Structural comparison of the Caenorhabditis elegans and human Ndc80 complexes bound to microtubules reveals distinct binding behavior

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ABSTRACT During cell division, kinetochores must remain tethered to the plus ends of dynamic microtubule polymers. However, the molecular basis for robust kinetochore–microtubule interactions remains poorly understood. The conserved four-subunit Ndc80 complex plays an essential and direct role in generating dynamic kinetochore–microtubule attachments. Here we compare the binding of the Caenorhabditis elegans and human Ndc80 complexes to microtubules at high resolution using cryo–electron microscopy reconstructions. Despite the conserved roles of the Ndc80 complex in diverse organisms, we find that the attachment mode of these complexes for microtubules is distinct. The human Ndc80 complex binds every tubulin monomer along the microtubule protofilament, whereas the C. elegans Ndc80 complex binds more tightly to β-tubulin. In addition, the C. elegans Ndc80 complex tilts more toward the adjacent protofilament. These structural differences in the Ndc80 complex between different species may play significant roles in the nature of kinetochore–microtubule interactions.

INTRODUCTION During cell division, the microtubule-based mitotic spindle forms direct connections with paired sister chromatids to capture and align them in the middle of the cell. Kinetochores assembled on sister chromatids are actively engaged with spindle microtubules during cell division. Kinetochore–microtubule attachments must be robust enough to harness the forces generated by microtubule dynamics and ensure chromosome motility. The conserved Ndc80 complex, a member of the KNL1/Mis12 complex/Ndc80 complex (KMN) network (Cheeseman et al., 2006), is essential to generate and maintain robust kinetochore–microtubule attachments (Wigge and Kilmartin, 2001; McCleland et al., 2004; DeLuca et al., 2006; DeLuca and Musacchio, 2012) and is required for spindle checkpoint signaling (Martin-Lluesma et al., 2002; DeLuca et al., 2003; McCleland et al., 2003). Although the Ndc80 complex is conserved throughout the vast majority of eukaryotes, the nature of the kinetochore–microtubule interface varies significantly between species. For example, whereas human kinetochores form at a single region on each chromosome, the nematode Caenorhabditis elegans is holocentric, forming kinetochores along the entire length of each chromosome (Maddox et al., 2004). Similarly, the checkpoint kinase Mps1 detects the presence of kinetochore–microtubule attachments by binding to Ndc80 in human cells (Hiruma et al., 2015; Ji et al., 2015), but Mps1 is absent from C. elegans (Espeut et al., 2015).

The Ndc80 complex is composed of Ndc80 (also known as HEC1 in humans; Chen et al., 1997), Nuf2, Spc24, and Spc25. Structural studies demonstrated that the globular domains of Ndc80/Nuf2 and Spc24/Spc25 heterodimers are separated by coiled-coil stalks linked by a tetramerization domain, which gives this heterotetramer an overall ~57-nm-long, dumbbell-shaped architecture (Ciferri et al., 2005; Wei et al., 2005; Wang et al., 2008). The Ndc80 complex binds to the microtubule lattice at an angle relative to the microtubule polarity, forming an arrowhead-like appearance (Wilson-Kubalek et al., 2008). Although this complex is highly extended such that it can bridge the inner kinetochore with the microtubule plus
Microtubule/ Human Ndc80 Complex

β-tubulin

α-tubulin

Ndc80 Complex

β-tubulin/ α-tubulin

Ndc80 Complex

To resolve these differences and define the molecular mechanisms by which the conserved Ndc80 complex associates with microtubules, we compared the binding of the C. elegans and human Ndc80 complexes to microtubules at comparable subnanometer resolution. We found that despite conserved sequences (Supplemental Figure S1A) and the conserved function of the Ndc80 complex, the nematode and human Ndc80 complexes have distinct binding and self-assembly modes on the microtubule.

