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Seeing is believing: our evolving view of kinetochore structure, composition, and assembly

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Abstract

This review highlights three recent trends in the field of kinetochore biology: the proliferation of structural data for kinetochore protein complexes (including CBF3, Dam1c, Mis12c^{MIND}, and CENP-NL$^{Chl4/Iml3}$); the growing consensus that the kinetochore is a dynamic structure whose composition changes as the cell cycle progresses; and the mounting evidence of multiple pathways whereby the microtubule binding elements of the outer kinetochore may be recruited by inner kinetochore proteins. Our focus is on the two best-studied systems in the field: human and budding yeast kinetochores. This review will demonstrate the remarkable similarity of these two systems, as well as their intriguing differences.

Introduction

How are duplicated chromosomes partitioned between daughter cells during mitosis and meiosis? How is this process regulated? How does it go awry in cancer? The kinetochore is central to all of these questions.

A macromolecular complex of about forty core proteins (not including regulatory proteins and motors) assembled on the centromere[1], the kinetochore mediates the process of chromosome segregation by coupling microtubule dynamics to chromosome movement (Figure 1). It also serves as a tension-sensitive regulatory hub for destabilization of improper microtubule-kinetochore attachments [2,3]. The kinetochore may be conceptually divided into the microtubule-proximal outer kinetochore and the centromere-proximal inner kinetochore.

Poised at the intersection of genetics, protein biochemistry, and molecular biophysics, pertinent to cancer biology and human genetic disease, the field of kinetochore research has a rich history, which is beyond the scope of this review. (Interested readers are directed to

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Musacchio and Desai 2017 [4].) Rather, this review will highlight recent trends in the field, specifically: a proliferation of structural data for kinetochore proteins; a growing consensus that the composition of the kinetochore changes as the cell cycle progresses; and the discovery of multiple means by which microtubule-binding elements are recruited by the inner kinetochore— paths whose primacy may vary between organisms and between mitotic stages.

Structures, structures, everywhere

Direct electron detector technology for cryo-electron microscopy (cryo-EM) has revolutionized the field of structural biology [5,6], bringing a wave of high-resolution macromolecular structures — including some protein subcomplexes of the kinetochore. In the past several years, these structures have provided unprecedented insight into the physical makeup of that molecular machine.

For years, kinetochore proteins have eluded structural analysis due to their flexible architectures. Severe truncations and simplifications were necessary to obtain constructs that would crystallize. An example of this approach is the creation and crystallization of the “bonsai” Ndc80c complex [7]. The N-terminus of Ndc80 was fused to the C-terminus of Spc25, and the N-terminus of Nuf2 was fused to the C-terminus of Spc24, abrogating the tetramerization domain and most of the coiled coils in order to allow crystallization of the globular microtubule-binding and kinetochore-binding elements of Ndc80c [7]. It was rare and hard-won triumph for kinetochore protein crystallographers and was instrumental to our understanding of *how* Ndc80c binds microtubules.

It was eight years before the intact tetramerization domain was crystalized, revealing a junction between the Ndc80-Nuf2 and Spc24-Spc25 coiled coils [8]. This junction may be subdivided into a region of three-chain overlap and one of four-chain overlap, which buries an aromatic side-chain stack to which all four proteins contribute [8]. A network of conserved polar contacts further stabilizes the junction. This structure of Ndc80c’s tetramerization domain, taken together with the globular head structures originally elucidated by “bonsai” Ndc80c, allow visualization of the intra-complex interactions that hold Ndc80c together, as well as the surfaces that interact with its binding partners— a significant step toward understanding the structures responsible for microtubule binding.

Just last year, cryo-EM cracked the structure of another key microtubule-binding complex (at least in fungi). A ~4.5 Å structure of the Dam1 complex (Dam1c) of *Chaetomium thermophilum* reveals how individual T-shaped heterodecamers come together at conserved polar and non-polar contact points to form a microtubule-encircling ring [9,10](Figure 2B). Orthogonal to both arms of the T, the protrusion domain is comprised of the C-terminal domains of Spc19 and Spc34; it is posited to contact the Ndc80-Nuf2 coiled-coil. In agreement with the conclusions of previously published cross-linking experiments [11,12], the C-termini of Dam1 and Duo1 are oriented towards the microtubule lattice, which they are predicted to bind. While the residues that actually contact Ndc80c (based on cross-linking data [13]) were not present in the reconstruction, Jenni and coworkers conclude that all three contacts with Ndc80c could be made by a single Dam1c ring— in contrast to Kim

et al.'s conclusion that Ndc80c bridges *two* Dam1c rings at the kinetochore [9,13]. Using electron cryotomography to examine the outer kinetochore *in vivo*, Ng et al. found that while a few kinetochore microtubules do have two rings, most have only one; and though some complete 17-membered rings were observed, the majority of Dam1c oligomers formed only partial rings [14].

