Deformations Within Moving Kinetochores Reveal Different Sites of Active and Passive Force Generation

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Kinetochores mediate chromosome segregation at mitosis. They are thought to contain both active, force-producing and passive, frictional interfaces with microtubules whose relative locations have been unclear. We inferred mechanical deformation within single kinetochores during metaphase oscillations by measuring average separations between fluorescently labeled kinetochore subunits in living cells undergoing mitosis. Inter-subunit distances were shorter in kinetochores moving toward poles than in those moving away. Inter-subunit separation decreased abruptly when kinetochores switched to poleward movement and decreased further when pulling force increased, suggesting that active force generation during poleward movement compresses kinetochores. The data revealed an active force-generating interface within kinetochores and a separate passive frictional interface located at least 20 nanometers away poleward. Together, these interfaces allow persistent attachment with intermittent active force generation.

Fig. 1. Tracking kinetochore probe pairs. (A) Simplified one-dimensional representation of kinetochore organization (6), with probe positions indicated by stars. (B) Experimental schematic. Kinetochore positions oscillate during metaphase. P denotes movement toward a pole, and AP denotes movement away. Probes are indicated as colored circles. (C) Representative images of EYFP-Cdc20 and mCherry-CenpC at one time point. Scale bar, 5 μm. (D) Enlarged image of the single kinetochore pair indentified in (C). Scale bar, 500 nm. (E) Representative tracks of a single kinetochore [circle-identified in (C)], with direction indicated. Dashed lines indicate direction reversals. (F) Inter-probe distance versus time from the tracks in (E). Dotted lines indicate direction reversals. (G and H) Histograms of all CenpC(N)—Cdc20 distance measurements during metaphase (G) and anaphase (H). The average of the means of P and AP distributions is consistent with the reported CenpC–KNL1 N-terminal distance in fixed cells (53 to 54 nm) (6, 17). (I) Histograms of all CenpC(N)—HeC1(C) distance measurements during metaphase. The noise in single traces and histogram widths (F) to (I) stemmed from both experimental noise (such as centroid determination and two-color registration) and biological variation. Stars indicate histogram distribution means.
mCherry-CenpC [N-terminal label, CenpC(N)] and EYFP-Cdc20, or mCherry-CenpC and Hec1EGFP [C-terminal label, Hec1(C)]. These probes did not perturb metaphase oscillations (17). The Cdc20 probe was brighter than the Hec1 probe and was used for most experiments. We imaged red and green probes simultaneously by means of confocal fluorescence microscopy with a dichroic beam splitter and a single camera. Cells were compressed with an agarose pad so as to keep kinetochores in focus (Fig. 1B), and compression did not perturb oscillations (table S1) (20). Occasionally, drastic compression was used to induce unusually large forces (17). Using a variant (3, 6) of SHREC (single-molecule high-resolution colocalization) (21) in vivo (Fig. 1C), we measured the distance between centroids of the probes (Fig. 1D) every 10 s during several oscillation cycles (Fig. 1E and movies S1 and S2) (17).

We first asked whether P and AP kinetochores were on average different. Graphs of interprobe distance over time for a single kinetochore (Fig. 1F), and a histogram of many kinetochores (Fig. 1G), revealed CenpC(N)–Cdc20 dista nces of 47 ± 20 nm in P kinetochores (n = 525 measurements), and 55 ± 19 nm in AP kinetochores (n = 569 measurements). These values differ with high significance (P = 10−10) (table S2). Of kinetochores imaged, 93% displayed a greater mean inter-probe distance in AP than in P state (fig. S1A). The 15% shorter CenpC(N)–Cdc20 distance in P as compared with AP kinetochores could stem from either a mechanically compliant CenpC(N)–Cdc20 linkage that responds to force or from biochemical changes that relocalize a probe molecule.

To distinguish mechanical from biochemical causes of inter-probe distance change, we first asked whether it also occurred during anaphase, which drastically changes kinetochore biochemistry (22). Anaphase kinetochores are biased toward P motion to segregate chromosomes, but AP transients still occur in PtK2 cells with similar velocities to metaphase (18, 23), probably because of polar ejection forces (17). Inter-probe distances in anaphase were statistically indistinguishable from those at metaphase (P = 0.2 for P, and P = 0.4 for AP). The mean CenpC(N)–Cdc20 distance in anaphase was 49 ± 22 nm in P kinetochores (n = 204 measurements) and 57 ± 20 nm in AP kinetochores (n = 89 measurements) (Fig. 1H and table S2). Just as for metaphase, anaphase P and AP inter-probe distances were statistically different from each other (P = 0.004), and all but one kinetochore displayed a greater mean inter-probe distance in AP than in P state.

Next, we measured the inter-probe distance at metaphase between CenpC(N) and Hec1(C), which is part of the main load-bearing complex, Ndc80. The mean CenpC(N)–Hec1(C) distance was 38 ± 15 nm in P kinetochores (n = 564 measurements) and 43 ± 17 nm in AP kinetochores (n = 487 measurements) (Fig. 1I and table S2). These values differ with high significance (P = 10−3), and their average was consistent with the localiza-

Fig. 2. Distance between sister kinetochores and between probes in one kinetochore during direction reversals. (A) Single kinetochore position (top) and inter-kinetochore distance (bottom) over time for a sister kinetochore pair (that of Fig. 1C). Black stars indicate all direction reversals, and dotted lines indicate direction reversals for the circle-identified kinetochore. (B) Mean kinetochore position, (C) inter-kinetochore distance, and (D) CenpC(N)–Cdc20 distance over time for metaphase P-to-AP (n = 104 traces) and AP-to-P (n = 98 traces) reversals (P in red, AP in blue). AP-to-P reversals are positioned 6 s later than P-to-AP reversals on the time axis so as to reflect the average time between them. Bars indicate SEM. Direction reversals cause abrupt changes in inter-probe distance within a kinetochore, which is consistent with a mechanical response to a change in force.

Fig. 3. Kinetochore velocity and CenpC(N)–Cdc20 distance at different inter-kinetochore distances. (A) Kinetochore velocity as a function of inter-kinetochore distance during metaphase P (n = 104 traces) and AP (n = 104 traces) movement before and after P-to-AP reversals, respectively, when the highest inter-kinetochore distances are visited. P velocity decreases and AP velocity increases with inter-kinetochore distance, as expected if P kinetochores generate force and inter-kinetochore distance is a metric for force. We include data from drastic spindle compression to probe even higher forces (17). (B) CenpC(N)–Cdc20 distance as a function of inter-kinetochore distance during metaphase P (n = 547 measurements) and AP (n = 529 measurements) movement. Inter-probe distance decreases with inter-kinetochore distance in P kinetochores, which is consistent with a force-deformation relationship. Lines are best linear fits.
time axis (Fig. 2, B to D). Inter-kinetochore distance tended to increase during coordinated movement because the leading P kinetochore moved slightly faster (on average) than did its trailing AP sister (Fig. 2A, A to D). P-to-AP reversal occurred on average 7 ± 15 s (n = 151 reversals) after the maximum inter-kinetochore distance had been reached; inter-kinetochore distance was 2.7 ± 1.0 μm at this reversal and decreased abruptly afterward as both sisters transiently moved toward each other (Fig. 2C). AP-to-P reversal occurred after this decrease in inter-kinetochore distance at 1.9 ± 0.6 μm, which is close to the global minimum (Fig. 2C and fig. S2D). The above data are consistent with a mechanical model (24) in which high centromere stretch favors P-to-AP reversal and low stretch favors AP-to-P reversal (14).

