In Vivo Protein Architecture of the Eukaryotic Kinetochore with Nanometer Scale Accuracy

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Summary

The kinetochore is a macromolecular protein machine [1] that links centromeric chromatin to the plus ends of one or more microtubules (MTs) and segregates chromosomes during cell division. Its core structure consists of eight multi-component protein complexes, most of which are conserved in all eukaryotes. We use an in vivo two-color fluorescence microscopy technique to determine, for the first time, the location of these proteins along the budding yeast kinetochore axis at nanometer resolution. Together with kinetochore protein counts [2, 3], these localizations predict the 3D protein architecture of a metaphase kinetochore-microtubule attachment and provide new functional insights. We also find that the kinetochore becomes much shorter in anaphase as metaphase tension is lost. Shortening is due mainly to a decrease in the length of the Ndc80 complex, which may result either from intramolecular bending of the Ndc80 complex at the kink within the stalk region of the Ndc80-Nuf2 dimer [4, 5] or from a change in its orientation relative to the microtubule axis. Conformational changes within the Ndc80 and Mtw1 complexes may serve as mechanical cues for tension-dependent regulation of MT attachment and the spindle-assembly checkpoint. The geometry of the core structure of the budding yeast kinetochore reported here is remarkably similar to that found in mammalian kinetochores, indicating that kinetochore structure is conserved in eukaryotes with either point or regional centromeres.

Results and Discussion

The budding yeast kinetochore is nucleated by one centromeric nucleosome containing the centromere-specific histone H3 variant Cse4 [6]. The centromere also binds the DNA-binding protein Mit2p and the CBF3 complex. Genetic, structural, and biochemical studies show that this assembly is stably linked to one microtubule (MT) plus end by a network of protein complexes comprising the Ctf19 complex [6], the Mtw1 complex [7, 8], the Spc105-Ydr532c complex [8], and the MT-binding Ndc80 complex [9, 10]. The MT-associated protein complex Dam1-DASH [11, 12] is also necessary for MT attachment. With the exception of the CBF3 and Dam1-DASH complex, these protein complexes are conserved in all eukaryotes [1, 13]. We have previously shown that the single MT attachment at the point centromere in budding yeast contains a specific number of each core structural protein complex [2]. Kinetochores at regional centromeres with 2–3 MT attachments in fission yeast also have nearly identical protein numbers per MT attachment (with the exception of the Dam1-DASH complex; see [3]), indicating that the protein architecture of individual MT attachment sites at these complex kinetochores is also conserved. The next critical task is determining the organization of these structural protein complexes within a kinetochore-MT attachment in living cells; this organization remains poorly understood because of poor visibility by electron microscopy methods [14].

We have used a two-color, in vivo fluorescence microscopy technique to determine the relative position of budding yeast kinetochore proteins along the kinetochore axis with ~10 nm resolution. Measurements are made pairwise, with one protein fused to EGFP (a green fluorescent protein) and the other fused to tdTomato (a red fluorescent protein [15]). Our technique is based largely on the in vitro method of Single-molecule High-Resolution Colocalization (SHREC [16]) and extends its scope to in vivo measurements. The ability to fuse fluorescent protein genes at the C terminus of budding yeast genes through homologous recombination—a technique not generally available in vertebrates—is critical for obtaining accurate localizations. The well-defined structure of the budding yeast mitotic spindle is also crucial. In metaphase, spindle, sister kinetochores on each chromosome are attached to MT plus ends from opposite poles and stretch their interconnecting chromatin apart by ~800 nm across the spindle equator [17]. The kinetochores from all 16 sister chromosome pairs form two well-separated clusters, on opposite sides of the spindle equator, that appear as nearly diffraction-limited spots when imaged with wide-field fluorescence microscopy (Figure 1A). After spindle elongation in anaphase, the sister kinetochore clusters become separated by >4 μm (average spindle length in our mid- to late-anaphase measurements was 5–6 μm; see Figure S1, available online). In both metaphase and anaphase, kinetochores within the same cluster face the same pole (Figure 1A). At metaphase, opposing pulling forces produced by each pair of sister kinetochores stretch the chromatin between sisters and thus align the kinetochores and the axes of their attached MTs closely with the central spindle axis (Figure S1). In mid to late anaphase, the kinetochore axes can be expected to be roughly perpendicular to the face of the spindle pole body to which they are connected by very short (~60 nm) MTs [17].

