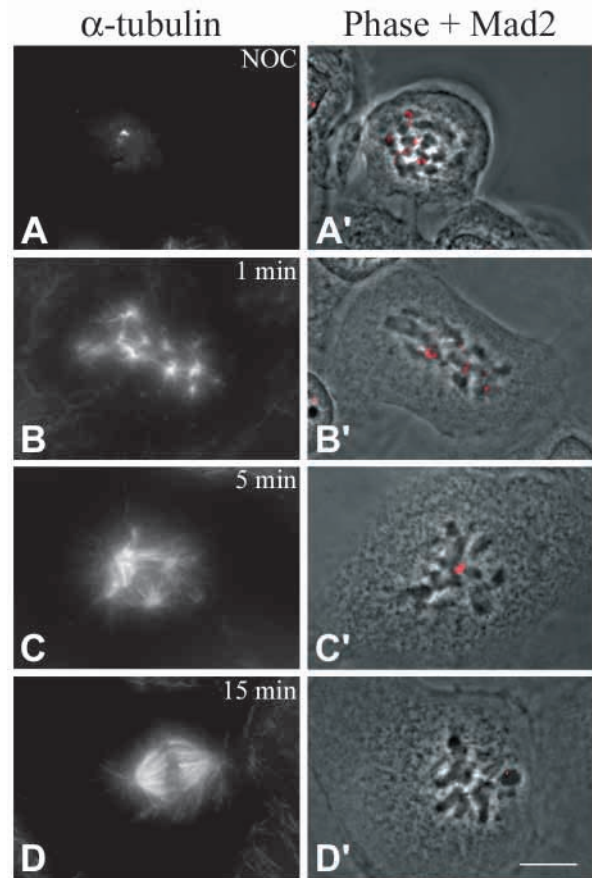


Fig. 5. PtK1 cells immunostained for Mad2 and α -tubulin. The α -tubulin staining is shown in the left column and the overlay of phase contrast images and Mad2 immunostaining is shown in the right column. (A,A') Cell fixed after a 3 hour nocodazole treatment. (B,B',D,D') Cells fixed after 1, 5 and 15 minutes recovery from the nocodazole-induced mitotic arrest. Scale bar: 10 μ m.

6A,B). Some sister pairs were aligned nearly parallel to the spindle axis (yellow arrowhead in Fig. 6A), while others were nearly perpendicular (white arrows in Fig. 6B). This perpendicular orientation of sister kinetochores may promote merotelic attachment as discussed below.

Prolonging metaphase significantly reduces lagging chromosomes in anaphase

Our microinjection experiments indicate that correction mechanisms must function to reduce the number of merotelic orientations and prevent anaphase lagging chromosomes. The next obvious investigation was to determine whether lengthening the time before anaphase enhances error correction. Both untreated cells and cells recovering from a nocodazole-induced mitotic block were treated for 2 hours with 5 μ M MG-132 to inhibit the proteasome and prolong metaphase (Hoffman et al., 2001; Rock et al., 1994; Topper et al., 2001). MG-132 induced a metaphase arrest, but did not affect spindle assembly. Chromosomes aligned at the metaphase plate, showing normal chromosome oscillations back and forth in the equatorial region (data not shown). At the end of the MG-132 treatment, the drug was washed out and

