Protein Architecture of the Human Kinetochore Microtubule Attachment Site

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SUMMARY

Chromosome segregation requires assembly of kinetochores on centromeric chromatin to mediate interactions with spindle microtubules and control cell-cycle progression. To elucidate the protein architecture of human kinetochores, we developed a two-color fluorescence light microscopy method that measures average label separation, Delta, at <5 nm accuracy. Delta analysis of 16 proteins representing core structural complexes spanning the centromeric chromatin-microtubule interface, when correlated with mechanical states of spindle-attached kinetochores, provided a nanometer-scale map of protein position and mechanical properties of protein linkages. Treatment with taxol, which suppresses microtubule dynamics and activates the spindle checkpoint, revealed a specific switch in kinetochore architecture. Cumulatively, Delta analysis revealed that compliant linkages are restricted to the proximity of chromatin, suggested a model for how the KMN (KNL1/Mis12 complex/Ndc80 complex) network provides microtubule attachment and generates pulling forces from depolymerization, and identified an intrakinetochore molecular switch that may function in controlling checkpoint activity.

INTRODUCTION

Kinetochore are protein assemblies at the periphery of centromeric chromatin that are required for segregating chromosomes in all eukaryotes (Maiato et al., 2004). Robust spindle microtubule (MT) plus-end attachment is “end-on,” and MTs bound to kinetochores are known as kinetochore microtubules (kMTs; Rieder, 1982). The number of MTs bound per kinetochore can be as few as one for budding yeast and up to 20 in humans (Rieder, 1982; Maiato et al., 2004). By electron microscopy (EM), human kinetochores show a multilayered disk structure (Maiato et al., 2004; Dong et al., 2007). The inner domain of the kinetochore is assembled from proteins constitutively present at centromeres during the cell cycle. First is CENP-A, a variant of histone H3, that replaces H3 in nucleosomes of chromatin at the base of the kinetochore (Blower et al., 2002). Following CENP-A are 14 proteins known as the constitutive centromere-associated network (CCAN) (Foltz et al., 2006; Cheeseman and Desai, 2008). Unlike the constitutively localized proteins, outer kinetochore proteins are assembled at kinetochores beginning at prophase and leave kinetochores at the end of mitosis. Important are three highly conserved protein
RESULTS

Delta Measurements of the Ndc80 Complex at Human Metaphase Kinetochores

We initially developed the Delta assay to analyze the four-subunit Ndc80 complex, which is the major MT-binding component of the outer kinetochrome. Electron microscopy and atomic structure studies indicate that the Ndc80 complex is 57 nm long and has a dumbbell shape (Figure 1A; Wei et al., 2007; Ciferri et al., 2008). MT-binding activity resides in the Hec1 and Nuf2 N-terminal regions at one end of the complex (Cheeseman et al., 2006; Wei et al., 2007; Ciferri et al., 2008). The 9G3 monoclonal antibody binds amino acids 200–215, roughly 2.5 nm inside the Hec1 N terminus (DeLuca et al., 2006; Ciferri et al., 2008) and localizes by immuno-EM to the kinetochrome outer plate (DeLuca et al., 2005). Globular C-terminal regions of Spc24 and Spc25 are at the opposite end of a long a-helical coiled-coil rod (Figure 1A). Previous work suggests that the 9G3 label is external to the Spc24 label along the inner-outer kinetochrome axis (DeLuca et al., 2006), which is consistent with binding orientation of the complex relative to MT polarity (Wilson-Kubalek et al., 2008).

We analyzed metaphase HeLa cells where kinetochrome inner-outager labels to kMTs are mostly parallel to the axis between sister kinetochores (Figures 1B and 1C). Two-color immunofluorescence was performed using the 9G3 antibody to Hec1 and polyclonal antibodies to the C-terminal heads of Spc24 or Spc25 (see Figures S1 and S2 available online). We acquired high-resolution, low-noise, 3D image stacks of cells where the metaphase spindle was parallel to the coverslip surface—ensuring that several sister kinetochrome pairs were in focus together within the same set of optical sections (Figure 1B). The image in each color is a convolution of the label distribution within the kinetochrome and the objective point spread function (Figure 1C; Maddox et al., 2003). As kinetochrome proteins are distributed across the width of kinetochores (Figure 1C), the “Airy disk” image is elongated into an elliptical cross-section whose major axis defines the orientation of the kinetochrome face and whose centroid defines the average position of the label along the inner-outer kinetochrome axis (Figures 1C, S3, and S4). Sister pairs in or close to the same image plane were selected for analysis. A 3D Gaussian fitting algorithm was used to obtain the four centroid coordinates needed to calculate the separation of the two color labels, Delta (Figures 1D and 1E). This calculation scheme automatically corrected for the considerable chromatic aberration—the green label was on average shifted down and to the left of red by ~30 nm, but exact shifts varied by position within the cell. Delta measurement simulations showed that staggering of attachment site linkages by up to 150 nm along the interkinetochore (K–K) axis (Figure S3Dii) had little effect on Delta accuracy. However, the tilt of the kinetochrome face that inclines attachment linkage to the K–K axis (Figure S3Dii) was a source of measurement error (1% in untreated cells) that was corrected to obtain the final average value of Delta (Supplemental Experimental Procedures).

The average Delta value (Figure 1E) measured between the 9G3 label near the Hec1 head and the Spc24/Spc25 C terminus was 45 ± 6 nm and 45 ± 4 nm, respectively (SD; n = 107 sister kinetochrome pairs for each combination). We also obtained the same value after correcting for the majority of lateral chromatic aberration between the 9G3 and Spc24 images (Supplemental Experimental Procedures; Figure S5). To assess the efficacy of the correction scheme and the accuracy of the Delta method using antibody labeling, we labeled Hec1 with 9G3 and used equal amounts of red and green secondary antibodies. The
Average Delta measured in this case was 0 ± 5 nm (SD; n = 91 sister kinetochore pairs). In a second test for accuracy, cells expressing GFP-Hec1 were labeled with anti-GFP and 9G3—in this case, the average Delta was 3 ± 7 nm, a value consistent with structure (Figure 1A; Ciferri et al., 2008). Average Delta values typically had 95% confidence intervals of ±1–2 nm (Table S1), and two averages that differed by 3 nm or more were significantly different (paired t test with p value < 0.02) because of the high signal-to-noise ratio (>30) of the kinetochore fluorescence (Figure S4) and the averaging of many (>100) individual Delta values. A major assumption of our Delta assay is that the two sister kinetochores have the same protein architecture and stiffness. We verified this assumption by direct measurements of label separation within individual kinetochores of sister pairs for the Ndc80 complex and several other proteins (Supplemental Experimental Procedures; Figure S5). These and other tests (Supplemental Experimental Procedures) establish the Delta assay as a technique for analyzing kinetochore architecture with an accuracy of <5 nm. The 45 nm Delta value measured for Spc24/25 and Hec1 indicates that the Ndc80 complex adopts an elongated shape along the inner-outer kinetochore axis.

Correlation of Delta Measurements to Centromere Stretch Indicates that the Ndc80 Complex Is Noncompliant

Sister kinetochores of metaphase bioriented chromosomes exhibit directional instability—oscillations characterized by abrupt switches between persistent phases of poleward and antipoleward movement (Skibbens et al., 1993; Inoué and Salmon, 1995; Maiato et al., 2005). This directional instability produces oscillations in the stretch of centromeric chromatins.
between sister kinetochores that are asynchronous between different chromosomes. Consequently, a fixed image of a metaphase cell has within it sister kinetochore pairs in different mechanical states (Figure 2A). It is straightforward to assess the mechanical state of each sister pair by measuring the K-K distance using the 9G3 label, which varies during metaphase oscillations between a minimum of 0.8 to a maximum of 2 μm in HeLa cells; the rest length in nocodazole-treated cells is 0.7 μm. Thus correlating Delta values with the interkinetochore K-K distance provides direct information on mechanical properties of specific protein linkages. The slope of the least-squares line through a plot of Delta versus K-K distance represents compliance during oscillation of a particular protein linkage at metaphase, which we refer to as “oscillation compliance” (Figure 2A).

When Delta values measured for 9G3 and Spc24 labels were plotted as a function of K-K distance, the slope was near zero, indicating that tension changes during sister kinetochore oscillations do not affect the conformation of the Ndc80 complex (Figure 2B). The slope was also near zero for cells labeled with 9G3 and equal amounts of red and green secondary antibody (Figure 2C), indicating that Delta measurements were insensitive

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**Figure 2. Correlation of Delta Values with Centromere Stretch: The Ndc80 Complex Is Not Compliant and Maintains a Constant Shape**

(A) Schematic of sister kinetochore pairs in different mechanical states (top); predictions of Delta measurements for noncompliant (middle) and compliant (bottom) protein linkages.

(B) Delta values of separations between Hec1-9G3 and antibody to Spc24 C terminus across the entire range of K-K distances.

(C) Delta values for 9G3 anti-Hec1 labeling with a mixture of red/green fluorescent secondary antibodies are individually plotted versus K-K distance. For (B) and (C), measurements are insensitive to K-K separation.

(D) Delta values of separations between Hec1-9G3 and GFP-CENP-A across the entire range of K-K distances show oscillation compliance. The whole data set of Delta values uncorrected for tilt are shown; average Deltas are within 1–2 nm of corrected values (Table S1).
to K-K separation. We conclude that the Ndc80 complex is a stiff mechanical entity within kinetochores, with one end attached to the plus ends of kMTs and the other end located toward the inner kinetochore.