While no vertebrate homolog of Dam1c has been identified, it was proposed a decade ago that the Ska complex might be Dam1c's "metazoan functional counterpart [15]." And despite the fact that the W-shaped, dimeric Ska complex looks nothing like the Dam1c ring [16], this was confirmed by recent reports that the Ska complex, like Dam1c, can bind Ndc80c [17], bear load at microtubule tips [18], and strengthen Ndc80c-mediated microtubule binding [18].

Moving away from the microtubule interface, we come to the Mis12c^{MIND} complex, which bridges the inner and outer kinetochore. In 2016, both the budding yeast *Kluyveromycces lactis (K. lactis)* [19] and human [20] Mis12c^{MIND} structures were published, revealing a conserved Y-shaped tetramer in which the N-termini of all four substituents (Dsn1, Ns11, Pmf1^{Nnf1}, & Mis12^{Mtw1}) are oriented towards the inner kinetochore (Figure 2C). The N-termini of Pmf1^{Nnf1} and Mis12^{Mtw1}1 comprise "head I" of the Y; the N-termini of Dsn1 and Ns11 comprise "head II." Inner kinetochore proteins CENP-U^{Amc1} and CENP-C^{Mif2} both bind to head I, while head II serves a regulatory function auto-inhibiting this interaction unless phosphorylated by Aurora B kinase [19]. The same mechanism regulates the Mis12c^{MIND}-CENP-C^{Mif2} interaction in humans [20], indicating that the interface between Mis12^{MIND} and the inner kinetochore is organized and regulated by a conserved mechanism.

Considerable progress has also been made towards visualizing the inner kinetochore. Building upon their previously published structures of the RWD domains of *K. lactis* CENP-P^{ctf19} and CENP-O^{Mcm21} [21], Schmitzberger and colleagues used crystallography, hydrogen-deuterium exchange, and mass spectroscopy to demonstrate that CENP-Q^{Okp1} binds CENP-U^{Ame1}, Nkp1/Nkp2, & CENP-PO$^{ctf19/Mcm21}$ through three distinct interfaces, which are separated by flexible elements [22].

CENP-NL$^{ch14/Im13}$ is an inner kinetochore protein complex of particular interest because like CENP-C^{Mif2} it binds directly and specifically to centromeric, CENP-A^{Cse4}-containing nucleosomes [23-25]. Since 2013, we have had a partial structure of the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae)* CENP-NL$^{ch14/Im13}$, including the heterodimer interface [26], but it was several years before the basis of centromeric nucleosome recognition by this complex was revealed by two independently obtained cryo-EM structures of a human CENP-A^{Cse4} nucleosome in complex with an N-terminal domain of CENP-N^{ch14} [27,28]. These structures revealed that CENP-N^{ch14} recognizes centromeric nucleosomes through specific interactions with charged residues in the L1 loop of CENP-A^{Cse4} that are absent from histone H3; this interaction is strengthened through electrostatic interactions between basic amino acids on CENP-N^{ch14} and the phosphate backbone of DNA [27,28](Figure 3C).

These structures facilitated residue assignment in a recent high-resolution (~4 Å) reconstruction of the 13-subunit budding yeast Ctf19 complex (Ctf19c) [29](Figure 3A). Two protomers containing one copy each of nearly every inner kinetochore protein (save CENP-C^{Mif2}) flank a central cavity. The CENP-A^{Cse4}-binding β3- β4 loop of CENP-N^{ch14} extends into this cavity, which is, however, too small to accommodate a CENP-A^{Cse4} nucleosome without Ctf19c dimer dissociation, significant conformational rearrangement, or partial nucleosome unwrapping [29]. The overall organization of the Ctf19c reflects published recruitment hierarchies, in which CENP-QU^{OA} is foundational, upstream of all other inner kinetochore components, while CENP-TW$^{Cnn1/Wip1}$ requires nearly all other Ctf19c proteins for proper kinetochore localization [29-31]. The organization may also be conserved; Pesenti and coworkers published a low-resolution (~22 Å) negative stain 3D reconstruction of a reconstituted 11-protein human CCAN and concluded that CENP-HIKM and CENP-OPQUR sandwich CENP-NL, just as the yeast homologs of CENP-HIK and CENP-OPQU sandwich CENP-NL$^{ch14/Im13}$ in the Ctf19c (Figure 3B) [32].