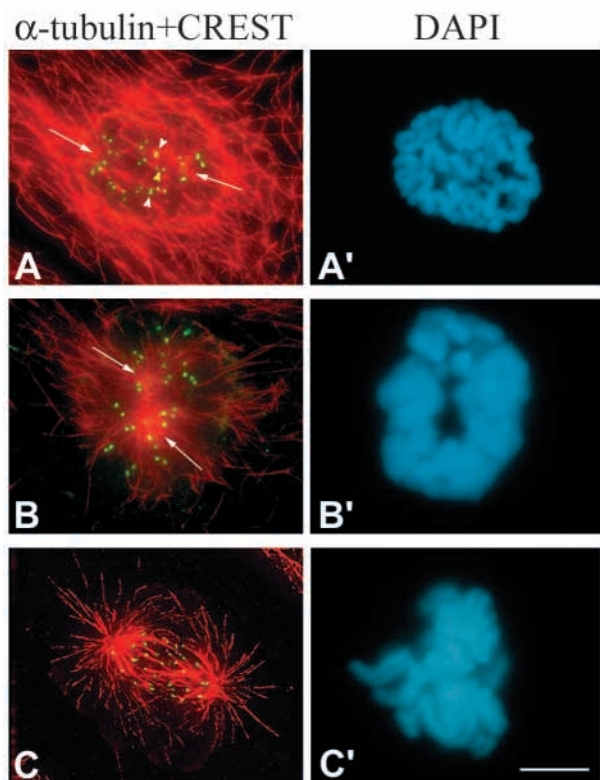


Fig. 6. PtK1 cells in early mitosis immunostained for CREST and α -tubulin (left column). DNA staining by DAPI is shown in the right column. (A,A',B,B') Cells fixed immediately after nuclear envelope breakdown. Two forming microtubule organizing centers can be seen (arrows) in these cells. The CREST staining shows that kinetochores are distributed around the spindle poles and are oriented in many different directions with respect to the poles (B). Furthermore, some chromosomes are oriented with their centromere axis through sister kinetochores nearly parallel to the spindle axis (yellow arrowhead in A), whereas other sister pairs are nearly perpendicular (white arrowheads in A). (C,C') Prometaphase control cell. Only two microtubule organizing centers can be identified (spindle poles) in this cell, as in the vast majority of untreated PtK1 mitotic cells. Scale bar: 10 μ m.

cells were fixed 75 minutes later for analysis of anaphase lagging chromosomes. The delay in metaphase produced by MG-132 reduced the incidence of lagging chromosomes in anaphase to 0.4% for untreated and 2.6% for nocodazole-recovering cells, representing 2.7- and 6.2-fold reduction, respectively, compared to cells progressing to anaphase without MG-132 delay (Fig. 7A). Fig. 7B shows that in nocodazole-released cells both single and multiple lagging chromosomes were substantially decreased. Taken together, these results indicate that time before anaphase is an important factor for the mechanisms correcting chromosome segregation errors produced by merotelic kinetochore orientation.

The frequency of merotelic kinetochores seen at late prometaphase/metaphase or after a metaphase delay is much higher than the frequency of lagging chromosomes in anaphase

In a previous study, we showed that merotelically oriented

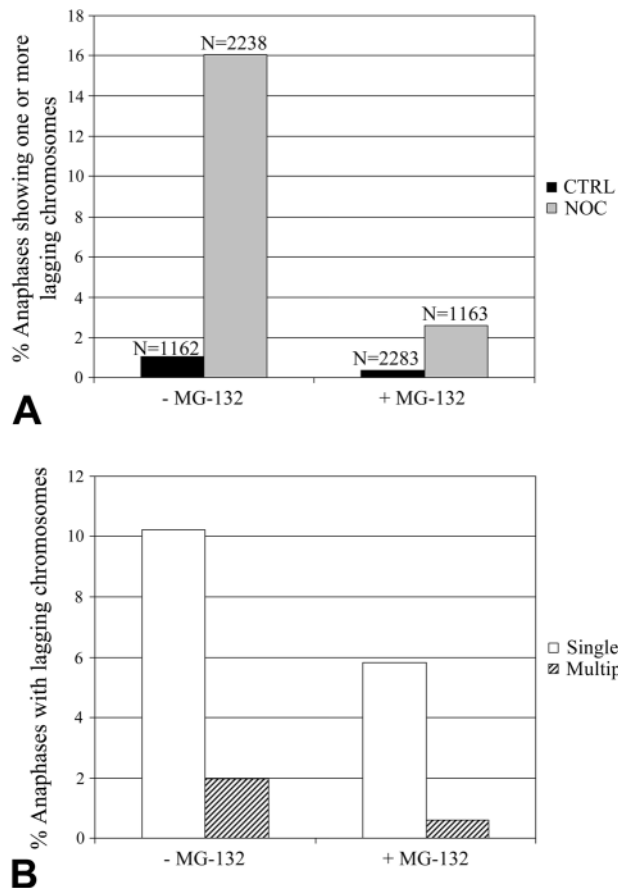


Fig. 7. Effect of a prolonged metaphase on the presence of anaphase lagging chromosomes. (A) Both control PtK1 cells (CTRL) and cells recovering from a nocodazole (NOC)-induced mitotic block were either fixed untreated or delayed in metaphase by a 2-hour MG-132 treatment and then fixed after recovery. Anaphase lagging chromosomes were analyzed by CREST staining. N, total number of cells analyzed. (B) Frequencies of single and multiple lagging chromosomes in anaphase PtK1 cells recovering from a nocodazole-induced mitotic block (-MG-132), or in which anaphase onset was delayed by a 2-hour MG-132 treatment (+MG-132) during recovery from nocodazole.

kinetochores can be seen in late prometaphase/metaphase cells by high resolution 3D confocal and deconvolution microscopy of fixed cells immunofluorescently stained for kinetochores and kinetochore microtubules (Cimini et al., 2001). Here we used the same 3D imaging technology (Fig. 8) to evaluate the actual frequency of merotelic orientation in prometaphase/metaphase cells and to test how the frequency of cells with merotelically oriented kinetochores before anaphase onset compares with the frequency of cells with lagging chromosomes in anaphase under our various experimental conditions.