We simultaneously recorded red and green images of kinetochore clusters in cells expressing a selected pair of fluorescently labeled kinetochore proteins (Experimental Procedures; Figure 1B). After red-green image registration, the distance separating the centroids of each pair of EGFP and tdTomato spots reflects the average distance separating the labeled kinetochore proteins within a cluster, even if the kinetochores themselves were staggered as much as 150 nm along the spindle axis (Figure S2). The centroids of the EGFP and tdTomato spots were determined within the in-focus plane with accuracy better than 10 nm by fitting of the intensity distribution with a 2D Gaussian function (Figure S3 [16, 18]). Residual error after red-green image registration was 6 nm or less (Supplemental Experimental Procedures, Figure S4). Image registration and the random orientation of spindle axes within the image plane within each data set suppressed any bias due to chromatic aberrations to negligible levels.*Correspondence: ajitj@unc.edu
Measuring the Distance Separating Two Kinetochore Proteins in a Budding Yeast Metaphase Spindle

(A) A budding yeast cell in metaphase (DIC) with fluorescently labeled kinetochores (green) and spindle pole body (blue).

(B) The cartoon depicts arrangement of kinetochores tagged with EGFP (white ovals with green dots) and the other with tdTomato (red dots). Tense chromatin connections (gray dotted lines) between sister kinetochores align them closely with the spindle axis. When such a cell is visualized with wide-field fluorescence microscopy, the two kinetochore clusters (each containing 16 kinetochores) appear as nearly diffraction-limited spots.

(C) Metaphase spindle in a strain that has two kinetochore proteins, one protein fused with EGFP (green dots) and the other with tdTomato (red dots). When such cells are imaged simultaneously in the EGFP and tdTomato channels (lower panel), the offset between the centroids of the EGFP and tdTomato images of a kinetochore cluster can be used for accurately determining the average distance separating the ends of the two proteins.

Scale bar represents ~1 μm in (A) and ~500 nm in (B) and (C); 1 pixel ~107 nm in (B) and (C).

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with both of these data. The Mtw1 complex is also linked to the centromere through the Ctf19 complex [23, 24]. We localized the C termini of three members of the Ctf19 complex [7]—Ctf19p, Ame1p, and Okp1p. Both Ame1p and Okp1p colocalized 13 nm inside the Spc24 C terminus, whereas Ctf19p was located 16 nm inside. These proteins were close to the N terminus of centromeric histone Cse4p, which was 17 nm inside the Spc24 C terminus.

In addition to depending on the Ndc80 complex, MT attachment within the kinetochore outer domain depends on Spc105p and the Dam1-DASH complex. The C terminus of Spc105p, a large, 100 kDa protein, colocalized with the Spc24p C terminus (1 nm inside; Figure 3A). The position of the N terminus of Spc105p could not be accurately determined with the use of the maximum likelihood method, but we estimate it to be 16 nm outside (toward the MT) the Spc24p C terminus (Table S1). This indicates that the protein probably extends outward along the microtubule axis. Surprisingly, we found that Ask1p, a key component of the Dam1-DASH complex, was 12 nm inside the microtubule-binding head domains of the Ndc80 complex (Figure 3A).

There were significant changes in the relative positions of kinetochore proteins from metaphase to anaphase. We found that the overall kinetochore length in late anaphase cells was reduced by 25 nm (Figure 3A, Supplemental Experimental Procedures, Table S2). The end-to-end length of the Ndc80 complex decreased from 55 nm in metaphase to 34 nm in anaphase. Within the Ndc80 complex, the separation between the two ends of the Ndc80-Nuf2 dimer was reduced by 13 nm, whereas the Spc24-Spc25 dimer showed a smaller decrease of 5 nm. Also important was the movement of the Spc24-Spc25 end of the Ndc80 complex 5 nm closer to the centromeric nucleosome (Figure 3A). Components of the Mtw1 complex also showed a significant redistribution; notably, Nsl1p moved closer to the centromeric nucleosome. On the other hand, the position of the Ctf19 complex with respect to the N terminus of Cse4p did not change significantly, suggesting a rigid coupling between this complex and the centromeric nucleosome. It is known that the CBF3 complex binds to the CDE III region [25] of the centromeric DNA via Ndc10p and Cep3p in metaphase. We found that the C terminus of Ndc10p was 35 nm inside the N terminus of Cse4p in anaphase. This large distance is probably due to the anaphase dislocation of Ndc10p from the kinetochore [26]. Finally, the position of the Dam1-DASH complex does not change significantly with respect to the Ndc80 head domain. It should be noted that the average number of Dam1-DASH complex molecules per budding yeast kinetochore decreases from 16–20 in metaphase to 9 in anaphase, which is insufficient for formation of Dam1-DASH rings around the late anaphase MTs [2].

