Pericentromere tension is self-regulated by spindle structure in metaphase

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During cell division, a mitotic spindle is built by the cell and acts to align and stretch duplicated sister chromosomes before their ultimate segregation into daughter cells. Stretching of the pericentromeric chromatin during metaphase is thought to generate a tension-based signal that promotes proper chromosome segregation. However, it is not known whether the mitotic spindle actively maintains a set point tension magnitude for properly attached sister chromosomes to facilitate robust mechanochemical checkpoint signaling. By imaging and tracking the thermal movements of pericentromeric fluorescent markers in Saccharomyces cerevisiae, we measured pericentromere stiffness and then used the stiffness measurements to quantitatively evaluate the tension generated by pericentromere stretch during metaphase in wild-type cells and in mutants with disrupted chromosome structure. We found that pericentromere tension in yeast is substantial (4–6 pN) and is tightly self-regulated by the mitotic spindle: through adjustments in spindle structure, the cell maintains wild-type tension magnitudes even when pericentromere stiffness is disrupted.

Results and discussion

In vivo characterization of pericentromere stiffness in yeast

To characterize pericentromere tension, it was first necessary to quantitatively characterize pericentromere stiffness. We measured stiffness by tracking the movements of pericentromeric fluorescent tags that occur as a result of thermal energy in the cell (Alexander and Rieder, 1991; Mickey and Howard, 1995; Marshall et al., 1997, 2001; Poirier et al., 2002; Levi et al., 2005). Here, thermally driven movements of a fluorescent pericentromeric tag will be large if the pericentromere is soft, whereas stiffer pericentromeres will lead to smaller displacements of the pericentromeric tag over time (Video 1).

In the work described here, we used an imaging-based approach to make quantitative estimates of pericentromere stiffness. These estimates were then used to evaluate pericentromere tension in wild-type (WT) cells and in mutant cells with disrupted pericentromere structure (Fig. 1 A, $F_{\text{tension}}$). We found that pericentromere tension in yeast is substantial (4–6 pN) and is tightly self-regulated by the mitotic spindle: the cell maintains WT tension magnitudes even when the pericentromere stiffness is disrupted. Our results suggest that maintenance of a metaphase tension set point may be an important characteristic of mitosis.
energy (in $k_B T$) to the maximum mean-squared displacement (MSD) of the fluorescent pericentromeric tag:

$$\kappa = \frac{k_B T}{<\sigma^2>}.$$  \hspace{1cm} (1)

Here, $\kappa$ is the spring constant, which in our assay represents the stiffness of the metaphase pericentromere, $k_B$ is Boltzmann’s constant, $T$ is the absolute temperature (in Kelvin), and $<\sigma^2>$ is the maximum MSD of the pericentromeric tag (Svoboda et al., 1993; Mickey and Howard, 1995; Kamiti and van De Ven, 1996; Bustamante et al., 2000).

We applied this approach in budding yeast metaphase spindles ($Saccharomyces cerevisiae$), by using a lacI (lac repressor)-GFP–expressing strain with 33 lacO (lac operon) repeats inserted 1.1 kb 3’ to CEN3 (Pearson et al., 2001). During metaphase in budding yeast, the pericentromeric lacO/lacI-GFP arrays are readily apparent as two separated spots, which we will refer to as lacO spots (Fig. 1 B, 1, green). In addition, red Spc110-mCherry–labeled spindle poles were used to specifically select in-plane metaphase mitotic spindles for analysis (Fig. 1 B, 1, red).

The movements of the green lacO spots were observed by collecting time-lapse videos of metaphase spindles (Fig. 1 B, 2). However, the movements of the lacO spots can only be used to quantitatively assess pericentromere stiffness ($\kappa$) if the lacO spot movements are caused by thermal energy alone: movements of the lacO spots that originate from kMT dynamics or from ATP-driven motor forces do not quantitatively reflect pericentromere stiffness. This is because the equipartition theorem (Eq. 1) relates lacO spot movements to pericentromere stiffness ($\kappa$) through the factor $k_B T$, which exclusively represents the thermal energy available in the cell. Therefore, to stabilize kMT and motor-driven dynamics over the time scale of the experiment, cells were treated with stabilizing drugs (azide ± deoxyglucose or low-dose benomyl), and/or rapid time-lapse videos were collected (32 frames per second [fps] and 48 fps). We predicted that even without the drug treatments, the movements of the lacO spots in the 32 and 48 fps videos would reflect thermal fluctuations because movements originating from yeast kMT dynamics and/or motors occur on a much slower time scale than would be apparent using this rapid frame rate (see Materials and methods; Maddox et al., 2000; Sprague et al., 2003; Gupta et al., 2006; Pearson et al., 2006).