Within our time resolution, changes in movement direction and CenpC(N)-Cdc20 distances coincided (17). CenpC(N)-Cdc20 distances increased abruptly after P-to-AP reversals and decreased abruptly after AP-to-P reversals (Fig. 2D; fig. S2, E and F; and tables S3 and S4), which was also true at anaphase (table S3). That change in forces exerted by P and on AP kinetochores—measured through inter-kinetochore stretch—coincides closely with changes in inter-probe distances within kinetochores supports the mechanical interpretation of inter-probe distances and constrains time scales associated with force transitions.

Extent of deformation is expected to correlate with magnitude of force in a mechanical model. To test this, we plotted inter-probe distance against inter-kinetochore distance. Here too, chromosome oscillations provided natural changes in kinetochore forces (fig. S2); to extend the range of forces, we included measurements from drastically compressed cells in which inter-kinetochore stretch was up to 6 μm. Force between kinetochores is due to active force from the P kinetochore opposed largely by friction from the AP kinetochore (14, 15, 24). Velocity of AP kinetochores increased with inter-kinetochore distance, as expected if AP movement is due to pulling by the P sister opposed by frictional drag at the AP kinetochore (P = 10⁻⁸) (Fig. 3A and table S5). Consistent with this view, velocity of P kinetochores decreased with inter-kinetochore distance (P = 0.08) (Fig. 3A and table S5) (13). Inter-probe distances in P kinetochores decreased with inter-kinetochore distance (P = 10⁻⁸) (Fig. 3B and table S5). This suggests that P kinetochores that exert more force are more compressed. No correlation between inter-probe and inter-kinetochore distances was detected in AP kinetochores (P = 0.6) (Fig. 3B and table S5). When little active force was generated at low inter-kinetochore distances, both P and AP kinetochores displayed similar inter-probe distances (Fig. 3B).

Because P kinetochores, where active force is generated, are internally compressed relative to AP kinetochores and because the larger the force generated at P kinetochores the more compressed they are, we developed a simple mechanical model in which frictional forces can evolve without the constraint of requiring persistent attachment. Together, both interfaces allow the kinetochore to harness force from depolymerizing microtubules without losing grip. That said, kinetochores may be able to function using only the passive interface—for example, in systems without anaphase A (25) or where microtubules polymerize continuously at kinetochores, even during anaphase (26). In these systems, segregation forces will be generated elsewhere in the spindle and presumably transmitted to chromatin via molecular friction.

Fig. 4. Mechanical model for kinetochore compliance. (A) Kinetochore and inter-kinetochore chromatin viewed as three springs in series, with probes indicated by stars. P kinetochores are compressed, on average, relative to AP kinetochores. We interpret this as indicating that an active force-generating interface that is only engaged in P kinetochores (orange) lies inward of the mean positions of Cdc20 and Hec1(C), whereas a passive frictional interface that is engaged in all kinetochores (blue) lies outward of these markers. (B) Preliminary structural interpretation, with inter-probe distances indicated for CenpC(N)-Cdc20 (mean ± SEM). The active and passive interfaces could differ because they comprise different molecules or because force generation is restricted to the end of the microtubule, whereas friction occurs all along the embedded lattice.

References and Notes
6. X. Wan et al., Cell 137, 672 (2009).
17. Materials and methods are available as supplementary materials on Science Online.
Regional Astrocyte Allocation Regulates CNS Synaptogenesis and Repair

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Astrocytes, the most abundant cell population in the central nervous system (CNS), are essential for normal neurological function. We show that astrocytes are allocated to spatial domains in mouse spinal cord and brain in accordance with their embryonic sites of origin in the ventricular zone. These domains remain stable throughout life without evidence of secondary tangential migration, even after acute CNS injury. Domain-specific depletion of astrocytes in ventral spinal cord resulted in abnormal motor neuron synaptogenesis, which was not rescued by immigration of astrocytes from adjoining regions. Our findings demonstrate that region-restricted astrocyte allocation is a general CNS phenomenon and reveal intrinsic limitations of the astroglial response to injury.

Fig. 1. Segmental distribution of fibrous and protoplasmic astrocytes in spinal cord. (A) Nkx2.2-creERT2 (tamoxifen induction E10.5 to E12.5):Rosa26-YFP fate map shows YFP+, GFAP+ cells at the ventral midline at P0. YFP, yellow fluorescent protein. (B) In P2 Olig2-tva-creCAG-GFP mice, astrocytes remain in register with pMN, whereas Olig2+ OPs distribute wide- (C) Ngn3-cre:Z/EG P1 cord shows intermediate wedge of astrocytes. (D, G, G') At P2, FAs and PAs in Pax3-cre animals remain dorsally restricted. DAPI, 4',6-diamidino-2-phenylindole. (E and F) Aldh1L1-GFP coexpression with GFAP+ (FAs) and AldoC+ (FAs and PAs) cells. (H to N) Astrocytes from Olig2cre/+ spinal cord have a restricted ventral distribution. In Olig2cre/- cells, we observe significantly (P < 0.0001) increased p2-type (GFAP+, Pax6+, AldoC+) astrocytes (fig. S1J), which fail to migrate from the ventral domain. Scale bars, 200 μm.

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Supplementary Materials
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Other Supplementary Material for this manuscript includes the following:
(available at www.sciencemag.org/cgi/content/full/science.1221886/DC1)

Movies S1 and S2

Correction: Extraneous references (77–79) were deleted from the reference list.
Materials and Methods

Cell culture and transfection
To measure the CenpC(N)-Cdc20 distance, Ptk2 EYFP-Cdc20 (gift from Jagesh Shah, Harvard Medical School) cells were cultured as previously described (20) and the mCherry-CenpC construct (gift from Christopher Carroll and Aaron Straight, Stanford University) was transfected using Fugene6 (Roche 11815091001). To measure the CenpC(N)-Hec1(C) distance, Ptk2 cells were cultured as above and both mCherry-CenpC and Hec1-EGFP constructs (gift from Jennifer DeLuca, Colorado State University) were transfected together as above. Cells were plated on #1.5 25 mm coverslips (HCl-cleaned poly-L-lysine coated, Sigma P1524) 24 h pre-transfection. Cells were imaged 36-48 h post-transfection in Leibovitz’s L-15 media as previously described (20).