**Human Metaphase Kinetochore Architecture from Delta Analysis of 16 Kinetochore Proteins**

We next extended the analysis of position and mechanical properties to 19 epitopes in 16 proteins representing all of the distinct groups comprising core kinetochore structure (Table S1). For three large proteins (hKn1, CENP-E, and CENP-F), we analyzed two different regions using independent antibodies (Table S2; Figure S6). The entire data set is summarized in Figure 3; the positions along the kinetochore inner-outer axis for all analyzed epitopes are plotted relative to Hec1-9G3. Positive values are outside (i.e., toward the spindle side) of the position of the 9G3 centroid, and negative values are inside (i.e., toward the centromeric chromatin). All average Delta values in this position map are corrected for tilt. Vertical lines indicate minimum and maximum Delta values measured during oscillations in centromere stretch—for most linkages that do not show significant compliance, the vertical lines do not extend beyond the symbol used to indicate the average.

![Figure 3. Summary of Delta Measurements in Control and Taxol-Treated HeLa Cells](image_url)

Summary of Delta measurements for 19 epitopes in 16 kinetochore proteins in control cells (left) and taxol-treated cells (right). The scale (red) on the far right is set equal to zero at the position of the Hec1-9G3 centroid; positive values are outward (toward the spindle MTs), while negative values are inward (toward the centromeric chromatin). Color-coded boxes indicate complexes. Colored dotted lines indicate proposed “arms” of the structural kinetochore. Black dots indicate average Delta values corrected for tilt. Vertical lines indicate minimum and maximum Delta values measured during oscillations in centromere stretch—for most linkages that do not show significant compliance, the vertical lines do not extend beyond the symbol used to indicate the average.
The data for control cells are on the left of Figure 3, with dots indicating average values. The mechanical properties of each Delta value are summarized by vertical lines through the average dots which indicate minimum to maximum variation with K-K separation. The average position data for taxol-treated cells are on the right, and insights derived from this perturbation are discussed in the next results section. With this overview of the entire data set, we describe specific aspects of measurements for each protein complex.

**Constitutive Centromere-Associated Proteins**

**CENP-A and CENP-C.** The centromeric histone H3 variant CENP-A and closely associated conserved CENP-C protein are present at centromeres throughout the cell cycle and provide a foundation for kinetochore assembly. We labeled CENP-A in two ways that gave similar results—in the first, CENP-A-GFP was stably expressed in HeLa cells (Gerlich et al., 2003) and GFP was detected with antibodies (Figure 3). In the second, a primary antibody to CENP-A was used. As this antibody required a different fixation condition (cold methanol) from the other epitopes, we focused on data acquired with the GFP fusion and the standard aldehyde-based fixation procedure. In control cells, the average Delta of CENP-A-GFP relative to the Hec1 head was 107 nm, and there was a pronounced upward slope of Delta with centromere stretch (Figures 2D and 3). The average Delta for CENP-C, which has direct DNA-binding activity (Politi et al., 2002) and lacks extended coiled coils, was 79 nm and the oscillation compliance was ~40% of that exhibited by CENP-A (Figure 3). From the entire set of proteins analyzed (Figure 3), these were the only two proteins that exhibited significant oscillation compliance relative to the Ndc80 complex.

**CENP-I and CENP-T.** In addition to CENP-A and CENP-C, 13 additional CCAN proteins (CENP-H, I, and K–U) localize to centromeres throughout the cell cycle and play important roles in chromosome segregation (Foltz et al., 2006; Okada et al., 2006; Hori et al., 2008). We analyzed CENP-I and CENP-T, two representatives of different subclasses of the CCAN (Okada et al., 2006). In control cells, the average positions of CENP-I and CENP-T were about 17 and 14 nm inside the Spc24/Spc25 end of the Ndc80 complex, respectively (Figure 3). In contrast to CENP-A and CENP-C, CENP-I and CENP-T did not exhibit significant oscillation compliance (Figures 3, 4C, and S8A). This result suggests that components of the CCAN (excluding CENP-C) assemble in the 34 nm gap between the centroid of CENP-C and the Spc24/Spc25 end of the Ndc80 complex and they exhibit a stiff linkage to the Ndc80 complex during oscillations.

**The KMN Network and the Spindle Checkpoint Kinase Bub1**

The KNL1/Mis12 complex/Ndc80 complex (KMN) proteins play a central role in kinetochore architecture and in MT binding. As described above, the two ends of the Ndc80 complex are ~45 nm apart, and this complex appeared stiff with no oscillation compliance (Figure 2B). The four subunits of the Mis12 complex extend from the Spc24/Spc25 end of the Ndc80 complex to about 11 nm inside it, next to the centroid of CENP-T (Figure 3), and also do not exhibit oscillation compliance. hKnl1 (also known as Blinkin/CASC5/AF15Q14) is a large (2342 aa) protein recruited to kinetochores by the Mis12 complex (Cheeseman et al., 2008).

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and is suggested to bind to the hDsn1 subunit of the Mis12 complex at its C-terminal end (Kiyomitsu et al., 2007; Cheeseman et al., 2008). hKnl1 has a predicted coiled-coil domain of 300 amino acids near its C terminus (Cheeseman et al., 2006). We measured the position of the central region of hKnl1 using an antibody to aa 1220–1440 and the position of the N terminus using a monoclonal antibody to the first 43 aa (Figure S6). These epitopes were found to be on average 34 nm and 25 nm inside of the Hec1 head (Figure 3) and neither exhibited significant oscillation compliance.

hKnl1 is part of the kinetochore-binding site for SAC kinase Bub1 (Kiyomitsu et al., 2007). A Bub1 antibody raised to a region near its kinetochore-targeting domain (Figure S6) was 26 nm internal to the Hec1 head and just inside of the N-terminal epitope in hKnl1 (Figure 3). The distance between hDsn1, a likely internal to the Hec1 head and just inside of the N-terminal globular tail domain (Kim et al., 2008). CENP-E has been local-ized primarily along the kinetochore inner-outer axis. That hKnl1 is stiff like the Ndc80 complex and that its long axis indicating lack of oscillation compliance. This result indicates that hKnl1 is stiff like the Ndc80 complex and that its long axis is oriented primarily along the kinetochore inner-axis.

Corona Proteins

The Motor CENP-E. CENP-E is a plus-end-directed kinesin motor protein with a very long (225 nm) coiled-coil stalk and a globular tail domain (Kim et al., 2008). CENP-E has been local-ized by immunogold EM to the outer plate and fibrous corona (Cooke et al., 1997) and contributes to kMT attachment and kinetochore movements toward the spindle equator (Kapoor et al., 2006; Kim et al., 2008). The C-terminal kinetochore-binding domain interacts with CENP-F and BubR1 (Chan et al., 1998), and CENP-E depends on Bub1 for targeting to kinetochores (Johnson et al., 2004; Liu et al., 2006). We found the centroid of an antibody to aa 1571–1859, about 100 nm from its C-terminal kinetochore-targeting domain (Figure S6), was 16 nm outside the Hec1 head (Figure 3). Surprisingly, the centroid of an antibody to aa 663–973, about 50 nm from the motor domain of CENP-E (Figure S6), was only ~13 nm outside the Hec1 head. Neither epitope on CENP-E exhibited any oscillation compliance (Figure S8A; Figure 3). This result indicates that the 225 nm long CENP-E molecule is bent with both its tail and motor domains located near or inside the Hec1/Nuf2 heads. The Coiled-Coil Protein CENP-F. CENP-F is a major component of the fibrous corona as determined by immunogold EM (Rattner et al., 1993). It is large (300 kDa, 3210 aa) and possesses extensive central α-helical coiled-coil domains (Figure S6). CENP-F localizes to kinetochores via its C terminus and requires hKnl1 and Bub1 (Johnson et al., 2004; Cheeseman et al., 2008). An antibody to the C-terminal 561 aa localized ~4 nm outside the Hec1 head, and a different antibody to a region in the middle of the molecule (Figures S6 and 3) was ~48 nm outside the Hec1 head, indicating that CENP-F is primarily oriented perpendic-ular to the outer plate.

The MT Polymerization-Promoting Protein CLASP. CLASP is an MT-binding protein that promotes polymerization and suppresses depolymerization (Maiato et al., 2005). CLASP is concentrated within the very periphery of the kinetochore during mitosis. We find that the centroid of CLASP is 29 nm outside the Hec1 head in control metaphase cells (Figure 3). This position is within the C-terminal half of CENP-F or the loop of CENP-E extending into the corona, but not near the location expected for the plus ends of the majority of kMTs, which is near the inner surface of the kinetochore outer plate (VandenBeldt et al., 2006; Dong et al., 2007).