Typically, a merotelically oriented kinetochore was shifted closer to the equator than normally oriented kinetochores of bi-oriented chromosomes at the metaphase plate, making the detection easier (Fig. 8B-E). Furthermore, the resolution in our images in combination with 3-D image rotation to check for parallax problems (Cimini et al., 2001) allowed for accurate detection of kinetochores that had attached bundles of fluorescent microtubules extending toward both poles.

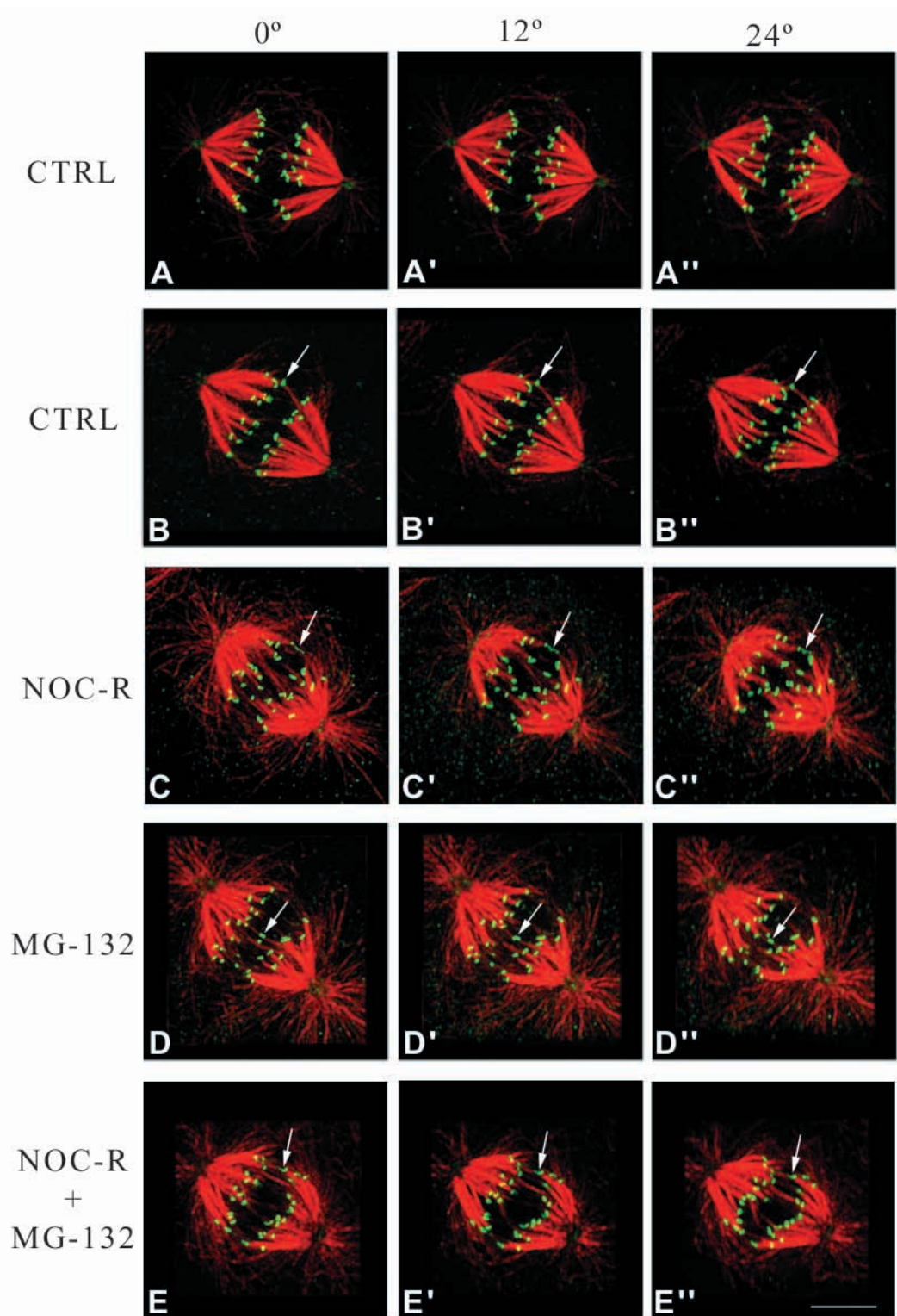


Fig. 8. Examples of merotelic kinetochore orientations in prometaphase/metaphase analyzed by confocal microscopy and 3D image deconvolution. Images on each row show three different angles of the same cell. Arrows indicate merotelically oriented kinetochores. Note that when a merotelically oriented kinetochore is present, it appears still connected to kinetochore microtubules coming from both poles when the image is rotated of 12° (middle column) and 24° (right column). CTRL, control; NOC-R, 30 minutes recovery from a nocodazole-induced mitotic arrest; MG-132, 2 hours treatment with 5 μM MG-132; NOC-R + MG-132, nocodazole (2 μM) mitotic arrest + 25 minutes recovery + 2 hours MG-132 treatment. Scale bar: 10 μm.

PtK1 cells were fixed untreated or after a 3 hour nocodazole block plus 15-30 minutes recovery. Late prometaphase and metaphase cells (prometaphases/metaphases) with bipolar spindles were analyzed for the presence of merotelically oriented kinetochores. The percentage of prometaphase/metaphase cells with at least one merotelically oriented kinetochore was 16.3% for untreated cells (Fig. 8B-B''; Fig.