Metaphase live imaging
Metaphase cells were imaged on a Nikon TE-2000 microscope with a Yokogawa CSU10 spinning disk and 491 nm and 561 nm lasers. A DualView (Photometrics, with Chroma 565dcrx, ET525/50M, ET630/75M) allowed simultaneous two-color acquisition. The lack of cross-channel bleed-through with EYFP/EGFP and mCherry was confirmed. Images were acquired on an Andor iXon 897 camera used without gain. Two-color cells were imaged by phase contrast (500 ms) and fluorescence (500 ms) every 10 s with a 100X 1.4 NA Plan Apo Ph3 oil objective through a custom 1.5X lens yielding 108 nm/pixel (bin=1, Metamorph 7.5.3.0, MDS Analytical Technologies). CenpC(N)-Cdc20 metaphase data was collected over 27 kinetochores in 10 different cells. CenpC(N)-Hec1(C) metaphase data was collected over 14 kinetochores in 8 cells. Cells were imaged at 29-30 °C through a water-heated coverslip holder using PerfectFocus.

Anaphase live imaging
Anaphase cells were imaged in identical conditions as metaphase ones. Fewer measurements at anaphase than metaphase were performed because anaphase is a shorter-lived state: CenpC(N)-Cdc20 anaphase data was collected over 9 kinetochores in 4 different cells. Similarly, fewer measurements during anaphase AP than P movement were performed because anaphase AP excursions are rare (Fig. 1H, Table S2) (23). While anaphase AP excursions are rare, AP movements in anaphase are very similar to AP movements in metaphase in terms of kinetochore dynamics: AP transients in anaphase occur at the same velocity as AP transients in metaphase (18), and photomarking of kinetochore-microtubules (27) and EB1 imaging (23) showed that they are due to directional switches at kinetochores, as in metaphase. The anaphase AP force likely comes from polar ejection forces, just as it does in monopolar metaphase (28). This polar ejection force increases as chromosomes approach the pole and the microtubule density decreases (29). Inhibiting the chromokinesin Kid in monopoles leads to a smaller chromosome-to-pole distance (30), and thus the polar ejection force may be mediated by chromokinesins engaging with non-kinetochore microtubules and walking to their plus-ends. Polymerization pressure of non-kinetochore microtubules against chromosomes may also contribute to the polar ejection force (20). Finally, the similar inter-probe changes we observe at metaphase (Fig. 1G) and anaphase (Fig. 1H)
indicate that inter-probe distance changes between P and AP states occur independently of a centromeric connection with the sister kinetochore.

**Spindle compression**

Spindle compression (20) was applied prior to imaging using a stage-mounted micromanipulator and held in place during imaging. Cell health was monitored through the ability to enter anaphase during imaging under compression. Mild spindle compression did not affect parameters of kinetochore movement (Table S1) or anaphase entry (20). Here, in addition to mild spindle compression, we have also included data from kinetochores where more drastic spindle compression was applied to increase the range of inter-kinetochore distances visited. More drastic spindle compression confines all chromosomes to one plane, and by doing so can prevent one kinetochore from moving, while its sister kinetochore is free to move towards its pole, yielding inter-kinetochore distances significantly above the normal range (Fig. 3). Above normal range inter-kinetochore distances are critical to the present experiment because they allow us to probe the inter-probe distance in cases of extreme force generation and centromere stretch, thereby improving the statistical significance of relationships between μm-scale and nm-scale observables and inter-kinetochore distance (e.g. Fig. 3 and Table S5).

While in Hela cells the metaphase inter-kinetochore distance typically ranges from 0.8-1.8 μm (6), in Ptk2 cells it typically ranges from 1-4 μm (Fig. 2A), and Ptk2 spindles under drastic spindle compression displayed inter-kinetochore distances of up to 6 μm (Fig. 3).

**Data analysis**

A Matlab program, Speckle Tracker (31) (Xiaohu Wan, UNC Chapel Hill), was used to track kinetochores as described (20) and determine their centroid by fitting a two-dimensional Gaussian (lsqcurvefit, Matlab) in a 10x10 pixel box (6). Only kinetochores vividly oscillating were tracked, whose metaphase plate alignment had been completed. The inter-probe distance was only measured on kinetochores with sufficient photon counts. For CenpC(N)-Cdc20, we typically collected 4000-7000 photons/kinetochore and the signal-to-noise ratio was SNR=I_{max}/√(I_{max}+b^2)=15-20 (with I_{max} the maximum pixel photon count and b the background photon standard deviation of about 8 photons). A home-written Matlab program measured parameters of sister kinetochore movement and inter-probe distance. The mean of the Gaussian fit of the inter-probe distance distribution was taken as the inter-probe distance. Because the inter-probe distance cannot be negative, this procedure overestimated distances by about 5% in the present datasets (32). Finally, because sisters were rarely in exactly the same focal plane, and because under drastic compression one sister will often be pinned down and immobile, we could not obtain inter-probe distances for both sisters in all pairs.

To colocalize red and green probes, red images were registered with green images using a position-dependent two-dimensional transform (cp2tform, polynomial degree 2, Matlab) accounting for chromatic aberrations (variant of SHREC (21)). Before each experiment, scattered TetraSpeck 100 nm microspheres (Invitrogen T-7279) bound to the coverslip surface were imaged to measure this transform (21). Bead Gaussian standard deviations were of 130-145 nm on the coverslip surface, and the mean kinetochore standard deviation was of around 160 nm along the microtubule axis (direction of
motion) in live cells (Table S6). Along the width of the kinetochore (metaphase plate axis), kinetochore Gaussian standard deviations were sometimes significantly larger (6) (e.g. Fig. 1D): Ptk kinetochores are attached to 20-25 microtubules (33) and are sometimes wider along the metaphase plate axis than they are deep along the microtubule axis.

Individual inter-probe distance traces displayed broad fluctuations (Fig. 1F), and the standard deviation of inter-probe distances was around 20 nm when combining all kinetochores (Fig. 1G-H, Table S2). Factors contributing to this standard deviation include centroid determination with limited photon counts, two-color registration map estimation errors and application to inhomogeneous environments, and biological variation. We estimated the centroid determination accuracy to be 4-6 nm (34) given the measured kinetochore Gaussian standard deviation, the range of photons per kinetochore, the pixel size and background photon standard deviation provided above. Testing the two-color registration transform of TetraSpeck microspheres, we estimated the target registration error (21) to be as high as 7 nm over the field of view for microspheres bound to the coverslip surface, and registration correction increased with distance from the center of the field of view. The same kinetochore traversed a given region in the field of view in both P and AP directions, serving as an internal control. The standard deviation for a single kinetochore was often smaller than that for all kinetochores combined, consistent with smaller regions of the field of view leading to smaller registration error fluctuations (see also Figure S1). Finally, the registration error must be higher inside cells than on the coverslip surface: despite spindle compression, kinetochores are undoubtedly at different distances from the coverslip, and can be in inhomogeneous environments. Thus, centroid determination error and two-color registration error at the coverslip account for only part of the inter-probe distance standard deviation we observe over all kinetochores, and registration errors deeper in the cell in inhomogeneous environments and biological variation are likely the main other contributors to the inter-probe distance standard deviation.