This study assembles the first in vivo, high-resolution map of kinetochore protein localization along the axis of a kinetochore-microtubule attachment (Figure 3A, [27]). It should be noted that these locations reflect average positions of kinetochore proteins. Furthermore, our technique can measure distances in the image plane, and it is insensitive to distance changes that may occur either along the optical axis or perpendicular to the spindle axis. Therefore, positional changes that take place within complexes of unknown shape (such as the Mtw1, Spc105-YDR532c, and Ctf19 complexes)
along these directions could not be detected. The position of
the MT plus end within the kinetochore could not be deter-
determined with our technique. The location of the MT-associated
Dam1-DASH complex suggests that the MT plus end extends
at least 10 nm beyond the contact point between the MT and
Nuf2p-Ndc80p head domains [28]. KNL-1, the C. elegans
homolog of Spc105p [22], and the N-terminal domain of
Spc7, the S. pombe homolog, show MT-binding activity [29].
These data suggest that the MT plus end may extend up to
the Spc24-Spc25 end of the Ndc80 complex.

The localization data can be combined with protein numbers
[2] and existing structural information to predict a 3D visualization
of kinetochore-MT attachment assuming a symmetric
distribution of proteins around the cylindrical MT lattice
(Figure 3B). The end-to-end measurement of the metaphase
length of the Ndc80 complex shows that it binds the MT lattice
while making a small angle with the MT axis, in contrast to the
40° angle made by unbound Ndc80 complexes observed
in vitro. This alignment of the Ndc80 complex and MT axes
can be expected, given that the Ndc80 complex is one of the
primary force generators at the kinetochore and that this force
acts along the MT axis. Available biochemical data suggest
that the contact between the Ndc80 complex and the inner
kinetochore is achieved through interactions of Spc24-Spc25
globular domains with the Mtw1 and Spc105 complexes [22].
Additional points of contact would be necessary for resisting
the pulling forces tending to align the Ndc80 complex along
the MT axis and maintaining its tilted orientation (with respect
to the MT) in metaphase. The model displays a possible mechanism that relies on bending of
the Ndc80-Nuf2p dimer at the kink as observed in vitro.

The possible conformations [11, 30] and functional mechanisms [31, 32] of the Dam1-DASH
complex in vivo are critical questions that remain unanswered.
The Dam1-DASH complex can form oligomeric rings (contain-
ing 16–23 copies) with an inner diameter of 35 nm and an outer
diameter of 45-54 nm around the MT lattice [11, 12].
Structural and theoretical studies also show that in this config-
uration, individual subunits within the ring interact with the MT
lattice via projections that span the ~5 nm gap between the
MT lattice and the inner surface of the Dam1-DASH ring [31,
33]. There are 16–20 DAM1-DASH complex molecules per
budding yeast kinetochore in vivo, enough to build one ring
[2]. If a persistent Dam1-DASH ring structure exists in vivo,
its location within the kinetochore would require the Ndc80

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complex molecules to attach the MT lattice at angles of 50° to 60° to accommodate the Dam1-DASH ring underneath. These large angles are inconsistent with the measured end-to-end length of 55 nm for the Ndc80 complex. Therefore, the Dam1-DASH ring will have to encircle both the MT lattice and the rod domains of Ndc80 complex molecules (Figure 3B). Individual Dam1-DASH monomers can still interact with the MT lattice via the projections spanning the gap between the inner surface of the ring and the MT lattice. This configuration may also promote rapid rebinding of Ndc80 heads to the MT lattice by limiting their diffusion. Alternatively, the Dam1-DASH complex may not form a single ring structure at the kinetochore. Instead, spiral oligomers that incompletely surround the microtubule lattice at several locations along the microtubule axis may assemble (Figure 3B). In this configuration, direct binding between the Dam1-DASH monomers and the Ndc80 complex becomes necessary for their stable association with the kinetochore. Although a direct biochemical link between the Dam1-DASH complex and other kinetochore complexes has not been established, such a linkage is necessary for transmitting the force generated through interactions between the Dam1-DASH complex and the MT lattice to the rest of the kinetochore for the participation of either configuration in force generation [31, 34].