9A, -MG-132) and 45.8% for cells recovering from the nocodazole-induced mitotic block (Fig. 8C-C''; Fig. 9A, -MG-132). Nocodazole-treated cells also exhibited high frequencies of cells possessing more than one merotelic kinetochore (Fig. 9B, -MG-132, diagonally striped bar). These results show that the frequency of merotelic kinetochore orientations remains high in late prometaphase/metaphase, much higher than seen

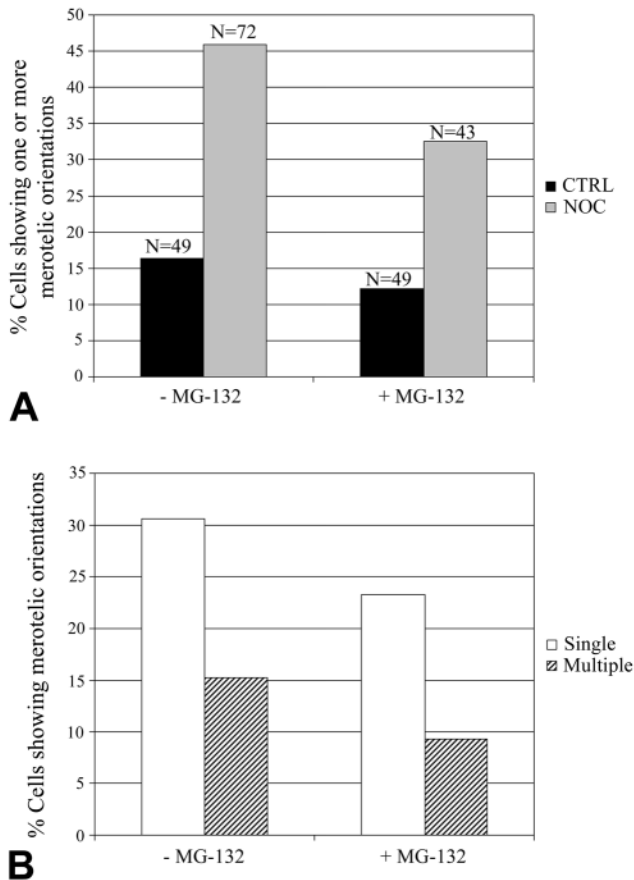


Fig. 9. Frequencies of merotelic orientations in late prometaphase/metaphase PtK1 cells and in 'delayed' metaphases (see text for details). (A) Frequencies of merotelic orientations in prometaphase/metaphase PtK1 cells (-MG-132), and metaphases 'delayed' by a 2 hour MG-132 treatment (+MG-132). Both in control (CTRL) and nocodazole-recovering (NOC) cells, the frequency of merotelic orientations after a 2-hour MG-132 treatment was lower than the frequency before the MG-132 treatment. N, total number of cells analyzed. (B) Frequencies of single and multiple merotelic kinetochore orientations in PtK1 cells recovering from nocodazole, with or without MG-132 treatment.

for the frequency of lagging chromosomes in control or nocodazole-released cells that progressed normally into anaphase (16.3% vs. 1.1% for controls, and 45.8% vs. 16% for nocodazole-released cells; compare Figures 7 and 9).