RESULTS AND DISCUSSION

Human Ndc80 complex bound to microtubules

Full-length human Ndc80 complex is poorly behaved biochemically and difficult to purify at high concentrations. Therefore previous studies of the human Ndc80 complex bound to microtubules used the Ndc80\textsuperscript{Bonsai} complex (Alushin et al., 2010, 2012), which binds to microtubules but lacks significant and potentially functionally relevant regions of Ndc80 downstream of its globular domain, including the “loop region,” which has been shown to have a critical role at the microtubule-binding interface. To visualize the structural basis for the interaction of Ndc80 with microtubules, in this study we used a human Ndc80\textsuperscript{Bonsai} complex construct (Schmidt et al., 2012), which contains the entire Ndc80 and Nuf2 microtubule-binding domains and downstream coiled-coil regions but lacks the Spc24/Spc25 domains.

We used cryo–electron microscopy (cryo-EM) to obtain a structure of microtubule–Ndc80\textsuperscript{Bonsai} complexes. The images were obtained using a K2 direct detector (Gatan, Pleasanton, CA) and the Krios microscope (FEI, Hillsboro, OR). To process the data, we used a single-particle approach and the iterative helical real-space reconstruction procedure (IHRSR; Egelman, 2007) with multimodel projection matching of microtubules with various numbers of protofilaments (Alushin et al., 2014). A final refinement of the 15-protofilament microtubule segment alignment parameters was performed in FRE-ALIGN (Sachse et al., 2007), resulting in the highest-resolution three-dimensional (3D) reconstruction of the human Ndc80 complex to date, at 4.17-Å resolution (Fourier shell correlation [FSC] 0.143 criterion; Supplemental Figure S1B and Supplemental Table S1). Our microtubule–Ndc80\textsuperscript{Bonsai} structure was at sufficient resolution to reveal secondary structure elements (Figure 1) and was similar to the 3D reconstruction of Ndc80\textsuperscript{Bonsai} complex generated previously (Alushin et al., 2010). We found that the human Ndc80\textsuperscript{Bonsai} complex bound equivalently to α- and β-tubulin, consistent with the Alushin et al. (2010) structure. The atomic structure of the human Ndc80\textsuperscript{Bonsai} (Protein Data Bank [PDB] ID 3IZ0; Ciferri et al., 2008; Alushin et al., 2010) and tubulin (PDB ID 1JFF; Lowe et al., 2001) fit well into our EM density map (r = 0.9; Figure 1). In addition, we observed density for the Ndc80 N-terminal tail, which is not present in the x-ray structure of soluble Ndc80 complex. Thus our analysis provides a high-resolution structure for the human Ndc80 complex bound to microtubules that is consistent with previous work, despite distinct constructs.
**C. elegans Ndc80 complex bound to microtubules**

Our previous structural analysis of the C. elegans Ndc80 complex bound to microtubules revealed a low-resolution structure (30 Å; Wilson-Kubalek et al., 2008), which prevented us from visualizing secondary structure elements. We therefore sought to generate a higher-resolution version of this structure using the C. elegans NDC-80/NUF2HIM-10 complex. We implemented the same strategy as before for the human Ndc80 complex. Preliminary 3D reconstructions for 13-, 14-, and 15-protofilament microtubules (unpublished data) indicated that the C. elegans Ndc80 complexes bound preferentially to the β-tubulin subunit, as we previously observed (Wilson-Kubalek et al., 2008). However, we were not able to obtain high-resolution data for this complex due to incomplete decoration of the microtubules. This likely reflects the lower affinity of the C. elegans Ndc80 complex for microtubules compared with the human Ndc80 complex (Schmidt et al., 2012). Encouraged that this difference in the binding behavior of the human and C. elegans Ndc80 complexes was not due to the processing method, we evaluated various freezing strategies and increasing protein concentration to increase occupancy of the Ndc80 complexes on the microtubule. However, none of these attempts significantly improved the binding of the C. elegans Ndc80 complex to microtubules; instead, they resulted in a higher background of unbound protein.