The anaphase measurements reveal tension and/or cell-cycle-dependent changes within the kinetochore (Figure 3C). The reduction in the end-to-end length of the Ndc80 complex in late anaphase indicates that the Ndc80 complex directly participates in force generation and transmits this force to the inner kinetochore components through the Mtw1 complex. The observed decrease may be explained through either intramolecular bending of the Ndc80 complex or a reorientation of molecular bending at the kink domain within the complex, observed in vitro (depicted in Figure 3C), or a reorientation of the entire complex so that it makes an angle of 45° to 50° with the axis of the MT. The latter configuration requires a large extension (~40 nm) of the inner kinetochore complexes perpendicular to the MT axis to stably link the Ndc80 complex back to the inner kinetochore and the centromere. The elongated shapes of the Mtw1 complex and the Ctf19 complex may facilitate such an alignment of the Ndc80 complex in anaphase. The total length of such a linkage in anaphase would predict a much longer distance between the centromere and the Spc24-Spc25 end of the Ndc80 complex under the metaphase pulling forces acting along the axis of the MT. We therefore show the simpler anaphase kinetochore configuration that relies on intramolecular bending of the Ndc80 complex.

Many of the structural proteins and protein complexes are conserved in all eukaryotes [1], although the complex architecture of the regional centromeres probably necessitates significant modifications, especially to the centromere-proximal proteins [35–37]. Architecture of the kinetochore-microtubule attachment site built on either the point or the regional centromere foundation, however, is probably conserved in all eukaryotes, as evidenced by the conserved stoichiometry of kinetochore proteins between point and regional centromeres [2, 3]. Indeed, kinetochore protein localizations obtained by antibody labeling in fixed HeLa cells show a strikingly similar pattern (E.D.S., unpublished data). This conservation of kinetochore protein structure and the protein architecture of the kinetochore-MT attachment demonstrate that the core structure of the kinetochore, along with its basic functional mechanisms in force generation and spindle assembly checkpoint signaling, are conserved throughout eukaryotic phyla.

Experimental Procedures

Strains and Growing Conditions

Strains (Table S3) were grown in complete media, with either glucose or galactose as the carbon source, at 32°C. Proteins were tagged with either EGFP or tdTomato through homologous recombination, mostly at the C terminus, with the use of PCR-amplified cassettes. Cells from mid-log phase cultures were resuspended in synthetic media and immobilized on concanavaline A (cat. no. 7279, Sigma, St. Louis, MO)-coated coverslips for imaging.

Imaging

Cells were imaged at room temperature on a Nikon TE-2000E (Nikon Instruments, Melville, NY) inverted microscope equipped with a 1.4 NA, 100× objective and 1.5× optovar lens (1 pixel ~ 107 nm). A dual-excitation filter set (FITC/TRITC ET set no. 59004, Chroma Technology, Rockingham, VT) was used for simultaneous excitation of both EGFP and tdTomato. Images were acquired with a DX-878B Xon camera (Andor Technology, South Windsor, CT), with the use of the conventional acquisition mode mounted on the bottom port of the microscope. The Dual-View attachment (MAG Bio-systems, Pleasanton, CA) was used for simultaneous acquisition of images at both wavelengths with prewoven dichroic and emission filters for EGFP and tdTomato. Before each experiment, 100 nm TetraSpek (cat. no. T-7279, Invitrogen, Carlsbad, CA) bead images were acquired for image registration (Figure S3). For each cell, 10 image slices were obtained through moving the piezoelectric Z-stage (MadCity Labs, Madison, WI) through 200 nm steps, and a 300×300-pixel-wide, centrally located region was recorded in each image. The exposure time was set at 800 ms per image, for maintenance of a high signal-to-noise ratio with minimal bleaching during image acquisition. The imaging and image acquisition hardware was run by Metamorph 7 (Molecular Devices, Sunnyvale, CA).

Image Analysis

Image analysis was carried out with custom software written in MatLAB 7 (MathWorks, Natick, MD). The tdTomato image stack was registered with the EGFP image stack (described in detail in Figure S3). For centroid determination, the area of interest for centroid localization was determined through placing an 8×8 x pixel region (for metaphase measurements) on an EGFP image such that the cumulative intensity within the centrally located 2×2 pixel square was maximized. The corresponding region from the registered tdTomato image was then extracted for centroid localization. A similarly selected 10×10 pixel region was used for analyzing anaphase cells.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, four figures, and three tables and can be found with this article online at http://www.current-biology.com/supplemental/2009/s0960-9822(09)00809-4.

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