In order to extract meaningful features of inter-probe distance evolution (Fig. 2D, S2E-F, Tables S2-3) otherwise obscured by a broad standard deviation, we used chromosome oscillations as a system where averaging can be performed over cyclical events. We only analyzed kinetochores which underwent several oscillation cycles to obtain meaningful values for each kinetochore. Normalized traces (Fig. S2B-F) were obtained by setting the variable start-point to 0 and its end-point to 1, and fitting the data \((\text{polyfit order 5, Matlab})\). The 95% fit confidence intervals correspond to \(\pm 2\) standard deviations of the fits obtained by resampling the same number of points randomly 1000 times. Normalized velocity (Fig. S2C) was calculated from the derivative of normalized position (Fig. S2A-B), and although by definition the velocity is zero at reversals (\(t=0\) and \(t=1\)), we did not impose it to be such during fitting.

Correlations are described by Pearson's linear correlation coefficient. Correlation parameters between the inter-probe distance and inter-kinetochore distance were measured over all inter-kinetochore distances above 2 \(\mu m\). The Ptk cell inter-kinetochore distance reaches up to 2 \(\mu m\) in prophase and taxol-treated metaphase (35), and thus inter-kinetochore distances above 2 \(\mu m\) ensure that kinetochores are under tension and favor the kinetochore-microtubule axis being parallel to the coverslip.
All p-values are for a two-tailed t-test. Unless otherwise noted, we use mean ± standard deviation.

Choice of mCherry-CenpC, EYFP-Cdc20 and Hec1-EGFP as sensor probes

CenpC(N) was chosen as an inner kinetochore marker. CenpC is conserved from yeast to humans (2) and is essential to kinetochore assembly (36). It is the second innermost known kinetochore component (6), is present at kinetochores throughout the cell cycle (2), binds DNA (16), localizes to centromeric DNA via its central domain and C-terminal regions (37), and is stably bound (38). Meanwhile, the N-terminal of CenpC (where mCherry is positioned) binds the Mis12 complex (39, 40), part of the KMN (KNL1/Mis12/Ndc80 complexes) network. Mis12 is an interior KMN network component that binds to the interior ends of the other two KMN network components, the Ndc80 complex and KNL1, which are highly elongated (Fig. 1A) and play critical microtubule binding roles at their outer ends. Thus, CenpC serves as the link between the inner and outer kinetochore, linking the constitutive centromere-associated network (CCAN) and the KMN network that is essential for load-bearing kinetochore-microtubule attachments (2, 41). It is not currently known how CenpC is oriented with respect to the microtubule axis in mammals. By labeling the N-terminal of CenpC, we can monitor the inner part of the KMN network and outer kinetochore, just outside the compliant centromere (6). mCherry-CenpC expression levels sufficient to yield high localization accuracy (5000-7000 photons/kinetochore) did not result in any detectable abnormalities in kinetochore movement or anaphase entry. Given that there are 20-25 microtubules at Ptk kinetochores (33), we estimate that there are about 500-600 CenpC copies per kinetochore (42).

Cdc20 was chosen as the first outer kinetochore marker. Cdc20 is conserved from yeast to humans (2) and is the target of the spindle assembly checkpoint. Combined data indicates that Cdc20 binds to the microtubule-binding protein (43) KNL1’s outer N-terminal through Bub1/BubR1: the N-terminal of KNL1 binds to Bub1 and BubR1 (44) and co-localizes within 1 nm of at least Bub1 (6), Cdc20 and BubR1 interact in vitro (45, 46), Bub1 is required for Cd20 kinetochore localization (47), Bub1 phosphorylates (48) and binds Cdc20 using a KEN box (49), and BubR1 and Bub1 directly interact (50). Cdc20 also directly binds Mad2 (e.g. (48)) at the kinetochore, and prior to metaphase half the Cdc20 kinetochore population requires Mad2 to bind there (22). However, we only imaged spindles in metaphase (or occasionally in late prometaphase, with weak pole Cdc20 localization), when full kinetochore localization (22) does not require Mad2 binding, and we only analyzed kinetochores whose alignment had been completed. For example, in Figure 1C the highlighted pair was analyzed (see also Fig. 1D-F, Fig. 2A, Movie S1). While BubR1 and Mad2 have largely left the kinetochore at anaphase (22), both Bub1 and Cdc20 remain at the kinetochore throughout anaphase (22). Because of its slow kinetics, Bub1 has been proposed to be a scaffold for other checkpoint proteins (22, 38). Cdc20 kinetochore levels are high from prophase to anaphase (22, 45), and EYFP-Cdc20 expression levels sufficient to yield high localization accuracy (5000-7000 photons/kinetochore) did not result in any detectable abnormalities in kinetochore movement (Table S1) or anaphase entry. Finally, we estimated that there are 400-500 KNL1 copies per kinetochore (42), which is consistent with a rough estimate of Cdc20 molecules at a metaphase kinetochore (22).
Finally, we note that in the present conditions EYFP-Cdc20 was a superior probe to Hec1-EGFP, which reports on the inner region of the load-bearing Ndc80 complex (Fig. 1A). Under the present experimental conditions, the number of photons collected from the CenpC(N)-Hec1(C) sensor probes, where both constructs were transfected together, was lower than that obtained from the CenpC(N)-Cdc20 sensor probes, where one probe was stably integrated and the other transfected. At the low Hec1-EGFP expression levels used here in conjunction with mCherry-CenpC expression, no detectable abnormalities in kinetochore movement were observed (Table S1). In contrast to Hec1-EGFP, EGFP-Hec1 (gift from Jennifer DeLuca) expression showed kinetochore movement abnormalities even at the lowest expression levels, limiting the use of the microtubule-binding end of Hec1 as a probe.

We validated sensor design and methodology as follows. We compared mean CenpC(N)-Cdc20 distance with the CenpC-Bub1 and CenpC-KNL1 N-terminal distances in fixed Hela cells averaged over sister pairs: both are similar, the former being 51 nm (Fig. 1G) and the latter were 53 nm and 54 nm, respectively (6). We compared the mean CenpC(N)-Hec1(C) distance with the CenpC-Hec1(9G3) distance (6), taking into account where the different ends of Hec1 are located.