To test whether spending longer times in metaphase before anaphase onset induces a reduction in the frequency of merotelic kinetochores, we assayed the frequency for cells delayed in metaphase for 2 hours. PtK1 cells were fixed after two hours in 5 μ M MG-132. Alternatively, PtK1 cells were arrested in mitosis by a 3 hour nocodazole treatment and then fixed after 25 minutes recovery from nocodazole plus 2 hours in MG-132. At the end of a 2 hour MG-132 treatment the 'delayed' metaphases analyzed showed only a slight decrease in merotelic kinetochore orientations compared to 'non-delayed' metaphases (12.2% vs. 16.3% in untreated cells; 32.6% vs. 45.8% in cells recovering from nocodazole; Fig. 9A). The frequency of merotelic orientations observed in these 'delayed' metaphases were still much higher than the frequency of anaphase lagging chromosomes (see Fig. 7A for

Table 1. Ratios between the fluorescence intensities of the two microtubule bundles connected to a merotelic kinetochore

	CTRL	MG-132	NOC	NOC+MG-132
<i>n</i>	7	6	52	19
Mean \pm s.d.	1.68 \pm 0.27	2.42 \pm 0.85	1.97 \pm 0.84	2.43 \pm 1.62

n, number of merotelic kinetochores analyzed.
 CTRL, untreated cells; MG-132, cells fixed after 2 hour treatment in 5 μ M MG-132; NOC, nocodazole (cells were fixed after 15-30 minutes recovery after a nocodazole-induced mitotic block); NOC+MG-132, nocodazole (2 μ M) mitotic arrest+25 minutes recovery+2 hour MG-132 treatment.

comparison). The analysis of merotelic orientations in nocodazole-released cells also showed that the delay in anaphase onset produced a similar reduction of merotelic orientations for cells possessing single and multiple merotelic kinetochores (Fig. 9B).

Taken together, the above results show that a second mechanism of error correction functions at or after the onset of anaphase to reduce the number of lagging chromosomes produced by kinetochores that are still merotelically oriented at the metaphase-to-anaphase transition. We do not know how this mechanism functions, but one possible factor is the ratio of microtubules attached to the kinetochore from opposite poles, with a ratio of 1 giving the highest probability and higher ratios showing lower probabilities of lagging chromosomes. To test this possibility, we measured the size of the two microtubule bundles connected to a merotelic kinetochore by measuring the fluorescence intensity of α -tubulin staining (see Materials and methods for details). For each merotelic kinetochore we calculated the ratio between the highest and the lowest intensity. In non-delayed metaphases the average ratio was smaller than 2 for both untreated cells and cells recovering from a nocodazole block (Table 1). After an MG-132-induced delay, the ratio increased to about 2.4 (Table 1). This increase was observed in both control cells (*t*-test, $P < 0.05$) and cells recovering from a nocodazole-induced mitotic block (*t*-test, $P = 0.1$) indicating that the ratio of microtubules from opposite poles is a critical factor in determining whether a merotelic kinetochore produces an anaphase lagging chromosome.

Discussion

Merotelic kinetochore orientation occurs at high frequencies in early mitotic stages

This paper provides for the first time a detailed, quantitative analysis of the frequencies of merotelic kinetochore orientation and its correction in unperturbed mitotic mammalian cells. A major finding of our study is that the frequency of merotelic orientation in early prometaphase is surprisingly high and it is much higher than measured by lagging chromosomes in anaphase as shown in Table S1 (<http://JCS.biologists.org/supplemental/>) and Fig. 10A, where we have converted our data from frequency per cell to frequency per kinetochore, assuming that each cell analyzed possessed the normal complement of 12 pairs of sister chromatids and 24 kinetochores. The values for early prometaphase are likely to be higher than those we calculated from the frequency of lagging chromosomes in precocious anaphase because the