There have also been not one but *three* recent structures of the CBF3 complex, an essential complex that binds the genetically specified centromere of budding yeast in the first step of CENP-A^{Cse4} deposition and kinetochore assembly. Leber, Nans, and Singleton published a 3.6 Å cryo-EM reconstruction of a Cep3 dimer in complex with Ctf13 and Skp1[33]. The entire complex is U-shaped, with a charged central channel in which DNA was postulated to bind. Ctf13 was characterized as an F-box-leucine-rich-repeat protein for the first time, a finding corroborated in 2018 by the publication of a similar “core CBF3” structure, this time including the essential DNA-binding protein Ndc10 [34]. Intriguingly, the central channel formed by Cep3 dimers and the DNA-binding domain of Ndc10 are perpendicular, leading to the proposal that CBF3 bends centromeric DNA into a loop, perhaps to facilitate loading of the centromeric nucleosome [34]. Yan and colleagues then published a structure of the complete CBF3 in complex with a 147-bp yeast centromere, revealing how Cep3, Ctf13, and Ndc10 interact with DNA [35] (Figures 4D and 4E). Of particular interest are the two sequence-specific Centromere Determining Element III (CDEIII)-binding sites, both of which occur in the same copy of Cep3 in one protomer of a CBF3 dimer; a zinc finger of this Cep3 interacts with a conserved CCG motif, while the α-MN helix contacts a conserved TGT motif [35].

Together these structures may be assembled into a near-atomic view of much of the kinetochore, although we cannot yet visualize what an entire yeast or human kinetochore might look like. It is unclear how many copies of each complex to place in such a reconstruction; absolute protein copy numbers remain controversial [36-40]. We also lack structural information on CENP-C^{Mif2}, the essential DNA-binding protein conserved even in highly simplified kinetochores [41]. CENP-C^{Mif2} has been described as the “blueprint” of inner kinetochore assembly [42], but comparatively little is known about what that blueprint might look like. Possibly this is because CENP-C^{Mif2} (except for its C-terminal dimerization domain) remains largely disordered and flexible when not in contact with its binding partners [43]. This has been suggested of other kinetochore proteins, including Ame1[22], Okp1 [22], Knl1 [44], Dad1 [45], and BubR1[46]. Thus the reconstitution of increasing complete kinetochore particles may be the necessary groundwork for another leap in our structural understanding of the kinetochore [47,48].

Cell-cycle dependent kinetochore composition

As a field, we have been unable to arrive at a single answer to one of the most basic questions about the kinetochore: how many copies of each protein complex are in it? Published estimates of the number of Ndc80c at a budding yeast kinetochore range from 6 to 20 [36,37,40]. A growing trend in the field is to view these divergent answers not merely as an indication of the inherent difficulty of counting individual proteins in a living cell, but as a reflection of a dynamic kinetochore whose composition and architecture change as the cell cycle progresses.

Controversy exists at the very foundation of the kinetochore: the CENP-A^{Cse4}-containing nucleosome. While there is consensus that a single Cse4-containing nucleosome is present at each budding yeast centromere, the exact composition of that centromere is a point of controversy [39,49-53]. A widely accepted model is the most conventional one: an octameric nucleosome containing two copies each of Cse4, H2A, H2B, and H4 wrapped with DNA in a left-handed manner [53-55]. But evidence has also been published supporting “hemisome [52],” “tetrasome [56],” “hexasome [57],” and “trisome [58]” models; in 2011, Black and Cleveland proposed to reconcile this confusion with a model in which centromeric nucleosomes mature throughout the cell cycle [59]. In this model, CENP-A^{Cse4} occupies a pre-nucleosomal trisome or hexasome during HJURP^{Scm3}-mediated deposition, a tetrasomal intermediate prior to H2A:H2B addition, and a standard octameric nucleosome for the rest of the cell cycle [59]. Within a year, biophysical evidence of centromeric nucleosomes undergoing cell cycle-coupled structural transitions in both yeast and humans was published [38,60]. However, in 2017 the Cleveland Lab — early proponents of centromeric nucleosome maturation — mapped all of the sequences bound by CENP-A onto individual α-satellite arrays in centromere reference models and concluded that CENP-A centromeric chromatin is made up of conventional octameric nucleosomes *throughout the cell cycle*, a conclusion supported by their biochemical, hydrodynamic, and solid-state nanopore analyses [61].