Large Changes in Kinetochore Protein Architecture Are Observed following Taxol Treatment

The anticancer agent taxol suppresses polymerization dynamics of MTs. Taxol treatment (10 μM) at metaphase eliminates tension at kinetochores and activates the spindle checkpoint (Waters et al., 1998; Clute and Pines, 1999). The mean interkinetochore distance in taxol-treated metaphase cells is about 0.75 μm (Figure 4A), only slightly less than the minimum 0.8 μm value that transiently occurs during oscillations (Figure 2). This similarity suggests that Delta measurements in taxol-treated cells should resemble values observed at lowest K-K distance for linkages with oscillation compliance (CENP-A and CENP-C) and similar to average values for stiff noncompliant linkages. Consis-tent with this, the dimensions of the Stewart Ndc80 complex remained constant in taxol (Figures 3 and 4B). However, somewhat surprisingly, there were several significant changes induced in kinetochore architecture in taxol-treated cells. Centroids of CENP-A, CENP-C, CENP-I, and CENP-T all moved ~16 nm closer to the position of the Spc24/25 end of the Ndc80 complex (Figures 3 and 4C). This movement did not change the relative separation distance between CENP-I and CENP-T centroids. In addition, separation between CENP-I/CENP-C centroids and CENP-I/CENP-A centroids was the same as that observed at minimal centromere stretch (K-K distance) during normal control oscillations. Thus, the entire inner kinetochore is ~16 nm closer to the Spc24/25 end of the Ndc80 complex in taxol-treated cells.

The Mis12 complex also exhibited a striking change in taxol-treated cells (Figure 3). In control cells, the distance along the inner-outer axis from the hNsl1 subunit at the interior end of the Mis12 complex and the hDsn1 subunit at the exterior end was constant during oscillations at ~9 nm; in taxol, this length became 19 nm. Two components at one end of this elongated complex, hNnf1 and hNsl1, did not significantly change position relative to Spc24 after taxol treatment; Mis12 showed modest (4 nm) outward movement. In contrast, a striking effect was observed for hDsn1, which shifted ~12 nm outward in taxol-treated cells.

hDsn1 likely directly binds to the C-terminal region of hKnl1 (Kiyomitsu et al., 2007; Cheeseman et al., 2008); consistent with this, hKnl1 also exhibited outward movement similar to hDsn1. The centroid of the Bub1 epitope also moved outward, but less than expected for its putative binding site near the N terminus of hKnl1; this lack of movement may indicate changes in Bub1-binding sites following taxol treatment.

The C-terminal regions of large corona proteins CENP-E and CENP-F also moved ~10 nm outward relative to Hec1/Nuf2 heads after taxol treatment (Figure 3). The most striking change observed was with CENP-E: the epitope close to the motor domain moved from near the Hec1 head (and its own C terminus) in controls to 33 nm beyond the Hec1 head. This result suggests that CENP-E changes from being bent in control metaphase cells to an extended conformation in taxol-treated cells.
A major pattern emerging from comparison of the taxol-treated and control cell Delta measurements suggests relative movement of two protein sets whose constituents behave as though they are coupled—we refer to each of these protein sets as “arms” in order to connote a multipart mechanical entity. The Ndc80 arm (whose average Delta values are linked using blue dotted lines between control and taxol treatment in Figure 3) is composed of the Ndc80 complex and hNnf1, hNsl1, and part of Mis12—none of these show significant taxol-specific changes in separation. The hKnl1 arm (whose average Delta values are linked using red dotted lines between control and taxol treatment in Figure 3) is composed of CENP-A, CENP-C, CENP-I, CENP-T, hDsn1 and part of the hMis12 subunits, hKn11, Bub1, CENP-F, and the C-terminal end of CENP-E—all of these move ~10–16 nm outward relative to the Hec1 head for compliant CENP-A and CENP-C linkages, this movement is relative to the Delta value measured at minimal K-K stretch, that is, the lowest tension state in control cells—see the point where lines linking the control and taxol data sets are drawn in Figure 3. As distances between the inner kinetochore proteins (e.g., CENP-I/CENP-T) and proteins in the Ndc80 arm decrease in taxol-treated cells, we conclude that the Ndc80 arm moves in toward the centromeric chromatin in taxol-treated cells whereas the hKnl1 arm does not. This shift, which is not observed at minimal K-K distance in control cells, suggests a mechanism in taxol-treated cells leading to uncoupling and separation of these normally coupled protein arms.

In summary, taxol treatment did not recapitulate kinetochore architecture at lowest K-K distance but instead revealed large-scale changes in different regions of the kinetochore. These changes may arise from persistent absence of mechanical tension (as opposed to transient absence in oscillating kinetochores), lack of depolymerization, activation of the SAC, or all.

**Location of the Plus Ends of Kinetochore MTs Relative to the Ndc80 Complex**

To determine the position of bound kMTs, we analyzed metaphase PtK2 cells cooled to 6°C. Under these conditions, only kMTs persist, a full complement of kMTs penetrating the outer plate is present (Rieder, 1981), and kMT fibers mainly orient perpendicular to kinetochores, which makes them remain in focus several micrometers beyond kinetochores toward their poles (Figures 5A and 5B). Such images could not be obtained in metaphase HeLa cells because of the high degree of spindle and kinetochore fiber curvature. In cold-treated PtK2 cells, centromeres were unstretched, with average K-K distance similar to that in nocodazole—the measurements of kMT position only apply to this low-tension state.

Preliminary experiments indicated that antibodies to tubulin do not penetrate kinetochores, as fluorescence ended in front of the Hec1-9G3 label (data not shown). Therefore, we directly imaged fluorescence of GFP-α-tubulin incorporated into kMTs (Rusan et al., 2001) and obtained intensity profiles along bundles of green fluorescent kMTs through the centroid of the red Hec1-9G3 label (Figures 5B and 5C). Different line scans (n = 92) were plotted on the same axes by setting the position of the 9G3 centroid to zero and normalizing intensity values. The position of the 50% intensity point was determined by fitting an error function to the cumulative data (Figure 5C). The average position of the kMT end determined with this method was 62.3 ± 15 nm (95% confidence interval) inside of the 9G3 label centroid (Figure 5C). The variance is large because fibers oriented at different angles within the field of view had different amounts of lateral chromatic aberration, which was averaged out by an

**Figure 5. A Two-Color Method for Locating the Plus Ends of kMTs Relative to the Hec1 Head in Cooled PtK2 Cells**

(A) Fluorescent image of PtK2 cells stably expressing GFP-α-tubulin cooled to 6°C, fixed, and stained with the Hec1-9G3 antibody and a red fluorescent secondary. The image shows a kinetochore fiber and its kinetochore in the same focal plane. At right is a magnified image of the boxed region, showing how line scans were drawn down centers of the fibers through the center of Hec1-9G3 fluorescence.

(B) Sample line scan of (A) showing GFP-α-tubulin intensity (green) and Hec1-9G3 (red) fluorescent intensity along the line scan.

(C) Plot of all normalized line scans (n = 92), with Hec1-9G3 centroid set to zero for each on the x axis and the error function (purple) that best fits the data set. Blue lines mark x and y positions of the 50% amplitude of the error function.
equal number of fibers facing in all directions (Figure S9). These results indicate that, on average at 6°C in PtK2 cells, the plus ends of kMTs extend 10–15 nm inside the Spc24/Spc25 ends of the Ndc80 complexes. In taxol-treated cells, this position would be at the periphery of the inner centromere (Figure 3).

DISCUSSION

The Chromatin-Proximal Region of the Kinetochore Has Distinct Structural and Mechanical Domains

At minimal centromere stretch, separation between centroids of CENP-I/T and CENP-C is ~11 nm and between CENP-I/T and CENP-A is ~30 nm (Figure 3). As biochemical studies support close associations between CENP-I/T and CENP-A nucleosomes (Obuse et al., 2004; Foltz et al., 2006; Okada et al., 2006), these values suggest that only a small fraction of chromatin-bound CENP-A and CENP-C is exposed at the peripheral surface of the centromere on a metaphase chromosome in a position to bind components of the CCAN. The depth of CENP-C and CENP-A chromatin from the base of the kinetochore increases with centromere stretch (from ~22 and ~60 nm, respectively, at minimal stretch to 46 and 128 nm, respectively, at maximal stretch: Figures 2 and 3), assuming a uniform distribution of each within chromatin (Figure S10A). These values are small compared to the 420 nm diameter of a green fluorescence microscopy. However, they are consistent with past results that CENP-C concentrates near the base of the kineto- chore (Saitoh et al., 1992), whereas CENP-A extends farther into the centromere (Blower et al., 2002; Amor et al., 2004), and that only 10% of the CENP-A is sufficient to build a functional kinetochore (Liu et al., 2006).

In contrast to the compliance of chromatin at the base of the kinetochore containing CENP-A and CENP-C, protein linkages between CENP-I and CENP-T and between these CCAN subunits and the outer kinetochore were stiff in control cells (Figure 3). Thus, for oscillating metaphase chromosomes, the CCAN is assembled at the very periphery of the CENP-A/C-containing centromeric chromatin (Figure S10A) and is stably linked to the outer kinetochore.

The Ndc80 Complex Is Likely Bent along Its Length and Connected to the Inner Kinetochore by a Flexible Linkage

During metaphase in both untreated and taxol-treated human cells, the Delta between markers at the two ends of the 57 nm long Ndc80 complex was a constant 45 nm. A previous study of isolated Drosophila chromosomes used a line scan method and reported ~22 nm separation between the ends of the Ndc80 complex (Schittenhelm et al., 2007). We have measured a Delta of ~18 nm between the Spc24 C terminus and 9G3 just inside the Hec1 head in nucodazole-treated HeLa cells (data not shown), whereas this distance from the structure is 54.5 nm. The lower numbers in nucodazole may indicate flexibility in orientation of the Ndc80 complex and bending of the rod domain at a kink site (Figure 1A; Ciferri et al., 2008; Wang et al., 2008) in the absence of attached kMTs. However, they may also result from measurement errors induced by severe tilt and/or curvature of the kinetochore face relative to the inner-outer kinetochore axis—such curvature has been observed following extended mitotic arrest in the absence of MTs (DeLuca et al., 2005).