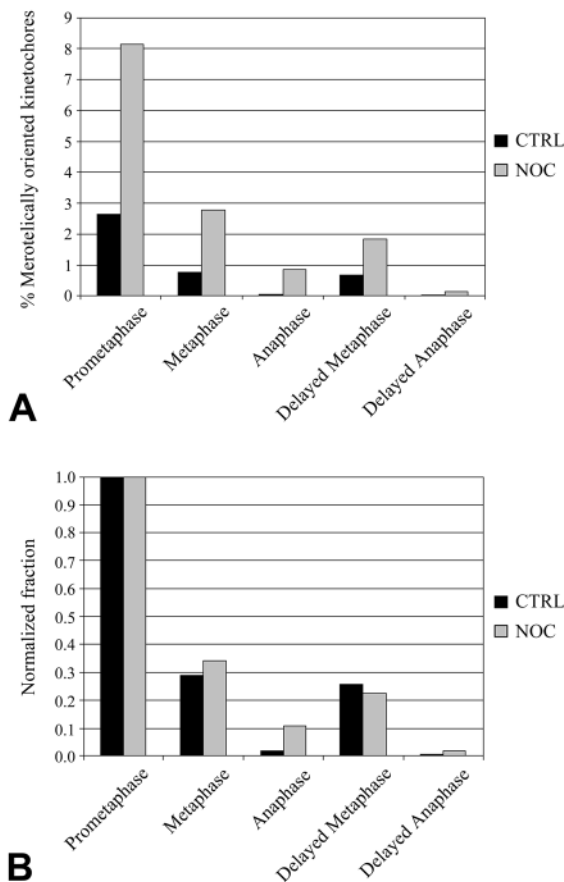


Fig. 10. (A) Frequencies of merotelically oriented kinetochores based on the assumption that every PtK1 cell analyzed had the normal complement of 12 pairs of sister chromatids and 24 kinetochores. (B) Percentage merotelic kinetochores data (shown in A) normalized by values measured in prometaphase.

percentage merotelic kinetochores measured in metaphase, whether normal or delayed, is higher than seen for lagging chromosomes in anaphase. We do not know how much these differences between metaphase and anaphase are applicable to the changes that occur when prometaphase cells are induced precociously into anaphase. Nevertheless, the initial error rate for merotelic kinetochores formation in early prometaphase in control cells is at least 2.65% (Fig. 10A; Table S1 at <http://JCS.biologists.org/supplemental/>).

This high incidence of merotelic orientations during prometaphase suggests that the back-to-back arrangement of sister kinetochores on opposite sides of the centromere does not guarantee their interaction with opposite spindle poles. However, the back-to-back geometry of sister kinetochores probably plays a relevant role in preventing even higher percentages of merotelic kinetochores, since perturbations of the centromeric region that produce single kinetochores associated to single chromatids or chromatid fragments, result in curled kinetochores that become merotelically oriented (Khodjakov et al., 1997; Wise and Brinkley, 1997; Yu and Dawe, 2000).

We also found that prevention of merotelic kinetochores orientation depends critically on establishing two and only two well-separated spindle poles before nuclear envelope

breakdown and spindle assembly. In cells recovering from a nocodazole block, our minimum estimate for the percentage of merotelic kinetochores in early prometaphase is 8.2%, about threefold higher than the minimum estimate for untreated cells (Fig. 10A; Table S1). Several factors may contribute to this enhancement, including the enlargement of the kinetochores outer plate in nocodazole (Cimini et al., 2001; Hoffman et al., 2001), a delay in centrosome separation, and the formation of multiple non-centrosomal centers around the chromosomes after nocodazole wash-out. The presence of multiple microtubule-organizing centers during recovery from a mitotic block had been previously described in different cell lines (De Brabander et al., 1981; Tousson et al., 1991), and a role of such ectopic centers in growing functional microtubules had been suggested by their association with the nuclear protein NuMA (Tousson et al., 1991), a protein that concentrates at functional spindle poles (Compton and Cleveland, 1994). However, the dynamics of mitotic spindle reassembly after nocodazole washout had never been directly related to kinetochores mis-orientation and chromosome mis-segregation. In our study the presence of multiple microtubule organizing centers during the initial stages of spindle reassembly provides an explanation for the fast disappearance of kinetochores crescents and Mad2 by interactions between kinetochores and growing microtubules (Hoffman et al., 2001; Waters et al., 1998). It also suggests that during recovery from the nocodazole-induced mitotic block, single kinetochores can interact with microtubules coming from two microtubule organizing centers that are initially close together and subsequently move apart into opposite spindle poles. This would strongly contribute to the higher incidence of merotelic orientations observed in nocodazole-recovering cells. In support of this conclusion, we have found that 19% of PtK1 cells exhibit one or more lagging chromosomes in anaphase (D. Cimini and E. D. Salmon, unpublished observation) when they are initially treated with the drug monastrol to produce monopolar spindles in prometaphase and then washed free of monastrol to allow spindle bipolarization and subsequent anaphase (Mayer et al., 1999).