Iterative Gaussian mixture model fitting was then used to track the distance between the lacO spots along the spindle axis with subpixel resolution ($\Delta x$; Thomann et al., 2002; Jaqaman et al., 2008), leading to a list of $\Delta x_i$ values corresponding to increasing time points in each video (Fig. 1 B, 2 and 3). To estimate the displacements of a single lacO spot, (a) $\Delta x_i$ values were divided by $\sqrt{2}$ (i.e., $s_i = \Delta x_i/\sqrt{2}$), which is equivalent to halving the

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**Figure 1.** In vivo pericentromere stiffness measurement. [A] Cartoon of yeast metaphase spindle denoting pericentromere location and tension. [B, 1] Gaussian-filtered experimental yeast metaphase spindle with fluorescent tags: LacO/lacI-GFP (lacO spots) and Spc110-mCherry (poles). Bar, 1 µm. [2] lacO spots were tracked using Gaussian mixture model fitting (blue lines). [3] The distance between the lacO spots $\Delta x$ over time. [4] Estimation of single spot motion and drift correction. [5] Eq. 2 converts $R_i$ values to MSD, and then, the maximum motion $<\sigma^2>$ is estimated. Error bars = SEMs.
The pericentromere stiffness estimates were statistically indistinguishable regardless of growth media, drug treatment, fast-imaging frame rate, or lacO spacing distance (at 32 fps: synthetic designed [SD] vs. azide, P = 0.18; H2O vs. azide, P = 0.11; t test assuming unequal variances; Fig. 2 B and Fig. S1, F–H), which justified the use of live-cell (no drug) fast imaging for our subsequent stiffness measurements. The mean value over all treatments was 15.5 ± 1.3 pN/µm, which is on the order of previously reported chromosome stiffness values that were measured with micromanipulation techniques (decatenated newt mitotic chromosomes ∼50 pN/µm; Kawamura et al., 2010) as well as similar to chromosome stiffness values estimated with viscoelasticity analysis after laser cutting of kMTs in Schizosaccharomyces pombe (∼42 pN/µm; Gay et al., 2012). However, our metaphase value of $\kappa = 16$ pN/µm is substantially higher than in a recent yeast interphase study, likely because the interphase values were measured using slow time-lapse imaging without drug treatments to stabilize active forces (Fig. S1, D and E; Verdaasdonk et al., 2013).

Figure 2. Substantial pericentromere stretching tension in budding yeast metaphase. (A) MSD versus time step size in WT cells (W303 strain background). (B) Mean pericentromere stiffness using different treatments. az = azide. (C) Estimating pericentromere rest length using nocodazole (see Materials and methods). Bars, 1 μm. (D) lacO spot separation distances in live cells (n = 390). (E) Pericentromere tension (n = 390). Error bars = SEMs.

Here, $t_{step}$ is the time step size between image frames in the movies, and $n$ is the number of displacements (Michalet, 2010). The MSD values were then plotted for increasing time intervals ($\Delta t$; Fig. 1 B, 5). As is expected for constrained diffusion, the plots of MSD versus $\Delta t$ reached a plateau (Fig. 2 A; Howard, 2001). Using these plots, the maximum MSD value ($<\sigma^2>$) was calculated as $<\sigma^2> = MSD_{plateau} - MSD_1$. To calculate pericentromere stiffness, $<\sigma^2>$ was then substituted into Eq. 1 (Fig. 2 B; see Materials and methods and Fig. S1 for details and extensive discussion of method validations).

The MSDs for the distance between two independently moving lacO spots (Fig. S1 A); (b) first differences were calculated ($d_i$); and (c) $d_i$ values were converted to drift-corrected residuals ($R_i$; Fig. 1 B, 4). Finally, MSDs were calculated for increasing time intervals ($\Delta t$) according to

$$\text{MSD}(\Delta t) = \frac{1}{n} \sum_{j=1}^{n} \left( \sum_{i=1}^{n/\Delta t} R_i \right)^2$$

Here, $t_{step}$ is the time step size between image frames in the movies, and $n$ is the number of displacements (Michalet, 2010). The MSD values were then plotted for increasing time intervals ($\Delta t$; Fig. 1 B, 5). As is expected for constrained diffusion, the plots of MSD versus $\Delta t$ reached a plateau (Fig. 2 A; Howard, 2001). Using these plots, the maximum MSD value ($<\sigma^2>$) was calculated as $<\sigma^2> = MSD_{plateau} - MSD_1$. To calculate pericentromere stiffness, $<\sigma^2>$ was then substituted into Eq. 1 (Fig. 2 B; see Materials and methods and Fig. S1 for details and extensive discussion of method validations).
In vivo metaphase pericentromere tension in budding yeast