A 45 nm average Delta value is predicted if the 57 nm long Ndc80 complex extends straight from the surface of the bound kMT at an angle $\theta = \sim 34^\circ$, similar to the angle that the rod domain of the Ndc80/Nuf2 dimer exhibits when bound in vitro to purified MTs (Cheeseman et al., 2006; Wilson-Kubalek et al., 2008). However, this inclined straight conformation puts the C-terminal MTs (Cheeseman et al., 2006; Wilson-Kubalek et al., 2008) at an angle that the rod domain of the Ndc80 complex or that the complex does not adopt a straight conformation. Anchorage by lateral linkages between adjacent kMT attachment sites (a “load-sharing mechanism”) that are of similar strength to the inner-outer linkages is unlikely because the kinetochore is weak laterally. For example, during merotelic attachments, when a single kinetochore is pulled toward opposite poles by kMTs, lateral stretch of kinetochore proteins and peripheral centromeric chromatin often occurs for ~1 μm (Cimini et al., 2001, 2004; Figure S10B).

An alternative explanation for the 45 nm separation of the labels at the two ends is that the Ndc80 complex is bent (Figure 6A). There are a number of reasons to favor this idea. There is a conserved break in the coiled-coil rod domain about 16 nm inside the Nuf2/Hec1 heads (Figure 1A; Ciferri et al., 2008). Recent EM analyses of purified Ndc80 complexes in vitro (Wang et al., 2008) indicate that flexible bending occurs at this site within the rod domain that connects the two globular ends of the Ndc80 complex (Figure 1A). The existence of a flexible bend does not, however, explain constancy of Ndc80 complex dimensions across the entire range of centromere stretch and in taxol—a fixed-angle bend would have to exist even under tension in order to account for this constancy and 45 nm separation of the two end labels. This consideration assumes that the majority of Ndc80 complexes are bound to kMTs, which is compatible with the requirement of this complex for the SAC and inactivation of the SAC at metaphase (Musacchio and Salmon, 2007).

A proposal we favor to account for the 45 nm distance is that there is a protein complex bound at the bend site that stabilizes the bend and links the Ndc80 complex to the inner kinetochore (Figure 6A). Upon binding of the Ndc80 heads to the kMT lattice, the coil-coiled region between the head and the bend/linker attachment site extends and transmits a pulling force in an outer-inner direction to the bend site and along the hypothetical linker protein to the inner kinetochore—in this case, there is no inward radial force, as the direction of force transmission is along the inner-outer axis. In this configuration, the Spc24/Spc25 end of the complex bends out toward the kMT lattice by ~20 nm to produce the ~45 nm inner-outer separation between the two end linkers.
labels. This model produces little to no radial force at either end of the molecule, and binding of the linker may force a constant bend angle at the otherwise flexible break in the coiled coil. It is possible that the CENP-H subunit of the CCAN, which is primarily coiled coil and has been shown to interact with the Ndc80 complex (Okada et al., 2006; Cheeseman et al., 2008; Hori et al., 2008), may constitute such a linker. Nevertheless, as discussed below, such a linker must be flexible.

Movement of the Ndc80 Arm Relative to the Inner Kinetochore and the hKnl1 Arm: A Low-Tension/Checkpoint-Activated Switch

We propose that the Ndc80 arm includes a flexible filament-like linkage between the bend in the Ndc80 complex and the inner kinetochore (Figures 6A and 6C–6F). Such a linker would explain why the Ndc80 arm is able to move 15 nm toward the inner kinetochore in taxol (the filament buckles either due to persistent low tension and/or due to SAC activation) but remains constant in position relative to the inner kinetochore at high tension (the extended filament is stiff). A flexible linker (not shown for clarity in Figure 6) may also exist between hNsl1 of the Mis12 complex and the inner kinetochore, as suggested by recent EM images (McIntosh et al., 2008). In contrast, the hKnl1 arm connects separately to the inner kinetochore and this connection is stiff and does not change in taxol-treated cells (Figure 6F).

Because the four subunits of the Mis12 complex are linked together (Kline et al., 2006), the best way to merge the control and taxol configurations is to assume that in controls the Mis12 complex extends mostly in a lateral direction (Figures 3 and 6C–6F). In taxol, when the Ndc80 arm moves relative to the hKnl1 arm, the Mis12 complex rotates such that the hDsn1 subunit makes the largest translation (Figures 6C and 6F). Movement of the Ndc80 complex toward the inner centromere can be explained by outward movement of the hKnl1 arm along stable kMTs until further movement is blocked by the kMT end (Figures 3 and 6F). This outward movement may be driven by the minus motor activity of cytoplasmic dynein linked to Knl1 by other proteins (Kiyomitsu et al., 2007; Stehman et al., 2007; Vergnolle and Taylor, 2007; Cheeseman et al., 2008).

The low-tension/SAC switch within the kinetochore may be part of the tension-sensing mechanism that controls the stability of kMT attachment and/or SAC signaling at kinetochores—the Ndc80 complex is required for both of these essential functions (Musacchio and Salmon, 2007; Maresca and Salmon, 2009). Another process the switch may regulate is the conformation of the CENP-E motor—in controls, the 225 nm long CENP-E molecule is bent with markers for its tail and motor domains located near the Hec1 heads, but in taxol the marker proximal to the motor domain of CENP-E moved more than 33 nm beyond the Hec1 heads into the coronal region (Figure 3). This dramatic conformational change is likely to involve kinase activity (Espeut et al., 2008; Kim et al., 2008), which in turn may be modulated by the switch that we describe here.

The Ndc80 Arm Could Contribute in Two Ways to MT Attachment and Pulling Force Generation

Multiple Ndc80 arms may contribute to a "Hill-like" mechanism, where the dynamic binding of their Hec1 and Nuf2 heads to a kMT helps hold the attachment site near either a growing or shortening plus end (Hill, 1985; Asbury and Davis, 2008; Figures 6D and 6E). In budding yeast, there are eight Ndc80 complexes...
per kMT (Joglekar et al., 2006), which supports this possibility—the number and distribution along the inner-outer axis in human cells are not yet established. The Ndc80 arm within HeLa kinetochores could also act as a force transducer. In budding yeast, recent analysis of kinetochores at metaphase indicates that the Ndc80 complex is extended its full length along the axis of kMTs (Joglekar et al., 2009). A DAM/DASH ring has received much attention as a force transducer in budding yeast for the rearward peeling of tubulin protofilaments (Efremov et al., 2007; Asbury and Davis, 2008; Tanaka and Desai, 2008). However, in mammalian kinetochores, no such rings have been identified and in fission yeast, the DAM/DASH proteins are nonessential (McIntosh, 2005; McIntosh et al., 2008). Within HeLa kinetochores, the bent configuration of the Ndc80 complex and its lateral linkage by an elongated Mis12 complex to the hKnl1 homolog (Figure 6E) could act in place of a ring for transmitting pulling forces generated by curling protofilaments to the inner kinetochore (Figure 6D).

EXPERIMENTAL PROCEDURES

HeLa cells grown on coverslips were typically fixed with 2%–4% formaldehyde, permeabilized with 0.5% Triton X-100, and labeled with primary antibodies to two different kinetochrome protein epitopes followed by Alexa 488 and Red-X-labeled secondary antibodies (Jackson Labs, West Grove, PA, USA), then immersed in mounting media (95% glycerol/0.5% n-propyl gallate) and sealed to a slide with nail polish (DeLuca et al., 2006). The source and application details for 19 different primary antibodies are given in Supplemental Experimental Procedures. PtK2 cells expressing GFP-tubulin were processed for sister kinetochores in HeLa cells grown on coverslips were typically fixed with 2%–4% formaldehyde, permeabilized with 0.5% Triton X-100, and labeled with primary antibodies to two different kinetochrome protein epitopes followed by Alexa 488 and Red-X-labeled secondary antibodies (Jackson Labs, West Grove, PA, USA), then immersed in mounting media (95% glycerol/0.5% n-propyl gallate) and sealed to a slide with nail polish (DeLuca et al., 2006). The source and application details for 19 different primary antibodies are given in Supplemental Experimental Procedures. PtK2 cells expressing GFP-tubulin were processed for the Delta calculations averaged from sister kinetochrome pairs are described in Results, and further details, including the method used for correcting the effects of tilt, are explained in Supplemental Experimental Procedures. As a test of our Delta assay, we measured the separation distances between red and green centroids of individual kinetochores using the SHREC method developed by Churchman and coworkers (Churchman et al., 2005) as described in Supplemental Experimental Procedures and Figure S5.

To measure the position of kMT ends relative to Hec1-9G3, PtK2 cells expressing GFP-α-tubulin were placed at 6°C for 4–6 hr to stabilize kMT fibers and depolymerize all non-kMTs (Rieder, 1981). For green kinetochrome fibers in the plane of focus, line scans were made down the axis of the fiber through the apparent center of the Hec1-9G3 red fluorescence. The centroid of the 9G3 fluorescence was accurately determined by the Gaussian fitting method, and its position along the line scan was set to zero nm. The amplitudes of line scans were normalized on a scale from 0 to 1 so that they could be plotted on the same graph. The average position of the ends of the kMTs relative to the 9G3 origin along the x axis was obtained by the best fit to the normalized line scans of the equation $y = (1 - erf[x - a])b/2$, where “erf” is the error function and the coefficient “a” is the mean distance where $y = 0.5$. See Results and Supplemental Experimental Procedures for more details.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, 3 tables, and 11 figures and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(09)00372-9.