Finally, the size of mCherry, EYFP and EGFP is on the order of 2 nm (51), significantly less than the changes observed here. mCherry, a monomer (52), and EYFP and EGFP, weak dimers (52), should explore space around their targets as they are linked to their targets with a flexible linker.

Time resolution and direction reversals: relating changes in microtubule dynamics and CenpC(N)-Cdc20 distance

To relate microtubule dynamics and CenpC(N)-Cdc20 distance changes at direction reversals, we must consider that microtubules flux towards the poles at about 0.4 µm/min in Ptk2 cells in the present conditions (20). Microtubule flux is neither required for chromosome oscillations nor for centromere stretch (53-55), and is superimposed to plus-end dynamics. Specifically, microtubule flux implies that direction reversals are not exactly coincident with changes in microtubule dynamics: the depolymerization-to-polymerization transition will occur just before the P-to-AP transition, and the polymerization-to-depolymerization transition will occur just after the AP-to-P transition. Transitions in microtubule dynamics states may thus be coincident with maximum inter-kinetochore distance (depolymerization-to-polymerization) and minimum inter-kinetochore distance (polymerization-to-depolymerization): indeed, we find that the inter-kinetochore distance maximum is reached just before the P-to-AP transition, and that the minimum is reached just after the AP-to-P transition (Fig. 2C, S2E). Based on velocity changes of about 2 µm/min in 10 s at reversals points (Fig. 2B), we estimate that the delay between the microtubule dynamics transition and direction reversal is at most a few seconds. Given the present time resolution of 10 s, it is a reasonable estimate that both microtubule dynamics transitions and direction reversals happen nearly simultaneously.

Examining a possible contribution of kinetochore tilt to apparent changes in average inter-probe distances
We address whether significant kinetochore tilting with respect to the imaging plane occurs, and whether it could significantly contribute to the inter-probe changes we observe. In Ptk cells, the kinetochore-microtubule fiber consists of 20-25 microtubules (33) arranged in a circle of about 400 nm in diameter at the kinetochore as viewed by electron microscopy (56, 57). The plus-ends of the microtubules do not all terminate at exactly the same position, and electron microscopy studies suggest that the majority of them terminate within a 30 nm-long band along the microtubule axis (58). Consequently, kinetochore proteins are expected to spread over about 30 nm along the microtubule axis, and fill a 400 nm-diameter circle around this axis. If the kinetochore tilted significantly in response to higher force, the standard deviation of the whole kinetochore should widen by a detectable level. To obtain a 15% reduction in inter-probe distance (Fig. 1G), a 32° tilt is required. If the 400 nm high kinetochore plate is tilted by 32° this should add nearly 200 nm to the width of the Gaussian. This corresponds to an increase of nearly 100 nm to the Gaussian standard deviation, a change which we are certainly able to detect. As revealed by Figure S4 and Table S7, we do not observe a significant change in standard deviation of both EYFP-Cdc20 and mCherry-CenpC kinetochore images as a function of inter-kinetochore distance. Furthermore, as revealed by Figure S3 and Table S6 the standard deviation of both EYFP-Cdc20 and mCherry-CenpC kinetochore images is statistically indistinguishable in P and AP movement. Thus, kinetochore probe standard deviation does not change with kinetochore state: it changes neither with P and AP movement, nor with force generated. The data does not support a significant contribution of kinetochore tilt to inter-probe distance changes.

Supplementary Text

Mechanical model for CenpC(N)-Cdc20 distance changes

The simplest interpretation of the data is that CenpC(N)-Cdc20 distance changes reflect structural kinetochore changes caused by force on a compliant linkage. However, we cannot strictly rule out the possibility that the data reflects changes between P and AP that are not caused by force, in particular that CenpC or Cdc20 could bind to additional or different sites in P vs. AP kinetochores. Clearly, non-force-mediated changes such as differential binding would only affect data interpretation if they were non-random and followed a different pattern in P and AP. Although non-force-mediated changes such as differential binding would provide invaluable insight into kinetochore function, we do not have any evidence for them. Instead, a body of data supports the simpler interpretation above and renders the latter unlikely.

1) Most important, we have tested the mechanical interpretation by repeating the experiment using a different probe, Hec1-GFP, that is part of the main tension-bearing complex in the kinetochore, Ndc80. If the response we observe is indeed mechanical, then the main tension-bearer between microtubules and chromatin should display the same response. We find that the inter-probe distance between mCherry-CenpC and Hec1-EGFP is 38±15 nm during P movement and 43±17 nm during AP movement (Figure 1I, Figure S1, and Tables S1-2). This strongly supports the mechanical picture we obtained for the major load-bearing KMN network using EYFP-Cdc20. While the behavior of one probe pair may in principle have reflected the probe’s local
rearrangement, obtaining the same results with two probes suggests that the findings reflect global mechanically relevant rearrangements rather than local chemical ones.

2) The negative correlation between CenpC(N)-Cdc20 and inter-kinetochore distances at the force-generating P kinetochore (Fig. 3, Table S5) suggests that mechanical force deforms the CenpC(N)-Cdc20 linkage in a titratable manner, as it does with the centromere. The lack of such correlation between CenpC(N)-Cdc20 and inter-kinetochore distances in AP (Table S5), where forces are not actively generated, suggests that such a correlation stems from active force generation. While non-force-mediated changes, including differential binding, could somehow also exactly mimic these force generation changes in a titratable manner, such a coincidence appears unlikely. These would not only have to be coordinated with P and AP changes (Fig. 1G-H, Table S2), but also with the amount of force generated by the P kinetochore (Fig. 3, Table S5).

3) The finding that CenpC(N)-Cdc20 distance changes coincide within <10 s (Fig. 2B-D, Table S3-4) with force-generating changes at the kinetochore suggests that these are directly linked. Although non-force-mediated changes, including differential binding, could also occur rapidly and in perfect coordination with force changes, the above observation limits the mechanisms by which such changes can take place.

4) The finding that CenpC(N)-Cdc20 distance changes between P and AP are the same at both metaphase and anaphase (Fig. 1G-H, Table S2) suggests that the observations reflect the binding of Cdc20 with a kinetochore platform component stable through anaphase. While Bub1 and Cdc20 remain throughout anaphase in Ptk2 cells, many checkpoint associated proteins have largely left the kinetochore by anaphase (e.g. (22)). The above finding thus limits the molecular mechanisms by which non-force-mediated changes, including differential binding, can take place.

5) CenpC is stably bound at the kinetochore (< 10% fluorescence recovered with t1/2 > 15 min (38)), while the CenpC(N)-Cdc20 distance changes we observe occur in ~ 10 s (Fig. 2D, Tables S2-3). Differential binding of CenpC is thus unlikely, as supported by statistically indistinguishable intensity and width of the CenpC spot in P and AP movement (Figure S3 and Table S6).