We previously demonstrated that addition of C. elegans Ska1 complex improves the binding of the C. elegans Ndc80 complex to microtubules (Schmidt et al., 2012). On mixing full-length C. elegans Ndc80 complex with full-length Ska1 complex before incubation with the microtubules, we observed a significant increase in fully decorated microtubules on the grid. To improve the resolution of C. elegans Ndc80 complex/Ska1 complex bound to microtubules, we collected a large cryo-EM data set using a direct detector and the Krios microscope. The resulting 3D reconstruction at subnanometer resolution of 4.06 Å (FSC 0.143 criterion; Supplemental Figure S1B and Supplemental Table S1) revealed that these complexes preferentially bind to the β-tubulin monomer. The apparent stronger density in this region of the map produces a clear strong–weak binding pattern along the protofilament (Figure 2), with stronger binding to β-tubulin and weaker binding to α-tubulin. This result is similar to the preliminary 3D reconstruction of the microtubule–NDC-80/NUF2HIM-10 complex in the absence of the Ska1 complex and to our previous report (Wilson-Kubalek et al., 2008). Although there is no atomic structure of the C. elegans Ndc80 complex, the atomic structure of the human Ndc80βtubais structure fit well into our EM density map (r = 0.8), leaving no density to accommodate the Ska1 complex, indicating that Ska1 is too disordered to resolve in these reconstructions.

We previously showed that Ska1 decorates microtubules but does not associate with the microtubule in a specific orientation (Wellburn et al., 2009). The apparent absence of the Ska1 complex is likely due to the averaging that occurs during image processing. Although the Ska1 density is not observed in the structure, the addition of the complex enabled us to produce a higher-resolution 3D reconstruction due to increased decoration of the Ndc80 complex on microtubules. Of importance, the addition of the Ska1 complex does not alter the structure of the Ndc80 complex on microtubules. Indeed, we obtained a similar 3D reconstruction of the full-length C. elegans Ndc80 complex bound to microtubules in the absence of Ska1, albeit at a lower resolution, 8.3 Å (FSC 0.5 criterion; Supplemental Table S1) due to incomplete decoration. We compared this map to the one with the full-length C. elegans Ndc80 complex in the presence of Ska1 complex (Figure 2) at a comparable resolution (Supplemental Figure S2, A–C). The maps are very similar at the threshold shown in Supplemental Figure S2. To verify that the full-length Ndc80 complex was not inhibiting access of the Ska1 complex to the microtubule interface due to the length and flexibility of the Ndc80 coiled-coils of the Spc24/25 domains, we also obtained a 3D reconstruction of the NDC-80/NUF2HIM-10 complex plus Ska1 complex bound to microtubules (Supplemental Table S1). The resulting map (unpublished data) was similar to that for the full-length Ndc80/Ska1 complex.

Taken together, these results represent a subnanometer 3D reconstruction of the C. elegans Ndc80 complex bound to the microtubule interface and provide a means to directly compare our results with the human microtubule–Ndc80 complex structure.

**Structural comparison of the human and C. elegans Ndc80 complexes**

The raw data for both the human and C. elegans Ndc80 complexes were similar (Supplemental Figure S1C). After 3D processing, the differences became clear. The
The overall resolution of the full-length C. elegans Ndc80 complex and human Ndc80<sup>Broccoli</sup> complex bound to microtubules was similar at 4.06 Å (FSC 0.143 criterion; Supplemental Figure S1B). The tubulin density in both maps was sufficiently resolved to clearly detect Taxol and surrounding loops (Figure 4, A and B). However, the density corresponding to Ndc80 was better resolved in the human Ndc80<sup>Broccoli</sup> map (Figures 3 and 4, C and D), suggesting that C. elegans Ndc80 could be more flexible and may require other proteins to keep it firmly attached to the microtubule polymer. It also remains possible that subtle differences in the primary sequence of tubulin (both between different species and in specific tubulin isoforms) or posttranslational modifications may further modulate the binding behavior of these complexes. Taken together, our data reveal that the binding mode of the Ndc80 complex is distinct between species, with different angles for their interaction with microtubules and differences in their ability to associate with every tubulin monomer.