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Supplemental Data

Protein Architecture of the Human Kinetochore Microtubule Attachment Site

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Supplemental Experimental Procedures

Cell Culture and Drug Treatment

HeLa and PtK2 cells were cultured as described previously (DeLuca et al., 2005; Howell et al., 2000). For nocodazole experiments, cells were incubated in DMEM (Gibco, Carlsbad, CA) containing 20μM nocodazole for 20 min before fixation. For taxol experiments, cells were incubated in DMEM (Gibco) containing 10μM taxol for 1 h before fixation. Reductions in K-K stretch and Delta measurements to their 1 hour values due to taxol were seen in as little as 5 minutes (data not shown).

Spc24 and Spc25 Antibody Epitope Mapping

Peptide arrays (containing peptides of 15 amino acids in length with a 7 amino acid overlap) covering the entire human Spc24 and Spc25 sequences were generated (New England Peptide, Gardner, MA). Peptides were spotted on nitrocellulose and
subjected to an immunoblot using polyclonal antibodies raised to Spc24 and Spc25 (McCleland et al., 2004).

**Antibodies**

Primary antibodies were diluted as follows in 5% boiled donkey serum: Hec1 9G3 monoclonal 1:500 (raised in mouse) (Abcam, Cambridge, MA); anti-Spc24 and -Spc25 (raised in rabbit) at 1:500 (Dr. P.T. Stukenberg, University of Virginia, McCleland et al, 2004); anti-CENP-A, and -CENP-T (rabbit) at 1:1000 (Dr. Aaron Straight, Stanford University Medical Center); anti-Bub1 (raised in sheep) and -CENP-F (rabbit) at 1:1000 (Dr. Stephen Taylor, University of Manchester); anti-hKnl1(Blinkin) mid-molecule epitope, -hKNL3h, and -hNsl1/DC31 at 1:700, anti-hKnf1/PMF1 (all rabbit) at 1:500; anti-Mis12, -CENP-I, and -CENP-C (all rabbit) at 1:500, anti-CENP-E 6A, -CENP-E Hx1, and -CENP-F at 1:1000; anti-hKnl1(Blinkin) N-terminus (mouse) at 1:20 (Dr. Mitsuhiro Yanagida, Kyoto University); and anti-GFP (rabbit) at 1:500 (Abcam). See Supplemental Table 2 for more details. All secondary antibodies (Jackson Immunoresearch, West Grove, PA) were used at 1:200 dilution in 5% boiled donkey serum.

**Immunofluorescence and Fluorescence Imaging**

Immunofluorescence using 4% paraformaldehyde as fixative was carried out as described previously (Howell et al., 2000), with the exception of primary antibodies to Spc24, Spc25, CENP-T, CENP-C, CENP-I, CENP-E 6A, CENP-E HX1, and CENP-F being fixed in 2% paraformaldehyde.
In short, cells were permeabilized in 0.5% Triton X-100 in PHEM buffer, fixed for 20 min in paraformaldehyde, rinsed, blocked in 5% boiled donkey serum at room temperature for 30 min, and incubated overnight in primary antibody diluted in 5% BDS at 4°C. The next morning, cells were rinsed, incubated in secondary antibodies at 1:200 dilution in 5% boiled donkey serum, rinsed, counterstained with DAPI, rinsed, and mounted on coverslips in 95% glycerol/0.5% n-propyl gallate mounting media (refractive index 1.46) (Cimini et al., 2001).

Anti-CENP-A staining required immunofluorescence using methanol as fixative as follows: Cells were placed in -20°C methanol followed by blocking in AbDil (1XTBS + 2% BSA, 0.1% Triton-X100, 0.1% NaN3). Primary antibodies diluted in AbDil were incubated overnight at 4°C. Coverslips were washed four times in AbDil. Secondary antibodies were applied to the cells for 45 minutes followed by three washes in AbDil. Cells were then placed in Hoechst diluted in AbDil followed by two washes in 1X TBS + 0.1% Triton-X. Coverslips were sealed in 95% glycerol/0.5% n-propyl gallate mounting media (Cimini et al., 2001).

For all samples, digital images were acquired using a Yokogawa (Tokyo, Japan) CSU10 Spinning Disk Confocal Fluorescence microscopy system (Maddox et al., 2003), a Nikon (Melville, NY) 100X 1.4 NA DIC Apochromatic objective, and a Hamamatsu (Hamamatsu, Japan) Orca AG cooled charge coupled device (CCD) camera at a magnification of 65 nm/pixel at the detector. Metaphase cells with metaphase plates perpendicular to the coverslip surface were identified by eye, and then red and green
image pairs were acquired at 200 nm intervals along the Z-axis through the cell to obtain two-color 3D image stacks.

**Delta Assay**

*Centroid analysis:* To obtain the centroids of red and green fluorescent labels at kinetochores of sister pairs, we used nonlinear curve-fitting methods (*lsqcurvefit* in MATLAB, The Mathworks, Natick, MA) that apply to our 3D image stacks least-square curve fitting with a 3D Gaussian function (Thomann et al., 2002). This was accomplished with a customized MATLAB program with graphical user interface (GUI) that was developed to make it easy to scroll along the z-axis through the 3D image stacks and identify sister kinetochore pairs near the same plane of focus for semi-automated analysis of the 4 centroid positions (i.e. a red and green one at each sister). The fitting volume for an individual kinetochore’s fluorescence was initially set by estimation, typically 7*7 pixels and 5 frames. After the first fitting, the area was adjusted based on size from the fitting results. Then the fitting was performed again under the adjusted area. The 3D Gaussian function reports independent variances for x, y and z dimensions. Rotation transformation was also introduced to the Gaussian function. The independent variances and rotation transformation made the fitting more accurate than a simple Gaussian function. Most kinetochore fluorescence had a peak intensity of 200-400 counts (equivalent to >16000 photons collected from the entire kinetochore) above a low noise background yielding high values for the peak signal to noise ratio, (SNR > ~30). That put the accuracy of an individual centroid measurement within less than 5 nm (Thomann et al., 2002, Churchman et al., 2005, Churchman and Spudich, 2008).
Primary Delta measurement with chromatic aberration correction:

In order to correct for chromatic aberrations of the microscope, which can vary between sister kinetochore pairs, the separation distance (Delta) was calculated as an average of a sister kinetochore pair as described in Fig. 1D, where Delta is calculated from the projections of the mean separation of protein labels within each kinetochore onto the inter-kinetochore axis, which was usually determined by a line through the Hec19G3 centroids.

Tilt correction: Fluorescent images of kinetochores were frequently elliptical in cross-section because the face of the kinetochore was sufficiently wider than the kinetochore depth along the inner-outer axis. They often appeared tilted to the inter-kinetochore axis (Fig. 1B; Supp. Fig. 3A).

The 3D Gaussian function reported the orientations of the major and minor axes of symmetry for each kinetochore fluorescent label. These orientations were used to determine the tilt of the face of the kinetochore, theta (θ), relative to the inter-kinetochore axis. We found for each kinetochore that the tilt angle for the red fluorescence was always nearly equal to the green fluorescence (Supp. Fig. 3B-C), indicating that all the protein linkages inbetween the red and green labels were oriented in the same way.

The mean value of θ for a sister kinetochore pair was calculated by averaging the four tilt angles, two green tilt angles and two red tilt angles. Each tilt angle was measured
by the angle between the perpendicular to the K-K axis and the long axis of the
kinetochore fluorescence. The orientation of the long axis was obtained from the
Gaussian fitting method, which also yielded the dimensions of the kinetochore
fluorescence. Some kinetochore fluorescent images appeared round. Some had a more
elliptical shape. The orientation of a round image was difficult to determine from the
fitting method. Therefore, the tilt angles from round images were excluded from the tilt
analysis. The threshold was set at 1.1 of the ratio between the dimensions of long axis
and short axis.

Tilt was a source of error in our Delta measurements only if it was produced by
inclination of kMTs and their linkages to the K-K axis (Θi in Supp. Fig. 3Di, inclination
tilt). On the other hand, tilt of the kinetochore face can be produced when kMTs and
their linkages within the kinetochore are parallel to the sister-sister axis, but the kMTs
end at different positions as shown in Supp. Fig 3Diii (sheared tilt). This tilt, nor random
differences in the relative positions of kMT ends within untilted kinetochores (Supp Fig.
3Dii) do not reduce Delta measurements from their true value if the stagger is less than
about 75% of the radius of the Airy Disk (~150 nm for green light). This is because the
linkages are parallel to each other and parallel to the K-K axis.

To obtain a mean value of Delta for a given pair of fluorescent labels corrected
for inclination tilt, we plotted Delta values from sister kinetochore pairs with non-round
fluorescent images versus their mean tilt angle (Supp. Fig. 7). We used least square
fitting of the plots with the function $x = A * \cos(\text{Flt} * \Theta)$, where A is the average Delta
value corrected for inclination tilt ($\Theta = 0$), and $Flt$ is the average fraction of tilt that is inclination tilt (Supp. Fig. 7, Supp. Table 1).