6) While Cdc20 is dynamic at the kinetochore (22, 45), three observations suggest that it binds to the same site in P and AP. i) The average intensity of the Cdc20 spot is indistinguishable in P and AP (Figure S3 and Table S6), indicating that either Cdc20 binds to the same site in P and AP, or to two different sites in P and AP with exactly the same affinity, or to more than one site while somehow preserving the total number of molecules bound. ii) The standard deviation of the Cdc20 spot along the motion axis is indistinguishable in P and AP (Figure S3 and Table S6), and over different inter-kinetochore distances (Figure S4 and Table S7), suggesting that Cdc20 binds to only one site at a time. iii) The Cdc20 spot recovers from photobleaching with dynamics best described by a single exponential at metaphase (22, 45), suggesting that either Cdc20 binds to one site, or to two sites with the same binding kinetics. These photobleaching dynamics did not depend on Mad2 or microtubule dynamics (45), indicating that such changes over P and AP would not affect Cdc20 binding. All together, these three observations suggest that Cdc20 binds to the same site in both P and AP. The MitoCheck project (59, 60) has identified a number of candidate Cdc20 interacting proteins. Six of them localize to kinetochores, of which four interact with more than Cdc20 there: these are Bub1, KNL1 (which binds microtubules (43)), BubR1 and Mad2. While Bub1 is
required for BubR1 and Mad2 kinetochore localization, BubR1 (61) and Mad2 (22) are not required for Bub1 kinetochore localization. Given that there is no known direct KNL1-Cdc20 interaction, this MitoCheck-displayed KNL1-Cdc20 interaction may well be mediated by Bub1, a known binder of both KNL1 (44) and Cdc20 (49). Together, these data suggest that the observations reflect Cdc20 binding through Bub1/BubR1, which in turn strictly require KNL1 for kinetochore binding (62) and both bind to the N-terminal of KNL1 (44). We conclude that differential binding of Cdc20 is unlikely to cause the CenpC(N)-Cdc20 distance change between P and AP observed at both metaphase and anaphase.

Although none of these six sets of observations is on its own sufficient to eliminate non-force-mediated changes, together they compel us to focus on the simplest interpretation of the data: CenpC(N)-Cdc20 distance changes reflect structural kinetochore changes caused by force on a compliant linkage.

Consideration of alternative mechanical models

In principle, mechanical changes between P and AP at any active or passive interface could lead to a different inter-probe distances in P and AP movement. We propose a mechanical model where active force generators engage in P to compress the inter-probe linkage, and where they disengage in AP (Fig. 4A-B). Indeed, active force generation only occurs during P movement (Fig. S2 and (14, 18)). There are two obvious alternative models to the one we propose (Fig. 4), and the data herein does not support either model.

In the first alternative model, microtubule polymerization pushing forces against the inner kinetochore during AP movement could cause the inter-probe distance to be greater in AP than P (63). While polymerizing microtubules are not found to push on Ptk kinetochores to move them (14) (and thus no active interface in AP is depicted in model Figure 4), we cannot a priori exclude that they deform the kinetochore without substantially moving it. However, observations herein indicate that microtubule polymerization forces are unlikely responsible for a greater inter-probe distance in AP than P. In particular, the CenpC(N)-Cdc20 distance in P changes in a titratable manner with active force generation, and thus the P state generates force to deform the CenpC(N)-Cdc20 linkage (Fig. 3B): at low force the CenpC(N)-Cdc20 distance in P is near that of AP, and at high force it is nearly 20 nm lower (Fig. 3B, Table S5).

In the second alternative model, different force generation states at the passive interface between P and AP could lead to different inter-probe distances. For example, this could occur if different proteins bound to the microtubule in P and AP (23, 64). However, while we cannot exclude that there are differences in P and AP passive interfaces, observations herein do not support a model where these differences are responsible for the observed inter-probe distance changes. First, if the AP kinetochore were more extended than the P kinetochore because of increased friction in AP, then the extension of the AP kinetochore should scale with its velocity (friction force ∝ friction coefficient × velocity). Instead, extension of the AP kinetochore increases (Figure S2E) as its velocity decreases (Figure S2C). Second, if the difference between kinetochore deformation in P and AP were due to different friction forces, this difference should decrease as kinetochore velocity diminishes. Instead, the difference increases (Figure S2E) as kinetochore velocity diminishes (Figure S2C). Third, if increased friction in the
AP state is the main reason why we observe different kinetochore deformation in P and AP, then this model does not explain why P kinetochore compression increases with inter-kinetochore distance (Figure 3B).

Microtubule depolymerization is likely the source of energy for active force generation (11, 12, 65), but the mechanism of such force generation remains unclear (10, 66). It is clear that on average microtubules depolymerize in P movement and polymerize in AP movement, but not all microtubules are depolymerizing in P and not all are polymerizing in AP (67, 68). Furthermore, electron microscopy reveals that the number of microtubules (33), their penetration depth (58) and their protofilament curvature (67) are not clearly distinguishable between P and AP movement. Whether protofilaments have the same stiffness and movement dynamics in P and AP is unknown.

Structural interpretation of the mechanical model

The structural interpretation of the mechanical model (Fig. 4B) has important implications for kinetochore structure-function. We emphasize that observed inter-probe distance changes need not reflect one-dimensional changes in protein conformation, but may also reflect the reorientation and reorganization of protein assemblies (e.g. angular changes in protein components in three-dimensional space).

The active interface, which transduces microtubule depolymerization into P force (11, 12) must lie inward of Cde20/KNL1-N terminal and Hec1(C). This makes the widely-discussed microtubule binding site in Hec1 (near 0 nm, Figure 4B) a poor candidate. The transducer of this active force on the kinetochore could either be a specific microtubule-binding protein (e.g. fibrils connecting microtubule tips with kinetochores (67), or CenpQ (64)), or proteins that resist microtubule protofilament curling (e.g. a non-specific kinetochore protein meshwork (69) or Mis12 (6)).

In turn, the passive frictional interface we infer lies at least 20 nm outward from the active one. Candidates include Hec1 and the Ska complex (also near 0 nm (70), Figure 4B). Both bind microtubules and are able to bear load in reconstitution experiments (70, 71). We favor Hec1 as this microtubule-binding site is conserved in evolution (72), and hyper-activating it with an antibody causes kinetochores to make a rigor bond to the microtubule lattice (73), which we interpret as increased binding affinity at the frictional interface. Modeling the Hec1-microtubule interface as likely passive and frictional rather than active and force-generating contradicts the assumptions of recent reviews (e.g. (10)). However, a passive interface is just as well suited as an active one for stabilizing kinetochore-microtubule interactions, and modulating their stability in response to Aurora B activity (73). An interface that is passive in kinetochores in vivo could become active in reconstitution experiments, since many molecules and objects that produce multiple weak interactions with the microtubule lattice (74) are able to transduce depolymerization into force (11, 75, 76).