It is important to note that our data show that neither a delay in centrosome separation, nor the presence of multiple microtubule organizing centers provides an explanation for merotelic kinetochores orientations in control cells. In normal prometaphase cells, unattached kinetochores can be wider and appear more curved, when seen by conventional electron microscopy, than kinetochores at the spindle equator with a full complement of microtubules (Rieder, 1982; Salmon et al., 1998). We also found that the orientation of the centromere axis through sister kinetochores was highly variable between different chromosomes relative to the spindle interpolar axis in early prometaphase. As shown in Fig. 11A, a curved kinetochores of a chromosome oriented with its inter-centromere axis perpendicular to the interpolar spindle axis will be more likely to capture microtubules from opposite poles, as previously proposed for *Drosophila* meiotic cells (Church and Lin, 1982). Given available information, this mechanism seems the most likely explanation for the high frequency of merotelic kinetochores formation in early prometaphase in control cells. However, another mechanism may also contribute to merotelic attachment. Kinetochores microtubules are infrequently assembled from kinetochores and their minus-ends recruited to the spindle poles (Khodjakov

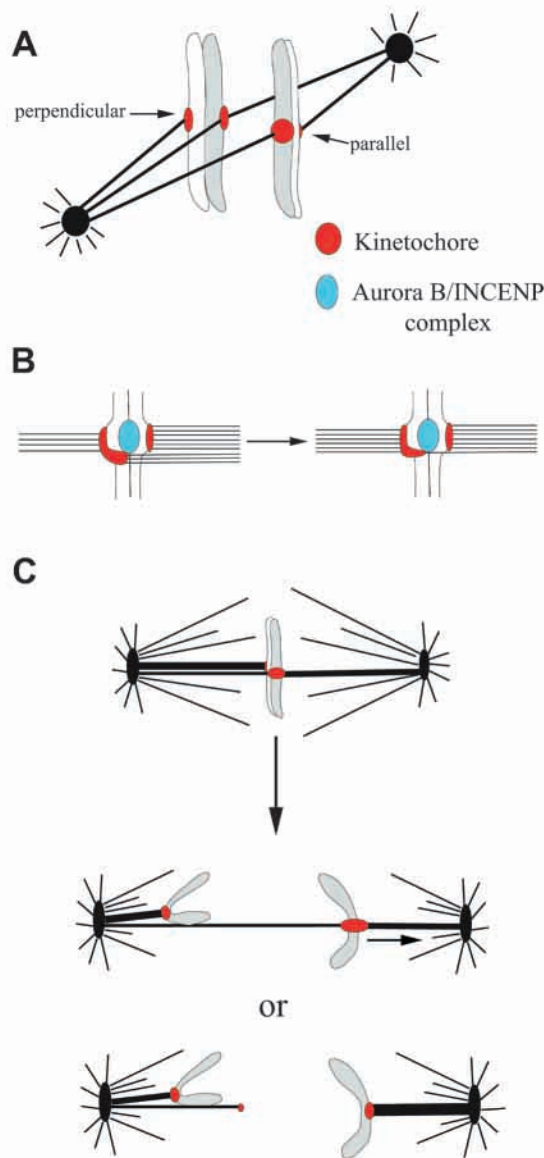


Fig. 11. (A) Diagram showing how merotelic kinetochore orientation might be favored in chromosomes oriented with their centromere axis perpendicular to the interpolar spindle axis. (B) The aurora B/INCENP complex might play a role in merotelic orientation correction because of its proximity to the part of the merotelic kinetochore attached to the incorrect pole. (C) Correct chromosome segregation in the presence of merotelic orientation can occur either because the pulling force in one direction prevails on the force in the opposite direction (middle) or because the pulling forces on the kinetochore induce its breakage (bottom).

et al., 2003). Merotelic orientation might occur if some minus ends are recruited to the wrong pole.

Correction mechanisms substantially reduce the frequency of lagging chromosomes produced by merotelic kinetochore orientation

Our results also identify the existence of at least two mechanisms that prevent anaphase lagging chromosomes during normal mitosis: one that functions before anaphase and

one or more that function at or after anaphase onset. To see better the dynamics of these correction mechanisms, we normalized the percentage merotelic kinetochore data (Fig. 10A) for both untreated and nocodazole treated cells by values measured in early prometaphase (Fig. 10B). The first information that we obtain from this normalization is that in general the major effect of nocodazole is the promotion of merotelic kinetochore orientation, and not the suppression of correction, this is particularly true for correction mechanisms operating before anaphase onset. The second significant information is that with or without nocodazole treatment, delaying metaphase before anaphase onset did not substantially decrease the percentage merotelic kinetochores, but did decrease substantially the incidence of lagging chromosomes in anaphase. This suggests that the correction mechanism operating before anaphase onset not only reduces the number of detectable merotelic kinetochores, but also reduces the ability of those persisting until anaphase to produce lagging chromosomes.