In controls, tilt of the face of the kinetochore occurred with an average $\Theta = 15^\circ$, with ~50% of that value due to inclination tilt. This generated only a 1% correction. In taxol treated cells, inclination-tilt was more significant and Delta corrections of as much as 15% were required in a few cases (Supp. Table 1).

**Accuracy:** In addition to the tests described for the Ndc80 complex in the Results (Fig. 1E), for each average Delta value corrected for tilt we obtained the 95% confidence limits about the mean using *t* test in MATLAB (Table 1, Supp. Fig. 11). For pairs of average Delta values separated by 3 nm or greater, the probability they were derived from the same data population was $p<.02$.

**Protein co-localization within individual kinetochores of sister pairs.** Our Delta calculation assumes that the separation between a pair of labels is the same for sister kinetochores. To test this assumption, we applied the single molecule high resolution co-localization (SHREC) methods developed by Churchman, Spudich, and colleagues (Churchman et al., 2005, Churchman et al., 2006, Churchman and Spudich, 2008) for protein complexes bound to coverslip surfaces. The accuracy of this measurement depends on correction of the lateral chromatic aberration by registration of the red and green images. We first did this by imaging multi-spectrum 175nm beads (TetraSpeck, Invitrogen) bound to the objective coverslip surface. Supp. Table 3 shows the average dx
and dy value between the centroids for red and green fluorophores. There was little variation within the center of the field of the camera (Supp. Table 3, SD = ~3nm) where we obtained cell images and the standard deviations were very small. We next fixed HeLa cells and then labeled 9G3 antibody with Rhodamine Red-X and Alexa-488 labeled secondary antibodies that produced nearly equal fluorescent levels. This specimen gave slightly different average dx and dy values from the beads (Supp. Table 3), probably because of the differences in fluorescent spectra. We used these average dx and dy values for the red and green labels of the 9G3 primary antibody to scale the corresponding bead values bound to the objective coverslip surface in the image registration procedure used for SHREC. As described by Churchman and colleagues, we used the lateral chromatic aberration of the bead field to develop a two-dimensional transform for correcting local chromatic aberration of our experimental images as described in Supp. Fig. 5 A-C. This transform, based on a local weighted mean, had a target registration error (TRE, Churchman et al., 2005) of 5.8 nm. Supp. Figure 5D shows a sub-sample of the vectors separating 9G3 and Spc24-C labels of individual kinetochores after this registration. The lengths of these vectors look very similar for sister kinetochores. To test this for the whole population of sister kinetochores (n=170), for each pair we took the center position between the red 9G3 labels as the origin and the line linking the 9G3 labels as the K-K, x-axis. We then plotted the position of the center between the Spc24-C labels relative to the center (origin) of the 9G3 labels along the K-K axis as diagramed in Supp. Fig 5E to test if the variance along the K-K axis was significantly different than expected from measurements of the distance between 9G3 and Spc24-C within each kinetochore. We
made similar tests for 9G3 vs. CENP-A-GFP, 9G3 vs. CENP-I, and the 9G3 Rhod Red-X/Alexa 488 double label experiment as described in detail in Supp. Fig. 5 legend.

**Localization of the Kinetochore Microtubule End**

For measurements of kMT ends relative to Hec1, PtK2 cells stably transfected with GFP-α-tubulin (Rusan et al, 2001) were seeded on glass coverslips in six-well plates approximately 72 hrs prior to fixation. On the day of processing, coverslips were placed in a 6°C cooler for 4-6 hours to stabilize kMT fibers and depolymerize all non-kMTs. (Rieder, 1981)

After cold-stabilization, cells were processed as above for immunofluorescence with 2% paraformaldehyde as fixative. All steps until blocking were at 6°C to prevent regrowth of microtubules. Coverslips were blocked for 30 min at 37°C in 5% boiled donkey serum. Primary antibody incubation for Hec1 at kinetochores followed for 30 min at 37°C at a dilution of 1:600 in 5% BDS. Cells were washed 3 times in PHEM + 0.5% Triton-X 100 for 5 min each. For secondary antibody labeling, cells were incubated for 30 min at 37°C at a dilution of 1:200 in 5% BDS. Cells were washed 3 times in PHEM + 0.5% Triton-X 100 for 5 min each, counterstained with DAPI to visualize chromatin for 2 min, and washed three more times for 5 min each.

Cells could not be mounted and sealed on slides because traditional mounting techniques alters the GFP signal. Instead, cells were imaged in modified Rose chambers.
minus the top coverslip. The chamber was filled with PHEM buffer and the top was sealed with a 25 mm circular glass coverslip. Cells were imaged as above.

**Data Analysis of kMT Fiber Linescans**

For analysis, image stacks were introduced into a custom MATLAB algorithm. Linescans were taken down the axis of microtubule fibers through the estimated center of the Hec1 fluorescence. The centroid of the Hec1 fluorescence was then determined by a Gaussian fitting method. For a bundle of kMTs with ends all occurring at the same position along the central axis of the bundle, ends are located at the point along the axial intensity profile where the fluorescence is 50% of the value above background along the axis. Axial spread in fluorescence intensity at ends is produced by diffraction within the objective that produces Airy Disk images of point sources of light. Ideally the end intensity profile will be a sigmoid of width equivalent to the diameter of the Airy Disk, which is ~420 nm in our system for green GFP fluorescence. Tilted fibers or fibers having variable ending positions produce further spreading of the fluorescence drop at the end of the kMT bundle, so they were excluded.

Since fluorescence intensity varied from cell to cell and coverslip to coverslip, the fluorescence intensity variations of the GFP-tubulin fibers over the length of the linescans were normalized on a scale from 0 to 1 so that they could be fairly compared to one another. Normalized fluorescent intensities for each kMT fiber sampled were plotted
together on the Y-axis versus the position of the Hec1 centroid on the X-axis. The
resulting sigmoid was fit using the equation $y = (1 - \text{erf}((x-a)/b))/2$, with “erf” being the
error function. Coefficient “a” when $y = 0.5$ is treated as the mean distance in nm of the
end of kMT fibers from the Hec1 centroid.
Table S1. Summary of Delta Measurements.

Results before tilt correction (Delta_uc and its SD) and after (Delta), as well as the tilt factor (Flt), 95% confidence intervals, and changes by tilt correction (|Delta| - |Delta_uc|) are listed.

|         | Delta_uc | SD  | n  | SEM | CI  | A  | Flt | Delta | SD  | n  | |Delta|  
|---------|----------|-----|----|-----|-----|----|-----|--------|-----|----|-----|
| control |          |     |    |     |     |    |     |        |     |    |     |
| Spc24-C | 44       | 7   | 176| 0.5 | 1  | 45 | 0.79| 45     | 6   | 107| 1   |
| Bub1-NM | 27       | 8   | 182| 0.6 | 1.2| 26 | 0   | 26     | 8   | 123| -1  |
| Cenp-A-GFP | 105 | 14  | 172| 1.1 | 2.2| 107| 0.49| 107    | 14  | 116| 2   |
| Spc25   | 44       | 4   | 147| 0.3 | 0.7| 45 | 0.69| 45     | 4   | 131| 1   |
| KNL3/hMis13/Dsn1-C | 46 | 7   | 276| 0.4 | 0.8| 47 | 0.62| 47     | 7   | 159| 1   |
| hNsl1/DC31/hMis14  | 48  | 5   | 97 | 0.5 | 1  | 49 | 0.84| 49     | 5   | 80 | 1   |
| hNnf1/PMF1        | 56  | 6   | 141| 0.5 | 1.1| 56 | 0.47| 56     | 7   | 64 | 0   |
| Mis12             | 47  | 6   | 149| 0.5 | 0.9| 48 | 0.71| 48     | 6   | 134| 1   |
| hKNL1/AF15q14/Blinkin-M | 34 | 6   | 77 | 0.7 | 1.4| 34 | 0   | 34     | 6   | 63 | 0   |
| CENP I            | 61  | 8   | 115| 0.8 | 1.5| 62 | 0.78| 62     | 7   | 69 | 1   |
| CENP T            | 59  | 5   | 210| 0.4 | 0.7| 59 | 0.59| 59     | 5   | 120| 0   |
| CENP C            | 79  | 10  | 231| 0.6 | 1.2| 79 | 0.52| 79     | 10  | 141| 0   |
| Cenp E-MC         | -5  | 18  | 122| 1.6 | 3.2| -3 | 1   | -3     | -16 | 64 | -2  |
| Cenp E-NM         | -11 | 14  | 209| 1   | 1.9| -13| 1.51| -13    | -15 | 107| 2   |
| CLASP             | -29 | 9   | 22 | 1.9 | 3.7| -29| 0   | -29    | -29 | 9  | 18  |
| 9G3 Red vs Green  | 34  | 4   | 117| 0.4 | 0.8| 0  | 0.02| 0      | 0   | 91 | 0   |
| Cenp F-C          | -4  | 10  | 172| 0.7 | 1.4| -4 | 0   | -4     | -4  | 97 | 0   |
| Cenp F-M          | -46 | 11  | 121| 1   | 1.9| -48| 0.19| -48    | -48 | 10 | 42  |
| Hec1-GFP          | 2   | 8   | 121| 0.7 | 1.4| 3  | -3  | 3      | 7   | 44 | 1   |
| taxol             | 0   | 4   | 117| 0.4 | 0.8| 0  | 0.02| 0      | 0   | 91 | 0   |
| Spc24-C           | 45  | 11  | 167| 0.8 | 1.7| 45 | 0.46| 45     | 11  | 46 | 0   |
| Bub1-NM           | 17  | 13  | 64 | 1.6 | 3.2| 18 | 0   | 18     | 14  | 28 | 1   |
| Cenp-A-GFP        | 72  | 17  | 129| 1.5 | 2.9| 78 | 0.88| 78     | 15  | 61 | 6   |
| Spc25             | 44  | 17  | 37 | 2.8 | 5.5|    |     |        |      |    |     |
| KNL3/hMis13/Dsn1-C | 30 | 12  | 157| 1   | 1.9| 35 | 1.14| 35     | 12  | 63 | 5   |
| hNsl1/DC31/hMis14 | 42  | 11  | 93 | 1.1 | 2.2| 49 | 1.03| 49     | 12  | 35 | 7   |
| hNnf1/PMF1        | 52  | 9   | 68 | 1.1 | 2.2| 54 | 0.94| 54     | 8   | 39 | 2   |
| Mis12             | 38  | 11  | 111| 1   | 2  | 44 | 1.34| 44     | 12  | 66 | 6   |
| hKNL1/AF15q14/Blinkin-M | 24 | 10 | 93 | 1 | 2 | 24 | 0.83| 24     | 10  | 40 | 0   |
| CENP I            | 47  | 9   | 83 | 1   | 2  | 47 | 0.88| 47     | 8   | 28 | 0   |
| CENP T            | 41  | 11  | 140| 0.9 | 1.8| 43 | 0.76| 43     | 11  | 29 | 2   |
| CENP C            | 56  | 11  | 106| 1.1 | 2.2| 57 | 0.38| 57     | 11  | 51 | 1   |
| Cenp E-MC         | -8  | 19  | 79 | 2.1 | 4.2| -12| 1.89| -12    | -12 | 1  |     |
| Cenp E-NM         | -35 | 17  | 63 | 2.1 | 4.2| -33| 0   | -33    | -33 | 18 | 19  |
| CLASP             | -40 | 11  | 11 | 3.4 | 6.6| -40| 0.83| -40    | -40 | 12 | 10  |
| Cenp F-C          | -14 | 15  | 127| 1.4 | 2.7| -14| 0   | -14    | -14 | 17 | 50  |
| Cenp F-M          | -60 | 20  | 98 | 2   | 3.9| -57| 0   | -57    | -57 | 18 | 39  |