We then used the pericentromere stiffness values (\(\kappa\)) to estimate in vivo pericentromere tension in budding yeast (Fig. 1A, \(F_{\text{tension}}\)). This was performed by applying Hooke’s law, which is

\[
F_{\text{tension}} = \kappa(\Delta x - \Delta x_{\text{rest}}),
\]

in which \(F_{\text{tension}}\) is the pericentromere tension, \(\Delta x\) is the lacO spot separation distance, \(\kappa\) is the pericentromere stiffness, and \(\Delta x_{\text{rest}}\) is the “rest” separation distance between lacO spots when \(F_{\text{tension}} = 0\) pN. To estimate \(\Delta x_{\text{rest}}\) for yeast, we treated cells with nocodazole, which depolymerizes kMTs and thus eliminates microtubule-associated pulling forces (see Materials and methods; \(\Delta x_{\text{rest}} \approx \sim 170\) nm; Fig. 2C; Waters et al., 1996; Ribeiro et al., 2009). We measured the range of lacO spot separations (\(\Delta x\)) in live cells (\(n = 387\) cells; Fig. 2D) and then used these values to directly calculate the distribution of pericentromere tension (Fig. 2E). We found that the pericentromere tension was 4.6 ± 0.1 pN in budding yeast (W303 strain background; mean ± SEM). This is nearly three orders of magnitude higher than is predicted for random thermal forces (\(F_{\text{thermal}} = \sim 0.01\) pN; see Materials and methods), suggesting that pericentromere tension could provide an important mechanical signal during mitosis.

Similar previously published in vivo mitotic and meiotic force measurements are summarized in Table S1. Here, measured forces ranged from 0.2 to 75 pN/kMT, with a mean value over all observations of \(\sim 16\) pN/kMT. The measurement that is perhaps closest to our work was performed in meiotic grasshopper spermatocytes, in which pericentromere tension was evaluated by measuring the extent of chromosome elongation during prometaphase (Nicklas, 1988). Here, tension was measured as \(\sim 7\) pN/kMT, which is similar to our pericentromere tension value in yeast of \(\sim 5\) pN/kMT. We note that larger estimated forces were generally required to halt microtubule-mediated chromosome motion (Table S1), suggesting that the maximum kMT depolymerization force (Grishchuk et al., 2005; Volkov et al., 2013) may be substantially higher than the mean pericentromere tension. This may be because large inwardly directed tension could overpower Kinesin-5–mediated outwardly directed spindle forces, leading to spindle collapse and, ultimately, a drop in pericentromere tension (Bouck and Bloom, 2007).

Evidence for maintenance of a set point tension magnitude in yeast

Our results suggest that pericentromere tension could provide an important mechanical signal during metaphase. Therefore, it may be that the cell maintains a robust tension signal even in the presence of chromatid stiffness alterations, as could naturally occur as a result of stochastic variations in chromatin packaging (Moser and Swedlow, 2011). We tested this hypothesis using \(top2-4\) cells, which have altered chromatin structure (DiNardo et al., 1984; Warsi et al., 2008). Thus, we performed the MSD analysis in isogenic strain background WT and \(top2-4\) mutant cells (15D strain background; Fig. 3, A and B). We found that the WT 15D strain had a similar stiffness to the WT W303 strain (\(\kappa_{\text{WT,15D}} = 14.2 \pm 1.4\) pN/µm and \(\kappa_{\text{WT,W303}} = 13.9 \pm 1.0\) pN/µm; both H2O at 32 fps). In contrast, pericentromeres in the mutant \(top2-4\) cells were nearly twofold less stiff than in WT cells (\(\kappa_{\text{top2-4}} = 7.5 \pm 0.7\) pN/µm; Fig. 3C).