The obvious hypothesis for the mechanism operating before anaphase onset is that it depletes merotelic kinetochores of microtubules from the incorrect pole while enhancing attachment to microtubules from the correct pole. When a kinetochore is merotelically oriented and aligned at the metaphase plate, part of it is pulled in the same direction as its sister (towards the incorrect pole) and part of it is pulled in the opposite direction (towards the correct pole) (Fig. 11B). This orientation might in some cases resolve itself simply through the instability of kinetochore microtubules, which have been shown to have a half-life of about 5 minutes in metaphase (Cassimeris et al., 1990; Shelden and Wadsworth, 1996; Zhai et al., 1995). If the kinetochore loses attachment to microtubules to the incorrect pole, that part of the kinetochore will have a tendency to face the correct pole and so attachments to the correct pole will be favored. Furthermore, the chromatin might shield the kinetochore, thus preventing re-attachment to microtubules from the incorrect pole. However, the correction of merotelic orientations might depend on other factors, rather than simply on the stochastic detachment and re-attachment process that characterizes kinetochore microtubules in prometaphase/metaphase. For example, the tension at each attachment site might play an important role. Work on grasshopper spermatocytes has shown that elevated tension at kinetochores stabilizes microtubule attachment (King and Nicklas, 2000; Nicklas and Ward, 1994). The force on the correctly attached side of a merotelic kinetochore is greater than at its sister kinetochore because of the force produced by the part of the merotelic kinetochore attached to the wrong pole. This makes microtubules attached to the correct pole more stable and consequently less likely to detach than microtubules to the incorrect pole. Finally, the Aurora B-INCENP complex may also have a role in promoting loss of incorrectly attached microtubules. This complex has been proposed to promote microtubule destabilization at kinetochores not under tension (Stern, 2002; Tanaka et al., 2002) and depletion of either Aurora B or INCENP have been found to produce lagging chromosomes both in *C. elegans* (Kaitna et al., 2000) and *Drosophila* embryos (Adams et al., 2001). Merotelic kinetochore orientation may reduce centromere stretch, as suggested by a shift of merotelic kinetochores closer to the equator than normal (Fig. 8).

Furthermore, the part of the merotelic kinetochore incorrectly attached is pulled back toward the centromeric region and closer to the inner centromere where the Aurora B-INCENP complex is localized (Adams et al., 2001; Canman et al., 2002b), whereas the part attached to the correct pole is pulled away from the centromeric region (Fig. 11B). In this way, the Aurora B-INCENP complex could destabilize attachment of microtubules from the incorrect pole. In fact, there is evidence in both yeast and higher organisms that Aurora B functions to destabilize microtubule attachment and promote re-orientation (Shannon and Salmon, 2002).

We found that anaphase correction plays a major role in preventing chromosome mis-segregation, since more than 90% of merotelic orientations present in late metaphase control cells do not produce a lagging chromosome in anaphase (Fig. 10B, Table S1). Previous studies of merotelic kinetochores on damaged or unreplicated chromosomes showed that only a fraction remained near the spindle equator (Khodjakov et al., 1997; Wise, 1999; Wise and Brinkley, 1997; Yu and Dawe, 2000). Our results are the first evidence that uncompromised kinetochores exhibit similar behavior. Microtubule attachment to kinetochores is more stable after anaphase onset than before anaphase (McEwen et al., 1997; Nicklas, 1983; Sheldon and Wadsworth, 1996; Zhai et al., 1995). The Aurora B-INCENP complex leaves the centromere at anaphase onset (Adams et al., 2001; Canman et al., 2002b), so it is likely that the 'anaphase' correction mechanism is Aurora B-INCENP-independent.

Our data suggest that the potential of a merotelic kinetochore to produce a lagging chromosome in anaphase decreases as the ratio of correct to incorrect microtubules increases. This hypothesis is supported by our findings that prolonging metaphase did not substantially decrease the number of merotelic kinetochores at metaphase, but increased the ratio of microtubules to opposite poles and substantially reduced the frequency of anaphase lagging chromosomes (Fig. 10, Table 1). A higher ratio of correct to incorrect microtubules might produce segregation of a merotelic chromosome to the pole in two ways. One is kinetochore breakage (Fig. 11C, bottom), because of the high degree of lateral stretching (Cimini et al., 2001), which, beyond a certain point, might break the kinetochore. The other is that the chromosome could move to the pole with the greater number of microtubules although remaining attached to both poles (Fig. 11C, middle). Although poleward movement of merotelic kinetochores does not occur before anaphase (Cimini et al., 2002), after anaphase onset the direction of chromosome movement might become strongly dependent on the ratio of microtubules going to opposite poles. This might depend on changes in kinetochore function, sister chromatid separation, loss of polar ejection forces on chromosome arms (Funabiki and Murray, 2000; Levesque and Compton, 2001) and strong pulling forces on kinetochore microtubules produced by spindle elongation (anaphase B).

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