Table S1. Summary of Delta Measurements.
Table S2 – Antibody/reagent detail

<table>
<thead>
<tr>
<th>Protein</th>
<th>Antibody</th>
<th>Antigen</th>
<th>MW (kDa)</th>
<th>Coiled-Coil Domains</th>
<th>AA #</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CENP-A</td>
<td>Rabbit anti CENP-A</td>
<td>Full Length</td>
<td>17</td>
<td>Weak or none</td>
<td>140</td>
<td>Aaron Straight (unpublished)</td>
</tr>
<tr>
<td>CENP-A-GFP</td>
<td>Rabbit anti GFP</td>
<td>GFP</td>
<td>17</td>
<td>Weak or none</td>
<td>140</td>
<td>Jan Ellenberg (Gerlich et al., 2003)</td>
</tr>
<tr>
<td>CENP-C</td>
<td>Rabbit anti CENP C</td>
<td>unknown</td>
<td>140</td>
<td>Weak or none</td>
<td>943</td>
<td>Tim Yen (Liu et al., 2006)</td>
</tr>
<tr>
<td>CENP-I</td>
<td>Rabbit anti CENP I</td>
<td>N-term 249</td>
<td>87</td>
<td>Weak or none</td>
<td>756</td>
<td>Tim Yen (Liu et al., 2003)</td>
</tr>
<tr>
<td>CENP-T</td>
<td>Rabbit anti CENP T</td>
<td>Full Length</td>
<td>34</td>
<td>320-360</td>
<td>561</td>
<td>Aaron Straight (unpublished)</td>
</tr>
<tr>
<td>hNnf1/PMF1</td>
<td>Rabbit anti hNnf1/PMF1</td>
<td>Full Length</td>
<td>23.3</td>
<td>Weak or none</td>
<td>220</td>
<td>Arshad Desai (Kline et al., 2006)</td>
</tr>
<tr>
<td>hNsl1/DC31/hMis14</td>
<td>Rabbit anti hNsl1/DC31</td>
<td>Full Length</td>
<td>22-281</td>
<td>32.2</td>
<td>281</td>
<td>Arshad Desai (Kline et al., 2006)</td>
</tr>
<tr>
<td>Mis12</td>
<td>Rabbit anti hMis12</td>
<td>Full Length</td>
<td>24.1</td>
<td>100-150, 175-205</td>
<td>205</td>
<td>Tim Yen (Liu et al., 2006)</td>
</tr>
<tr>
<td>KNL3/hMis13/Q9H410/Dsn1-C</td>
<td>Rabbit anti hKNL3 (hDsn1)</td>
<td>Full Length</td>
<td>181-356</td>
<td>40.1</td>
<td>356</td>
<td>Arshad Desai (Kline et al., 2006)</td>
</tr>
<tr>
<td>hKNL1/AF15q14/Blinkin-M</td>
<td>Rabbit anti hKNL1</td>
<td>~1220-1440</td>
<td>265.3</td>
<td>1900-2200</td>
<td>2342</td>
<td>Arshad Desai (Cheeseman et al., 2008)</td>
</tr>
<tr>
<td>Knl1/Blinkin/AF15q14-N</td>
<td>Mouse anti Blinkin</td>
<td>N-term 22</td>
<td>265.3</td>
<td>1900-2200</td>
<td>2342</td>
<td>M. Yanagida (Kiyomitsu et al., 2007)</td>
</tr>
<tr>
<td>Bub1-NM</td>
<td>Sheep anti Bub1</td>
<td>336-489</td>
<td>123</td>
<td>275-300, 1050-1100</td>
<td>1085</td>
<td>Steve Taylor (Taylor et al., 2001)</td>
</tr>
<tr>
<td>Spc24-C</td>
<td>Rabbit anti Spc24</td>
<td>Full Length</td>
<td>22.4</td>
<td>20-130</td>
<td>197</td>
<td>Todd Stukenberg (McCleland et al., 2004)</td>
</tr>
<tr>
<td>Spc25</td>
<td>Rabbit anti Spc25</td>
<td>Full Length</td>
<td>26.1</td>
<td>50-150</td>
<td>224</td>
<td>Todd Stukenberg (McCleland et al., 2004)</td>
</tr>
<tr>
<td>Hec1</td>
<td>Mouse Mab9G3</td>
<td>200-222</td>
<td>73.9</td>
<td>240-420, 460-580,600-642</td>
<td>642</td>
<td>Abcam</td>
</tr>
<tr>
<td>GFP-Hec1</td>
<td>Rabbit anti GFP</td>
<td>GFP</td>
<td>73.9</td>
<td>240-420, 460-580,600-642</td>
<td>642</td>
<td>Walt Gall (DeLuca et al., 2006)</td>
</tr>
<tr>
<td>CENP-F-M</td>
<td>Sheep anti CENP F</td>
<td>1363-1640</td>
<td>367</td>
<td>1-1400, 1550-1700, 1800-2900</td>
<td>3114</td>
<td>Steve Taylor (Hussein et al., 2002)</td>
</tr>
<tr>
<td>CENP-F-C</td>
<td>Rabbit anti CENP F</td>
<td>C-term 561</td>
<td>367</td>
<td>1-1400, 1550-1700, 1800-2900</td>
<td>3114</td>
<td>Tim Yen (Liao et al., 1995)</td>
</tr>
<tr>
<td>CENP-E-NM</td>
<td>Rabbit anti CENP E 6A</td>
<td>663-973</td>
<td>312</td>
<td>300-400, 500-2600</td>
<td>2663</td>
<td>Tim Yen (Zecevic et al., 1998)</td>
</tr>
<tr>
<td>CENP-E-MC</td>
<td>Rabbit anti CENP E Hx1</td>
<td>1571-1859</td>
<td>312</td>
<td>300-400, 500-2600</td>
<td>2663</td>
<td>Tim Yen (Chan et al., 1998)</td>
</tr>
</tbody>
</table>

Table S2. Antibody and Reagent Detail.
Sources of antibodies as well as regions labeled within proteins are shown and cited where applicable (see Supplementary References).
Table S3. Average Chromatic Aberration Measurements.

<table>
<thead>
<tr>
<th></th>
<th>dx (SD) (nm)</th>
<th>dy (SD) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beads (coverslip surface)</td>
<td>-28 +/- 3.2</td>
<td>28.4 +/- 3.4</td>
</tr>
<tr>
<td>Centroids of RedX and Alexa 488 bound by antibodies to 9G3</td>
<td>-31 +/- 9.6</td>
<td>32.9 +/- 9.7</td>
</tr>
</tbody>
</table>

Chromatic aberration was measured for beads and for Red-Green labeled Hec1 9G3 kinetochores. See Supp. Methods for details.
Figure S1. Epitope Mapping of Spc24 Antibody.