We then calculated and compared pericentromere tension in WT and \(top2-4\) metaphase spindles. Here, Eq. 3 (Hooke’s law) was used to calculate tension, which depends on both the stiffness (\(\kappa\)) as well as the net pericentromere stretch (\(\Delta x - \Delta x_{\text{rest}}\)), such that an increase in stretch could potentially compensate for softer stiffness to maintain tension. As was previously reported, we found that lacO spots were indeed farther apart in \(top2-4\) cells as compared with WT cells (\(P < 0.001, t\) test; Fig. 3D). Importantly, because of the decreased \(top2-4\) stiffness, this corresponding increase in stretch resulted in tension magnitudes that were statistically indistinguishable between WT and \(top2-4\) cells (\(F_{\text{tension, WT,15D}} = 5.8 \pm 0.24\) pN and \(F_{\text{tension, top2-4,15D}} = 5.4 \pm 0.13\) pN; \(P = 0.12, t\) test; Fig. 3E). Thus, even though stiffness was reduced by approximately twofold in the \(top2-4\) mutant relative to WT, tension was similar in both cases (Fig. 3E). In addition, analogous tension maintenance was observed in \(bub1\Delta\) cells, which displayed a more moderate pericentromere stiffness phenotype (Fig. S2). Together, these results suggest that pericentromere tension is regulated by the spindle during mitosis to maintain a set point tension level (Maresca and Salmon, 2009).

Pericentromere tension is regulated by changes in spindle structure

Because our results suggested that the metaphase spindle acts to maintain a set point tension, we then explored possible mechanisms for how cells could maintain WT tension in the presence of altered pericentromere stiffness. By imaging cells with labeled spindle poles (SPC110-mCherry) and kinetochores (Nuf2-GFP; Fig. 4A), we found that cells compensate for softer pericentromeres by altering spindle structure: spindle lengths were increased by 18% in \(top2-4\) cells as compared with WT cells (Fig. 4B), and kMTs were 20% shorter (Fig. 4C). Therefore, the net effect of a simultaneous increase in spindle length and a decrease in kMT length is that a set point WT tension is maintained even with a significant disruption in pericentromere stiffness (Fig. 4D).

Because kMTs were shorter in the presence of softer \(top2-4\) chromatin, we then asked whether dynamic kMTs were required for tension regulation in yeast. This was performed by treating WT and \(top2-4\) spindles with low-dose benomyl, which has been previously reported to stabilize kMT dynamics (Fig. 4E; Pearson et al., 2003). We found that tension regulation was not efficient in \(top2-4\) spindles treated with benomyl: the mean tension in benomyl-treated \(top2-4\) spindles was 27% lower than in benomyl-treated WT cells (Fig. 4F, red arrows, \(P < 0.0001\)). In contrast, the mean tension was statistically indistinguishable between untreated WT and \(top2-4\) cells (Fig. 4F, blue arrows, \(P = 0.12, t\) test). Importantly, the difference in spindle lengths between strains was similar in controls and in benomyl treatment (Fig. 4B and G), meaning that the loss of tension regulation in benomyl-treated \(top2-4\) cells was specifically attributable to stabilization of kMT dynamics (Fig. 4C, H, and I). We conclude that dynamic kMTs are required for tension regulation in yeast.
Ftension = \kappa_{\text{sim}}(\Delta x - \Delta x_{\text{rest}}) \quad (\Delta x = \text{distance between simulated sister kinetochores, and } \kappa_{\text{sim}} = \text{pericentromere stiffness}; \text{Fig. 5 A}).

Initially, WT simulations were run to obtain agreement between WT experimental and simulated images (see Materials and methods). The WT simulation value for pericentromere stiffness was thus established as \kappa_{\text{sim},\text{WT}} = \kappa_{\text{exp},\text{WT}} = 14.2 \text{ pN/\mu m} (Fig. 5 B). Then, the simulations were used to test hypotheses for tension regulation in yeast. We first asked whether increasing the WT-simulated spindle lengths to match the experimentally observed \textit{top2-4} spindle lengths (i.e., without changing \kappa_{\text{sim}}) would be sufficient to reproduce the experimental \textit{top2-4} kinetochore distribution. However, these simulations did not recapitulate the \textit{top2-4} experimental results (Fig. 5 C), suggesting that a passive whole-spindle force balance (leading to longer spindle lengths in \textit{top2-4} mutants) is insufficient by itself to explain tension regulation.

Simulations predict that tension-dependent kMT dynamics can explain tension regulation

It has been previously proposed that kMT dynamics are regulated by tension (Gardner et al., 2005; Asbury et al., 2006; Ribeiro et al., 2009; Akiyoshi et al., 2010). Thus, a possible mechanism for tension regulation is that kMT dynamics are responsive to pericentromere stiffness changes, so that kMTs would maintain proper tension by self-adjusting their lengths. To test whether tension-dependent kMT dynamics can explain pericentromere tension regulation, we performed metaphase spindle simulations in which each kMT “sensed” tension (Gardner et al., 2005). Here, the probability of a kMT rescue event (i.e., a switch from shortening to growing) increased with increasing pericentromere tension, in which tension was calculated as

\[ F_{\text{tension}} = \kappa_{\text{sim}}(\Delta x - \Delta x_{\text{rest}}) \]
stiffness and thus act to maintain a set point pericentromere tension during metaphase.