**Structural differences in Ndc80 complex–microtubule interactions between species might underlie distinct functional features**

The Ndc80 complex is conserved across species and is the main facilitator of dynamic attachments between the kinetochore and microtubule plus ends. It is therefore surprising that the N-terminal tail, which is crucial for binding to the microtubule for the human protein, is highly divergent among species. The N-terminal tail domain of Ndc80 is essential for viability in human cells but is not essential in budding yeast (Guimaraes et al., 2008; Miller et al., 2008; Kemmler et al., 2009) or C. elegans (Cheerambathur and Desai, 2014). The N-terminal tail of human Ndc80 plays a role in the reported cooperative binding behavior of the complex to microtubules, such that previous models suggested that the human Ndc80 complex oligomerizes along an individual microtubule protofilament (Alushin et al., 2010, 2012). In contrast, the apparent strong/weak binding pattern suggests that the CH domains of the C. elegans Ndc80 complexes to adjacent tubulin monomers cannot be anchored to microtubules in this manner. This suggests that either Ndc80 oligomerization is not a conserved or critical functional property of the Ndc80 complex or other binding partners are required for robust binding of the C. elegans Ndc80 complex to microtubules. Future studies of the specific interactions of the Ska1 complex with the Ndc80 complex and the microtubule surface will further our understanding of the different binding mechanisms between these species. Taken together, our results reveal that despite the strong functional conservation of the Ndc80 complex, its structural interaction with microtubules has evolved between species. The nature of these diverse binding interfaces is critical for considering structural and biophysical models for the mechanisms of Ndc80 complex interaction with microtubules and the basis by
A Microtubule (From Human Ndc80 Complex)

B Microtubule (From C. elegans Ndc80 Complex)

C Human Ndc80 Complex

D C. elegans Ndc80 Complex

FIGURE 4: Local resolution calculation. (A, B) Taxol density observed in β-tubulin of maps of (A) human Ndc80-bound microtubule complex and (B) C. elegans Ndc80 complex plus Ska1 complex bound to microtubule. The Taxol density is highlighted in both maps with a dashed spherical outline. (C, D) The tubulin dimer and associated Ndc80 complex is colored according to its local resolution calculated using the bsoft blocres function. Dark blue density corresponds to 3.5-Å resolution, with a continuum of colors indicating increasingly lower resolution, ending with red at 7.5-Å resolution. The local resolution calculation reveals variability in resolution within the reconstructions, with the Ndc80 densities substantially less resolved than the tubulin density.

which kinetochores associate with and harness the force from depolymerizing microtubules to drive chromosome movement.

MATERIALS AND METHODS
Cryo-EM sample preparation
For grid preparation, full-length C. elegans Ndc80 complex and full-length C. elegans Ska1 complex (Schmidt et al., 2012) were mixed in a 1:1 ratio at 6 μM. Human Ndc80Brocolli complex (Schmidt et al., 2012) was used at 3 μM. All proteins were present in BRB80 (80 mM 1,4-piperazinediethanesulfonic acid [PIPES], pH 6.8, 1 mM MgCl₂, 1 mM ethylene glycol tetraacetic acid [EGTA]). Bovine brain microtubules were prepared by polymerizing 5 mg/ml bovine brain tubulin (Cytoskeleton, Denver, CO) in polymerization buffer (80 mM PIPES, pH 6.8, 1 mM EGTA, 4 mM MgCl₂, 2 mM GTP, 12% dimethyl sulfoxide) for 30 min at 36°C. Paclitaxel was added at 250 μM before further incubation of 30 min at 36°C. The polymerized microtubules were then incubated at room temperature for several hours before use.