A peptide array comprising the sequences of human Spc24 and Spc25 were adsorbed onto nitrocellulose and immunoprobed with Spc24 antibody. As a control, HeLa extract was adsorbed onto the nitrocellulose at region G12. The antibody recognized 3 spots in the C-terminal region of Spc24 sequence as well as the positive control. At right is the hSpc24 sequence with recognized peptide sequence in red. Two experiments are shown.
Spc25 antibody

*Spc25 antibody recognizes 2 major spots on the blot: one peptide from Spc24 at amino acids 1-15 and one peptide from Spc25 at amino acids 91-105 (in blue). These two spots were of equal intensity. The antibody recognizes a fainter 2-spot series at amino acids 156-176 (pink) within Spc25. These results are consistent with the second blot, although the spots at C8, C9 (aa 156-176, pink) are more intense.

Spc24

1 maafdieey sagpialga mraearqor ilqheorver lietodgak oretlnmek
61 evaqslinak eqiyhggqve qgpleagfoga geesfirisf kalgtrelka ikeiaadler
121 gekveveddtt vitpsavvy a Wynihvkve welwceopmgm vgkihgoxva qphldstol
181 srlfslhwh sultlew

Spc25

1 mvedelatdf ksnicfvmk fksitsogma girdtykksi kafaektisvkg kkeermvem
61 fleycngqis qhktlgekkd niklliaevx gikqeleult aniqfikeeey srikketsta
121 nkansaenkr kksiedyld rileiky yokhlfith sdpknopsgsf mfsilhneah
181 dyevedsasph leglafljqen virktnatsf lanvkflaf tryn

Expt 1 (G-12 = HeLa XT)

Expt 2 (G-12 = HeLa XT)

Supplementary Figure 2
Figure S2. Epitope Mapping of Spc25 Antibody.
A peptide array comprising the sequences of human Spc24 and Spc25 were adsorbed onto nitrocellulose and immunoprobed with Spc25 antibody. As a control, HeLa extract was adsorbed onto the nitrocellulose at region G12. The antibody recognized 1 spot at the N-terminus of Spc24 sequence and 4 spots near the C-terminus of Spc25. At right are the hSpc24 and hSpc25 sequences with recognized peptide sequences in red, blue, and pink. Two experiments are shown. By immunofluorescence, the Spc25 antibody does not appear to recognize the N-terminal epitope of Spc24 since Delta measurements for anti-Spc24, which recognizes only C-terminal epitopes (Supp Fig. 1) vs. Hec1 9G3 were identical to measurements for anti-Spc25.
Figure S3. Analysis of Tilt for Red vs. Green Labels.
(A) Tilt detection from 3-D Gaussian fitting, shown by blue lines. (B) Tilt angles of 9G3 and Spc24-C of control cells. (C) Tilt angles of red and green labels from all the control cells. (D) Potential tilting of protein linkages: (i) Inclination tilt, $\Theta_i$; (ii) Randomly staggered ends with $\Theta_i = 0$; (iii) Sheared staggered ends with $\Theta_i = 0$. 
Figure S4. Signal Intensity.

(A) Images of 9G3 and Spc24-C. (B) Line scan of 9G3 signal along K-K axis. (C) Line scan of Spc24-C along K-K axis. The images and line scan show a peak signal of 250-350 counts above background noise (SD = 8). This yields a high signal-to-noise ratio of 31-44.
Supplementary Figure 5
Supplementary Figure 5
Figure S5. Co-localization of Fluorescent Labels within Individual Kinetochores of Sister Pairs by SHREC to Test if Distances are the Same and Equal to Values Measured in the Delta Assays.

(A) Red to green vectors for bead array bound to the objective coverslip surface before registration. The vector length was drawn 20 times its actual length for better visualization. (B) Vectors after registration (80 times actual vector length). (C) Polar plot for both unregistered (blue) and registered (red) vectors. (D) Vectors of 9G3 to Spc24 for individual kinetochores of sister pairs in a sample of the population analyzed after correction for lateral chromatic aberration as described in Supp. Methods (8 times actual vector length). (E) A diagram showing the vector (Vm) between the middle point of the centroids of 9G3 labels on sister kinetochores and the middle point of the centroids of Spc24-C labels for the same pair; the K-K axis extends between the 9G3 labels for each sister pair. (F) Vm vector plot for 9G3 and Spc24-C. (G) Vm vector plot for 9G3 and CENP-A-GFP. (H) Comparison among different measurements. The distance between 9G3 and the three other protein labels was measured for individual kinetochores by the SHREC method (Churchman and Spudich, 2005, 2008) with Maximum Likelihood (Churchman et al., 2006) correction. The results were very similar to the Delta measurements obtained from sister kinetochore pairs. However, errors introduced by the registration to correct for lateral chromatic aberration (TRE = 5.8, see Supp. Methods) made the SD of the SHREC distance higher than the SD of Delta for all 3 protein measurements. The standard deviation (SD) of the Vm vector projection on the K-K axis (Vm_kk) was calculated and compared with the standard deviation of Delta. Similar tests were also conducted for Spc24 and CENP-I. Almost identical results for Spc24 and CENP-I (rigid by Delta analysis) shows no clear difference within a sister pair showing they exhibit the same values. The lower SD for CENP-A-GFP (compliant by Delta analysis) is particularly significant. This shows that the sister kinetochores exhibit the same compliance since the middle point of CENP-A-GFP tends to stay in the same place over a wide range of stretch and the SD for center movement is smaller than the SD of Delta.
Figure S6. Maps of Key Epitopes on Large Proteins.
Graphical representations of antigens and key structural features relevant to our measurements for (A) hKnl1/Blinkin; (B) CENP-E; (C) CENP-F; and (D) Bub1.
For further antibody information, see Supplementary Methods and Supplementary Table 2.
Supplementary Figure 7A
Delta vs theta (control)
Figure S7. Tilt Plots for All Measurements.
Delta before tilt correction (nm, Y-axis in all plots) was plotted as a function of tilt angle theta (degrees, X-axis in all plots) for a subset of the data where tilt angle could be identified. Least-square fitting lines for each protein linkage to function $x = A \cdot \cos(\text{Flt} \cdot \text{theta})$ are plotted onto figures, with $A$ corresponding to intercept at theta = 0. $A$ equals the average value of Delta corrected for inclination tilt. (A) Control measurements. (B) Measurements for cells treated with 10 μM taxol for 1 hr prior to fixation.
Supplementary Figure 8A
Delta vs K-K distance (control)
Figure S8. Delta Plots for All Measurements.
Delta before tilt correction (nm, Y-axis in all plots) was plotted as a function of K-K measured between Hec1 9G3 centroids of sister kinetochores (μm, X-axis in all plots). (A) Control measurements. (B) Measurements for cells treated with 10 μM taxol for 1 hr prior to fixation.
Supplementary Figure 9

Figure S9. KMT Fiber Orientation.
Histogram showing uniform distribution of kMT fiber orientation angle relative to the horizontal image axis for the data set. Even sampling of fibers from all orientations ensured additional measurement errors due to chromatic aberrations in the optics were minimal.
A  Half-Centromere Length During Oscillations

- KMN-CCAN Minimum
- CENP-A + CENP-C Average
- CENP-A Maximum

1000 nm

B  Adjacent Attachment sites at Low Magnification

- Inner Plate
- GAP
- Outer Plate

Supplementary
Figure 10
Figure S10. Centromeric Chromatin Compliance and Assembly of Multiple Attachment Site Kinetochores.

A. Depth and Compliance of CENP-A and CENP-C within the peripheral centromeric chromatin in comparison to the stiff kinetochore during chromosome oscillations. Using CENP-I as a marker for the peripheral surface of the centromeric chromatin, at minimal centromere stretch, the separation between the centroids of CENP-I and CENP-C was ~11 nm and the centroids of CENP-I and CENP-A ~30 nm. Assuming uniform distribution, these numbers indicate total depths of 22 nm for CENP-C and ~60 nm for CENP-A. At maximal centromere stretch, these depths increase to 46 nm and 128 nm respectively. The region of the centromere containing CENP-A appears to be about twice the stiffness of the bulk of the centromeric chromatin.

B. Speculative Model for how the Kinetochore is Built from Multiple kMT Attachment Sites. Kinetochores with multiple attachment sites are constructed from a two-dimensional parallel array of chromatin fibers that extend along the K-K axis at metaphase, each with kinetochore protein complexes assembled at their peripheral tips. To account for the anisotropic properties of the kinetochore and peripheral centromere (strong along the inner-outer axis and weak laterally), we suggest that a kinetochore microtubule attachment site is primarily linked to one or a few chromatin fibers at their peripheral tips where the path of the DNA changes from an outside to an inside direction (Yeh et al., 2008). Mechanical anisotropy at the centromere periphery results from weak lateral linkages (yellow rectangles) between neighboring chromatin fibers and potentially in between their kMT attachment sites. There are several points of similarity of the proposed side-by-side attachment sites to published high resolution tomographs (Dong et al., 2007). These include the low contrast gap, and a 45 nm thick outer plate mainly defined by the 45 nm axial length of the bent Ndc80 complex. The horizontal arms of the Ndc80 and Mis12 complexes could produce the horizontal filaments reported near the inner surface of the outer plate in electron micrographs (Dong et al., 2007).
Supplementary Figure 11

Figure S11. Statistical Analysis of Average Delta Values.
Statistical significance between any two different delta measurements was calculated by paired t-test (*ttest2* in MATLAB). The difference between the two measurements was characterized by their mean value difference. Statistical significance was represented by a number between 1 and 0 (the p-value).
Supplemental References