In addition to tension-dependent kMT dynamics, the balance of forces in the spindle between outward force-generating molecular motors and passive inwardly directed pericentromere stretch also likely contributes to tension regulation, primarily through changes in total spindle length. However, although it is clear that this type of passive response tension regulation mechanism (i.e., through a whole-spindle force balance) could respond

tension regulation. In contrast, simulations in which the pericentromere stiffness was reduced to a value that was similar to the experimentally measured top2-4 value ($k_{sim, top2-4} = \sim 6.5$ pN/µm) produced a much better fit to the top2-4 kinetochore distributions, both in simulations that used WT spindles lengths (Fig. 5 D) and in simulations with the top2-4 spindle lengths (Fig. 5 E). Therefore, from the simulations, we conclude that tension-dependent kMT dynamics provide a simple, robust mechanism to explain how kMT lengths could self-adjust to changes in pericentromere stiffness and thus act to maintain a set point pericentromere tension during metaphase.

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compensate for heterogeneity in stiffness across different chromosomes in the spindle or to compensate for stiffness changes in an individual chromosome over time (Chacón and Gardner, 2013; Stephens et al., 2013). It has long been suggested that mechanical tension at the kinetochore could modulate kMT to global changes in chromosome stiffness, it is less clear that this mechanism could compensate for stochastic variation in individual chromosome stiffness (Moser and Swedlow, 2011). In contrast, tension-dependent kMT dynamics could effectively regulate tension for each individual chromosome pair, to compensate for heterogeneity in stiffness across different chromosomes in the spindle or to compensate for stiffness changes in an individual chromosome over time (Chacón and Gardner, 2013; Stephens et al., 2013). It has long been suggested that mechanical tension at the kinetochore could modulate kMT
stability inside of cells (Nicklas, 1988; Skibbens et al., 1993, 1995; Rieder and Salmon, 1994, 1998; Inoué and Salmon, 1997; Skibbens and Salmon, 1997; Maddox et al., 2003; Cimini et al., 2004; Ribeiro et al., 2009). Thus, we propose that yeast kMTs sense tension, such that high forces promote net kMT polymerization, whereas low forces promote net depolymerization. As demonstrated by our simulations, this effect naturally leads to shorter kMTs in the presence of softer pericentromeres, as is experimentally observed for the yeast top2-4 mutants, and thus provides a physically reasonable explanation for tension regulation in yeast metaphase.

**Conclusions**

In this work, we used a novel imaging-based method to measure pericentromere stiffness during metaphase and then applied our stiffness measurements to determine the magnitude of pericentromere tension in budding yeast. We found that the cell compensates for reductions in stiffness: the spindle length was increased, whereas the mean kMT length was decreased, to restore WT pericentromere tension in cells with reduced pericentromere stiffness (Fig. 5 F). This suggests that tension is regulated by the integrated dynamics of spindle length and kMT length during mitosis to achieve precise regulation of metaphase tension. This process could act to prevent transient reactivation of the spindle checkpoint during metaphase and thus ensure a timely anaphase onset.

**Materials and methods**

**Yeast strains and imaging**

Yeast strains are shown in Table S2. For imaging of the yeast cells, metaphase cells were imaged using a microscope (Eclipse Ti; Nikon) using 488- and 561-nm lasers. To optimize the signal to noise ratio in our assay, we used pseudo-total internal reflection fluorescence (TIRF; pseudo-TIRF) microscopy of cells that were adhered to coverslips in a flow chamber (i.e., TIRF microscopy but with the laser angle adjusted to increase evanescent field depth). A rapid switching FireWire setup allowed for near-simultaneous TIRF microscopy but with the laser angle adjusted to increase evanescent phase cells were imaged using a microscope (Eclipse Ti; Nikon) using Yeast strains and imaging

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To measure \( \Delta \text{MSD}_{\text{base}} \), cells treated with nocodazole were continuously imaged (no delay) until complete bleach in the green (lacO) channel. Total magnification was 64 nm/pixel. We then used freely available ImageJ plugins (National Institutes of Health) to analyze the image stacks, as previously described (Burnette et al., 2011): (a) to correct drift in the images, we used the translational setting of StackReg, (b) bleaching/blinking off events and blinking on events were identified with the Delta F down setting and Delta F up setting, respectively, (c) bleaching-assisted localization microscopy molecules were localized, and (d) image reconstructions were rendered by using QuickPALM. Single molecule localization was estimated by measuring dot locations in the final rendered images.