Our data show that the active and passive interfaces must be physically separate, but they tell us little about their nature. They might be made of different molecules. Alternatively, the different mechanics at the two sites could stem from the same molecules interacting with different parts of the microtubule, with lattice binding causing friction and end binding causing force generation. Such spatial partitioning of mechanics is seen in the classic “Hill sleeve” model, for example, where all bound sites (which are towards the outside) generate friction, while force generation comes from unbound sites.
(which are more internal) moving into contact with the microtubule (74). Currently, we are unable to probe whether different molecules or the same molecule constitute active and passive interfaces. Super-resolution imaging of individual kinetochores and their component proteins may help address this question.
Fig. S1
Most individual kinetochores have a larger mean inter-probe distance in AP than P movement. (A) Mean AP and P CenpC(N)-Cdc20 distances for each of 27 metaphase kinetochores. (B) Mean AP and P CenpC(N)-Hec1(C) distances for each of 14 metaphase kinetochores. Each point on the figures corresponds to a single kinetochore, and kinetochores from the same cell are represented by the same symbol.
Fig. S2

The kinetochore experiences different forces and different CenpC(N)-Cdc20 distance changes during metaphase chromosome oscillations P and AP movement. (A) Kinetochore positions, mean (B) position, (C) velocity, (D) inter-kinetochore distance, and (E) CenpC(N)-Cdc20 distance over normalized P (n=1066 points) and AP (n=1067 points) movement. (F) Color-coded mean CenpC(N)-Cdc20 distance superimposed on a plot of mean normalized kinetochore position over normalized P and AP movement (as in (B) and (E) above). Normalization removes absolute value from the time axis, but reveals average details of full excursions with high precision. Dashed lines = 95% confidence intervals.
Fig. S3
Test of correlation between kinetochore probe Gaussian fit parameters and kinetochore P and AP movement direction. Gaussian standard deviations of (A) EYFP-Cdc20 and (B) mCherry-CenpC as a function of P and AP movement. Gaussian maximum amplitude of (C) EYFP-Cdc20 and (D) mCherry-CenpC as a function of P and AP movement. The data does not support a model where one or the other kinetochore spot changes significantly in dimension or intensity as a function of P and AP movement (Table S6). Gaussian standard deviations were measured along the inter-kinetochore axis, reflecting fluorescence width along the inner-outer axis of the kinetochore. Because the expression level of EYFP-Cdc20 and mCherry-CenpC varied over cells, for each kinetochore the maximum amplitude of the Gaussian fit was normalized over the mean amplitude for that kinetochore.
Fig. S4
Test of correlation between Gaussian fit standard deviation and inter-kinetochore distance. The data does not support a model where kinetochore tilt increases with inter-kinetochore distance or where additional binding sites become available at higher force (Table S7). Gaussian standard deviations were measured along the inter-kinetochore axis, reflecting fluorescence width along the inner-outer axis of the kinetochore.
Table S1
Chromosome oscillation dynamics are not detectably affected by mild spindle compression and outer kinetochore protein expression under the present experimental conditions.

<table>
<thead>
<tr>
<th>Cell label</th>
<th>Spindle compression</th>
<th>Mean P velocity (µm/min)</th>
<th>Mean AP velocity (µm/min)</th>
<th>Mean P half-period (min)</th>
<th>Mean AP half-period (min)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYFP-Cdc20</td>
<td>None</td>
<td>0.8±0.3(20)</td>
<td>0.7±0.2(20)</td>
<td>2.7±0.8</td>
<td>2.8±0.8</td>
<td>8</td>
</tr>
<tr>
<td>EYFP-Cdc20</td>
<td>Mild*: during compression</td>
<td>0.8±0.2(20)</td>
<td>0.8±0.2(20)</td>
<td>2.9±0.6</td>
<td>3.0±0.5</td>
<td>10</td>
</tr>
<tr>
<td>EYFP-Cdc20</td>
<td>Mild*: compressed steady-state</td>
<td>0.7±0.2(20)</td>
<td>0.8±0.2(20)</td>
<td>3.2±0.4</td>
<td>3.0±0.7</td>
<td>5</td>
</tr>
<tr>
<td>Hec1-EGFP</td>
<td>Mild*: compressed steady-state</td>
<td>0.9±0.3</td>
<td>0.9±0.3</td>
<td>3.0±1.0</td>
<td>2.8±1.1</td>
<td>8</td>
</tr>
</tbody>
</table>

Fluorescence imaging

Phase contrast imaging**

<table>
<thead>
<tr>
<th></th>
<th>Mean P velocity (µm/min)</th>
<th>Mean AP velocity (µm/min)</th>
<th>Mean P half-period (min)</th>
<th>Mean AP half-period (min)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.9±0.4</td>
<td>0.8±0.3</td>
<td>2.4±0.7</td>
<td>2.7±0.6</td>
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</tr>
<tr>
<td>EYFP-Cdc20</td>
<td>0.9±0.3</td>
<td>0.9±0.3</td>
<td>2.5±0.8</td>
<td>2.7±0.7</td>
<td>10</td>
</tr>
</tbody>
</table>

* While mild spindle compression does not affect chromosome oscillation dynamics, drastic spindle compression importantly alters chromosome oscillation dynamics, which allows us to probe the relationship between kinetochore deformation and inter-kinetochore distance (see Materials & Methods).

** Since Ptk2 kinetochores are not directly visible by phase microscopy, the outer edge of the chromosome was tracked for the smallest chromosomes where kinetochore position along the chromosome can be easily identified.
Table S2
Mean inter-probe distances are significantly smaller in P than AP movement for CenpC(N)-Cdc20 at metaphase and anaphase, and for CenpC(N)-Hec1(C) at metaphase.

<table>
<thead>
<tr>
<th>Probes and mitotic stage</th>
<th>P movement</th>
<th>AP movement</th>
<th>p-value between P and AP movement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean distance (nm)</td>
<td>s.d. (nm)</td>
<td>s.e.m. (nm)</td>
</tr>
<tr>
<td>CenpC(N)-Cdc20 Metaphase</td>
<td>47</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>CenpC(N)-Cdc20 Anaphase</td>
<td>49</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>CenpC(N)-Hec1(C) Metaphase</td>
<td>38</td>
<td>15</td>
<td>1</td>
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Table S3
Measured µm-scale observables (inter-kinetochore distances) and nm-scale observables (CenpC(N)-Cdc20 distances) at metaphase P-to-AP and AP-to-P reversals are significantly different.