All microtubule samples were prepared on 400-mesh C-flat grids (Protochips, Morrisville, NC) containing 2.0-μm holes separated by 2.0-μm spacing. Grids were glow-discharged before sample application. The cryosamples were prepared using a manual plunger, which was placed in a homemade humidity chamber that varied between 80 and 90% relative humidity. A 4-μl amount of the microtubules at ∼0.5 μM in 80 mM PIPES, pH 6.8, 4 mM MgCl₂, and 1 mM EGTA supplemented with 20 μM Taxol was allowed to absorb for 2 min, and then 4 μl of the Ndc80 preparations in BRB80 was added to the grid. After a short incubation of 2 min, the sample was blotted (from the back side of the grid) and plunged into liquid ethane.

Electron microscopy and image processing
Data were collected on the FEI Krios at the Scripps Research Institute or the FEI Krios at the University of California, Los Angeles. All images were recorded at 300 keV on a Gatan K2 direct electron detector with a pixel size of 1.31 Å at the specimen level using the automated Leginon software (Suloway et al., 2005). Image processing was performed within the Appion processing environment (Lander et al., 2009). The contrast transfer function was estimated by using CTFFIND3 (Mindell and Grigorieff, 2003), and the best-quality micrographs were selected for further processing. Microtubules were manually selected, and overlapping segments were extracted with a spacing of 80 Å along the filament. The boxed images were binned by a factor of two for two-dimensional analysis and 3D refinement. The particle stacks were subjected to iterative multivariate statistical analysis and multireference alignment. Particles in classes that did not clearly show Ndc80 density were excluded from further processing.

Cryo-EM 3D reconstruction
Undecorated 14- and 15-protofilament microtubule densities (Sui and Downing, 2010) were used as initial models for all preliminary reconstructions. We used the IHRSR procedure (Egelman, 2007) for multimodel projection matching of microtubule specimens with various numbers of protofilaments (Alushin et al., 2014), using...
libraries from the EMAN2 image processing package (Tang et al., 2007). After each round of projection matching, an asymmetric back-projection is generated of aligned segments, and the helical parameters (rise and twist) describing the monomeric tubulin lattice are calculated. These helical parameters are used to generate and average 14 and 15 symmetry-related copies of the asymmetric reconstruction, and the resulting models were used for projection matching during the next round of refinement. In most reconstructions, we had a higher percentage of 15-protofilament segments. The resulting 14- and 15-protofilament reconstructions were similar, however; we used 15-protofilament segments only in the final refinement, since they do not have a seam, allowing for helical refinement. Final refinement of the 15-protofilament microtubule segment alignment parameters was performed in FREALIGN (Grigorieff, 2007) without further refinement of helical parameters. FSC curves were used to estimate the resolution of each reconstruction, using a cutoff of 0.143 (Supplemental Figure S1B). To estimate more accurately the resolution of each region of the reconstructed density, we performed a local resolution calculation using the blockes and blockfit functions in the Bsoft processing package (Heymann and Belnap, 2007). This analysis revealed that the majority of the tubulin density is in the range of 3.5- to 5-A resolution, whereas the Ndc80 portion ranges from 4.5- to 7.5-A resolution (local resolution map; Figure 4, C and D). To better display the high-resolution features, we applied a B-factor of 100 Å^2, using the program bfactor (http://grigoriefflab.janelia.org). Atomic models were obtained through rigid-body docking of the electron crystallographic structure of tubulin (PDB ID 1JFF; Lowe et al., 2001) and the Ndc80aphoscrystal structure (PDB ID 3Z20; Ciferri et al., 2008; Alushin et al., 2010) into the cryo-EM density maps, using UCSF Chimera (Pettersen et al., 2004).

**Accession numbers**

The Electron Microscopy Data Bank (EMDB) accession code for the human Ndc80:microtubule complex structure is EMD-6594. The EMDB accession code for the C. elegans Ndc80:microtubule complex structure is EMD-6595.

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