Pericentromere stiffness calculation method

To calculate yeast chromatin stiffness, we found the MSD of the lacO/lacI-GFP tags and then applied the equipartition theorem formula (Eq. 1), as follows (Fig. 1): (a) We collected time-lapse videos of metaphase yeast cells at the specified time interval while holding the stage at a constant temperature. (b) We found the distance between the lacO spots using our custom MATLAB script as described under Image analysis and then divided this distance by \( \sqrt{T} \) to represent the motion of a single lacO spot. (c) We then calculated first differences and removed the effect of drift by fitting a line through a plot of the first differences over time for each cell. Residuals from this fit were used for the remainder of the analysis. In most cases, the slope of the line was not significantly different than 0, implying that little drift occurred. (d) To find the MSD for the cells within one treatment, we pooled the residuals from all cells. Next, we calculated the MSD using Eq. 2. We verified that pooling information across cells did not affect our results by randomly reordering the cells in the pool and then by verifying that the results were consistent regardless of order.

For the fast imaging experiments, our MSD plots typically achieved a plateau by \( T = 0.5 \text{ s} \). Therefore, we estimated the MSD plateau value by averaging the MSD values from \( T = 1 \text{ s} \) to \( T = 2 \text{ s} \). Some cells did not appear to plateau or had plateaus that were large outliers. These cells were identified by (a) finding the MSD plateau value from each individual cell, (b) making a histogram of the plateau MSD values from all cells, and then (c) fitting a two-mixture Gaussian model to this histogram for each treatment. In every case, the two-Gaussian mixture fit better than a single Gaussian, and there was usually a clean separation between the peaks. We discarded cells whose plateau was >2 standard deviations past the first peak, which when compared with the individual cell MSD plots, was indicative of an unstable maximum or a failure of the drug treatments.

The stiffness of an elastic polymer dictates the maximum amount a probe bound to the polymer is able to move at a particular temperature. The maximum MSD (\(<\text{cor}^2>\)) is the plateau in our MSD plots minus the intercept. Intuitively, an object cannot move in zero time, so any nonzero motion estimate at the intercept is erroneous (Michalet, 2010). In this study, we used the first MSD point as an estimate for the intercept, which is a combination of noise in the images, imperfect Gaussian fitting, and movement of the GFP tags during the time the shutter is open. This noise correction explains why some plateaus may appear similar (Fig. S2 B) and yet have different stiffness estimates (i.e., the lacO spots would “bump” into the kinetochore), artificially inflating our stiffness estimates. Here, there would be a higher stiffness estimate near the kinetochore barrier, whereas the stiffness away from the kinetochore would be represented by the movement of tags that are unconstrained by bumping into a barrier (Verdaasdonk et al., 2013). However, for our metaphase measurements, we stabilized the motor and microtubule dynamics over the time course of the experiment, the total observed displacements were very small. Therefore, it is unlikely that our metaphase lacO displacements were large enough to bump the tether point at the kinetochore. Regardless, because we measured 1D lacO distances along the spindle axis, we expect that if the lacO spot movements were indeed restricted in one direction by the proximity of the lacO marker to the kinetochore, a statistically significant skew would be present in the lacO displacement data. This is because displacements toward larger lacO spacings would be limited by the presence of the kinetochore.
and so the distribution would be cut off at larger displacements, skewing the distribution of lacO displacements toward smaller lacO spacings. We tested this by calculating the skew in our largest dataset, the W303 WT cells imaged in H2O (Fig. S1 C), and found that the data do not significantly deviate from a normal distribution when assessed using a D’Agostino skewness test (residuals: \( P = 0.22 \); raw lacO spacing data: \( P = 0.88 \)). We conclude that by eliminating the effects of motor and microtubule dynamics via drugs and fast imaging, and also by ensuring that the motions of the lacO spot were not restricted by the proximity of the kinetochore-attached microtubule tip, we have eliminated potential position-dependent artifacts of the stiffness measurement in our study.

**Spindle axis positioning of lacO spots.** The lacO spots were generally well in line with the mCherry-labeled spindle poles, and no consistent spindle offset was observed. Regardless, because we measured the fluctuations in lacO spacing along the spindle axis, we expect that stiffness along the spindle axis would still be reflected even if the lacO spots were contained in an off-axis chromatin loop. Specifically, if the lacO spots were off axis, their along-axis spacing fluctuations would still reflect along-axis thermal fluctuations of the pericentromere because the tag would travel along with fluctuations in the pericentromere itself. Therefore, regardless of whether the tags were directly in line with the spindle axis or slightly offset, as long as they are associated with the pericentromere, their motion as a result of thermal forces will be dictated by the mean stiffness of the pericentromere.