<table>
<thead>
<tr>
<th>Metaphase</th>
<th>P-to-AP reversal (n=104)</th>
<th>AP-to-P reversal (n=98)</th>
<th>p value for two reversals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inter-kinetochore distance at reversal (µm)</td>
<td>2.7±1.0</td>
<td>1.9±0.6</td>
<td>10^{-13}</td>
</tr>
<tr>
<td>CenpC(N)-Cdc20 distance at reversal (nm)</td>
<td>45±23</td>
<td>56±20</td>
<td>10^{-4}</td>
</tr>
<tr>
<td>Change in CenpC(N)-Cdc20 distance in 20 s before &amp; after reversal (nm)</td>
<td>5±14</td>
<td>-4±15</td>
<td>10^{-6}</td>
</tr>
<tr>
<td>Anaphase</td>
<td>P-to-AP reversal (n=16)</td>
<td>AP-to-P reversal (n=17)</td>
<td>p value for two reversals</td>
</tr>
<tr>
<td>Change in CenpC(N)-Cdc20 distance in 20 s before &amp; after reversal (nm)</td>
<td>8±13</td>
<td>-8±12</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Table S4
Metaphase CenpC(N)-Cdc20 distance changes significantly at P-to-AP and AP-to-P reversals and within P and AP movement phases.

<table>
<thead>
<tr>
<th></th>
<th>P-to-AP reversal (n=208)</th>
<th>AP-to-P reversal (n=196)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CenpC(N)-Cdc20 distance in 20 s before reversal (nm)</td>
<td>45±21</td>
<td>56±19</td>
</tr>
<tr>
<td>CenpC(N)-Cdc20 distance in 20 s after reversal (nm)</td>
<td>50±20</td>
<td>51±20</td>
</tr>
<tr>
<td>p value between before and after reversal periods</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>CenpC(N)-Cdc20 distance in first 20 s of movement (nm)</td>
<td>51±20 (n=196)</td>
<td>50±20 (n=208)</td>
</tr>
<tr>
<td>CenpC(N)-Cdc20 distance in last 20 s of movement (nm)</td>
<td>45±21 (n=208)</td>
<td>56±19 (n=196)</td>
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<tr>
<td>p value for first and last 20 s of movement</td>
<td>0.001</td>
<td>0.003</td>
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</table>
Table S5
Comparison of correlations between inter-kinetochore distance, and CenpC(N)-Cdc20 distance and velocity over metaphase P and AP movement.

<table>
<thead>
<tr>
<th>Metaphase oscillation phase</th>
<th>Correlation coefficient between velocity and inter-kinetochore distance</th>
<th>p value for correlation</th>
<th>Slope of linear fit (min⁻¹)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Last 20 s of P movement</td>
<td>-0.2</td>
<td>0.08</td>
<td>-0.1</td>
<td>104</td>
</tr>
<tr>
<td>First 20 s of AP movement</td>
<td>0.4</td>
<td>10⁻⁴</td>
<td>0.4</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Correlation coefficient between CenpC(N)-Cdc20 and inter-kinetochore distances</td>
<td>p value for correlation</td>
<td>Slope of linear fit (nm/µm)</td>
<td>n</td>
</tr>
<tr>
<td>P movement</td>
<td>-0.2</td>
<td>10⁻⁸</td>
<td>-7.0</td>
<td>547</td>
</tr>
<tr>
<td>Last 20 s of P movement</td>
<td>-0.3</td>
<td>0.003</td>
<td>-7.5</td>
<td>76</td>
</tr>
<tr>
<td>AP movement</td>
<td>-0.02</td>
<td>0.6</td>
<td>-0.7</td>
<td>529</td>
</tr>
<tr>
<td>Last 20 s of AP movement</td>
<td>0.05</td>
<td>0.7</td>
<td>2.3</td>
<td>56</td>
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Table S6

Test of correlation between kinetochore probe Gaussian fit parameters (standard deviation and maximum amplitude) and kinetochore P and AP movement direction. The data below does not support a model where one or the other kinetochore spot changes significantly in dimension or intensity as a function of P and AP movement (Fig. S3). Gaussian standard deviations were measured along the inter-kinetochore axis, reflecting fluorescence width along the inner-outer axis of the kinetochore. Because the expression level of EYFP-Cdc20 and mCherry-CenpC varied over cells, for each kinetochore the maximum amplitude of the Gaussian fit was normalized over the mean amplitude for that kinetochore.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Direction of movement</th>
<th>n</th>
<th>Gaussian standard deviation (nm)</th>
<th>p value between P and AP Gaussian standard deviations</th>
<th>Gaussian maximum amplitude (a.u)</th>
<th>p value between P and AP Gaussian maximum amplitudes</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYFP-Cdc20</td>
<td>All</td>
<td>1488</td>
<td>163±25</td>
<td>0.5</td>
<td>1 by design</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>752</td>
<td>163±26</td>
<td></td>
<td>0.99±1.27</td>
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<tr>
<td></td>
<td>AP</td>
<td>736</td>
<td>162±24</td>
<td></td>
<td>1.01±1.10</td>
<td></td>
</tr>
<tr>
<td>mCherry-CenpC(N)</td>
<td>All</td>
<td>1488</td>
<td>160±28</td>
<td>0.4</td>
<td>1 by design</td>
<td>0.3</td>
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<tr>
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<td>159±28</td>
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<td>AP</td>
<td>736</td>
<td>160±28</td>
<td></td>
<td>0.97±0.92</td>
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Table S7
Test of correlation between kinetochore probe Gaussian fit standard deviation and inter-kinetochore distance. The data below does not support a model where kinetochore tilt increases with inter-kinetochore distance or where additional binding sites become available at higher force (Fig. S4). Gaussian standard deviations were measured along the inter-kinetochore axis, reflecting fluorescence width along the inner-outer axis of the kinetochore.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Direction of movement</th>
<th>n</th>
<th>Correlation coefficient between Gaussian standard deviation and inter-kinetochore distance</th>
<th>Slope of linear fit (nm/µm)</th>
<th>p value for correlation</th>
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<tr>
<td>EYFP-Cdc20</td>
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<td>1488</td>
<td>-0.01</td>
<td>-0.3</td>
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<td>P</td>
<td>752</td>
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<td>736</td>
<td>-0.03</td>
<td>-1.1</td>
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<td>mCherry-CenpC(N)</td>
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<td>1488</td>
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<td>-1.7</td>
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<td>AP</td>
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<td>-0.01</td>
<td>-0.6</td>
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</table>
**Movie S1**
Kinetochore movement simultaneously visualized with mCherry-CenpC and EYFP-Cdc20 probes. Fluorescence imaging of kinetochore oscillations in a compressed spindle (same cell as Figures 1C-F and 2A), with EYFP-Cdc20 imaged on the left of the camera and mCherry-CenpC on the right. Non-concurrent image portions are shown. Time in min:s.

**Movie S2**
Kinetochore movement simultaneously visualized with mCherry-CenpC and Hec1-EGFP probes. Fluorescence imaging of kinetochore oscillations in a compressed spindle, with Hec1-EGFP imaged on the left of the camera and mCherry-CenpC on the right. Non-concurrent image portions are shown. Time in min:s.
References and Notes


17. See Supporting Online Materials on Science Online.