**Estimating single spot motion from the distance between both lacO spots.**

Division of the lacO spot separation distance by \( \sqrt{2} \) to estimate single spot motion was justified as follows: (a) by generating simulated data of two independently moving lacO spots, we demonstrated that halving the MSD of the distance between the two spots i.e., dividing the \( \Delta x \) displacement by \( \sqrt{2} \) was equivalent to estimating the MSD of each single spot (Fig. S1 A), and (b) by calculating cross-correlation between individual lacO spot positions, we demonstrated that the experimental sister lacO spot movements were independent and uncorrelated (Fig. S1 B), as is required for estimation of single spot MSDs from the \( \Delta x \) displacements.

To demonstrate that the MSD of each individual lacO spot can be estimated by dividing lacO spacing displacements (\( \Delta x \)) by \( \sqrt{2} \), we simulated 10^4 time steps of constrained diffusion of two independent spots, which were centered at different locations but had the same diffusion coefficients and level of constraint. We then calculated \( \Delta x/\sqrt{2} \). Plots of these are shown in Fig. S1 A (left). The MSD curve of \( \Delta x/\sqrt{2} \) is similar to the individual spot curves, which demonstrates that the distance between the spots can be used to estimate the mean single spot motion (Fig. S1 A, right).

To demonstrate that the experimental sister lacO spot movements were independent and uncorrelated, we calculated cross-correlation of lacO spot locations in the same cell and across cells (Fig. S1 B). The lacO spot motions are similarly uncorrelated in each case, demonstrating that sister lacO spot motions are uncorrelated.

**Linearity of pericentromere stiffness**

We also asked whether our measured pericentromere stiffness values depended on the separation distance, or amount of pericentromere stretch, between sister lacO spots. To perform this analysis, we noted that the lacO spot separation distances used for the MSD analysis varied from 400 to 800 nm (Fig. S2 F). We compared the stiffness of three groups that were distinguished by increasing lacO spot separation distances using the MSD analysis (Fig. S2 G) and found no statistically significant change in stiffness between the groups of cells with the smallest and largest lacO spot separation distances (\( P = 0.11 \) for fast time-lapse data of untreated cells imaged in water; Fig. S2 H). Consistent with this result, the force-extension curves of chromosomes pulled with needles were also linear over biologically relevant forces (Pandey and Koch, 1969; Kawamura et al., 2010). However, because there is a trend in the direction of lower stiffness at higher amounts of stretch, we also calculated tension forces using the specific stiffness values for each lacO spot separation distance (Fig. S2 I). The mean tension was not significantly altered by this approach (4.5 vs. 4.6 pN), and therefore, we conclude that the use of a mean stiffness is a reasonable approximation for our study.

**Statistical analyses**

To compare stiffness between treatments, we conducted two-sided \( t \) tests using Welch’s \( t \) test assuming unequal sample size and unequal variance. Variance and degrees of freedom were determined as described under Pericentromere stiffness calculation method. We only conducted one \( t \) test to determine whether WT yeast stiffness changed as a function of chromatin stretch (Fig. 1 F), because the greatest difference was not significant, we did not conduct further tests and therefore did not need to correct for multiple comparisons. Two-sided \( t \) tests using Welch’s \( t \) test assuming unequal sample size and unequal variance were also used to compare tension, spindle length, and kMT length between treatments. To examine how the effect of benomyl treatment on tension or kMT length may be different depending on genotype, we conducted a two-way analysis of variance. To determine whether there was significant skew in histograms of residual values, we used the D’Agostino test implemented in the moments package in R. Analyses were conducted in R.

**Estimation of thermal forces in metaphase spindles**

To calculate the thermal tension value for yeast spindles, we assumed that the total energy available to an individual pericentromere during mitosis was \( \Delta x kT \). This amount of energy is equal to \( -4 \) pN-nm. Therefore, taking into account a mean stretch distance in yeast of \( \Delta x = 499 - 170 = 329 \) nm, the expected \( F_{\text{tension}} \) as a result of thermal energy alone would be \( F_{\text{tension, thermal}} = 4/329 \approx 0.01 \) pN.

**Simulation methods**

The computational simulation used for mitotic kMT dynamics was based in large part on that described in Gardner et al. (2005). The model was composed of 16 oppositely oriented pairs of dynamic kMTs within a preset spindle length (\( x \) axis), and whose minus-end-\( x \) positions were randomly chosen within a circle of radius of 125 nm, to simulate spindle poles. The kMTs’ behavior was dictated by three rules. First, individual kMTs were governed by dynamic instability parameters, such that kMT growth and shortening occurred at a rate of \( 2 \mu \text{m/min} \), and the basal catastrophe frequency (switch from growth to shortening) and rescue frequency (switch from shortening to growth) were 0.25 and 0.14 \( \text{s}^{-1} \), respectively. The second rule affected how the catastrophe frequency changed with the length of the kMT. In the previous model, catastrophe frequency increased with a nonlinear gradient toward the spindle midzone (Gardner et al., 2005), but more recent work suggests that catastrophe frequency increase linearly with kMT length (Gardner et al., 2008). Therefore, the catastrophe frequency of a specific growing kMT was determined by \( k_c = k_{c,0} + \alpha L_{\text{MT}} \), in which \( k_c \) was the catastrophe frequency (seconds \(^{-1} \)), \( k_{c,0} \) was the basal catastrophe frequency (seconds \(^{-1} \)), \( L_{\text{MT}} \) was the kMT’s length (micrometers), and \( \alpha \) was a free parameter with units of micrometers \(^{-1} \text{s}^{-1} \), which established how relating how kMT length affected catastrophe.

The final rule in the model dictated how tension caused by stretching of elastic chromatin between paired kMTs affected the kMT’s chance of rescue from a shortening state. Gardner et al. (2005) assumed that tension modifies the baseline chance of rescue by \( k_R = k_{R,0} e^{-F_{\text{tension}}/F_0} \), in which \( k_R \) was the rescue frequency (seconds \(^{-1} \)), \( k_{R,0} \) was the basal rescue frequency (seconds \(^{-1} \)), and \( \Delta x \) was the amount by which the chromatid was stretched (micrometers). \( k_* = k_c/F_0 \), which incorporates both the experimentally measured stiffness of the pericentromere \( k \) (piconewton/micrometers) as well as \( F_0 \) (piconewtons), which is the characteristic force at which the chance of rescue increases e-fold.

To constrain the free parameters in the model that are not of direct interest \( (k_R \) and \( F_0 \) ), we created simulated spindle images from our models and compared these directly to experimental data. First, we simulated spindles with spindle lengths equal to those measured in vivo using WT cells (same WT data as in Fig. 4, B and C). At the end of the simulation, the positions of the plus ends of kMTs, as well as the positions of the spindle poles, were convolved using the point-spread function of our microscope, and simulated images were generated from these convolutions (Sprague et al., 2003; Gardner et al., 2010). Simulated fluorescence intensity along the spindle axis was calculated for such models over many values of \( F_0 \) and \( k_R \). We directly compared these simulated fluorescence curves to data from live cells whose kMT plus ends were labeled with GFP (Nuf2-GFP), and whose spindle poles were labeled with mCherry (Spcl10-mCherry). Once we obtained a good fit to WT images, which occurred at \( k_* = 2.75 \text{ pN} \mu\text{m}^{-1} \) and \( \alpha = 120 \text{ pN} \mu\text{m} \), we fixed \( k_* \) to our experimental value \( k_{\text{MT}} = 14.2 \text{ pN} \mu\text{m} \) and therefore held \( F_0 \) and \( \alpha \) as constants (\( F_0 = 5.15 \) and \( \alpha = 120 \)) in subsequent simulations.

Once we found parameter values that led to a reasonable fit between the model and the experimental WT data, we tested the hypothesis that in top2Δ4 cells, softer pericentromeres led to the shortened kMTs that were experimentally observed in vivo. We did this by simulating spindles...
whose spindle lengths matched those observed in top2Δ cells or in WT cells and by varying k, creating simulated images, and finding the best match to the experimental data.

**Online supplemental material**

Fig. S1 provides online data to justify assumptions inherent in the chromosome stiffness calculations and a linear spring analysis. Fig. S2 includes an experiment, which demonstrated that tension self-regulation occurred in a bub1Δ mutant strain. Table S1 provides a summary of in vivo mitotic force measurements. Table S2 provides a list of strains used in this study. Video 1 is an animated cartoon, which demonstrates how thermal fluctuations of lacO spots can be used to estimate metaphase chromosome stiffness (equation therefor). A ZIP file is also provided containing the MATLAB script used to measure distance between lacO spots for stiffness measurements. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201312024/DC1.

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