But the centromeric nucleosome maturation hypothesis still profoundly influences kinetochore research. And because CENP-A^{Cse4} fluorescence has been used as the calibration standard for estimates of kinetochore protein copy number in methods using genetically encoded fluorescent proteins [40,62], reevaluating the number of CENP-A^{Cse4} present at the kinetochore means reevaluating all estimates of kinetochore protein copy numbers. Indeed, building on their finding that a second CENP-A^{Cse4} is not deposited at the centromeric nucleosome until anaphase, the Gerton Lab has recently published that nearly all kinetochore components (save Dam1c) double in abundance during anaphase [36].

While this finding has yet to be corroborated, there has long been consensus that levels of Ndc80c receptor CENP-T^{Cnn1} increase at kinetochore during anaphase, at the same time that an Ndc80c- CENP-T^{Cnn1} becomes detectable [31,63,64]. This phenomenon was recently explained by the Musacchio Lab’s demonstration that CENP-T^{Cnn1} binds two Ndc80c and one Mis12c^{MIND} at three distinct sites upon phosphorylation by the CDK1:Cyclin B complex [65]. That a cyclin-dependent kinase should regulate the association of CENP-T^{Cnn1} with outer kinetochore components explains neatly *how* levels on Ndc80c at the

kinetochore increase as mitosis progresses (although it is puzzling that Ndc80c-CENP-T^{Cnn1} levels increase in anaphase, when Cyclin B is being degraded [66]). As to the *why,* the fact that CENP-T^{Cnn1} recruits additional Ndc80c to the kinetochore only after the spindle assembly checkpoint (SAC) is satisfied and cells have irreversibly committed to chromosome segregation suggests that the relative importance of different Ndc80c recruitment paths may change as the cell cycle progresses.

Thus the kinetochore may change its organization and composition throughout the cell cycle in order to perform in turns its mitotic functions as tension sensor, regulatory hub, and molecular tether.

Parallel paths of Ndc80c recruitment

Over the past 15 years, the Davis and Asbury Labs have demonstrated using optical tweezers that Ndc80c and Dam1c in cooperation are the load-bearing, microtubule-binding elements of the budding yeast kinetochore and that their binding to the microtubule tip and one another is multivalent and phospho-regulated [14,31,65,67-71]. This year, Volkov and coworkers recapitulated the finding that Ndc80c multivalency is required for efficient microtubule coupling using a novel Ndc80c oligomerization platform [72]. This highlights another persistent puzzle in our field: how does a single centromeric nucleosome (in the case of budding yeast) recruit many copies of Ndc80c?

The final trend we wish to highlight in this review is the growing consensus that there are multiple, parallel pathways of Ndc80c recruitment in both yeast and human kinetochores.

In the previous section, we highlighted recent work demonstrating that each CENP-T^{Cnn1} recruits up to three Ndc80c [65]. Using a novel kinetochore assembly assay to examine recruitment dependencies, Lang, Barber, and Biggins further elucidated this pathway in budding yeast; they determined that CENP-T^{Cnn1} is downstream of most inner kinetochore proteins save CENP-C^{Mif2} [31]. As both of these inner kinetochore proteins can recruit the outer kinetochore (either directly or through Mis12c^{MIND}), Lang, Barber, and Biggins conclude that they represent two distinct Ndc80c recruitment pathways [31].

Recent work complicates this picture further. Building on the demonstration that *K. lactis* CENP-C^{Mif2} and CENP-U^{Amc1} both bind to Mis12c^{MIND} [19], Fischboeck and colleagues demonstrated that *S. cerevisiae* CENP-QU$^{Okp1/Ame1}$ (like CENP-C^{Mif2}) also binds specifically to Cse4-containing nucleosomes [73]. The Ehrenhofer-Murray Lab corroborated this finding, further demonstrating that two post-translational modifications to the N-terminus of Cse4 (R37Me and K49Ac) inhibit its interaction with CENP-QU$^{Okp1/Ame1}$ [74]. In light of the Musacchio Lab's report that *H. sapiens* CENP-QU$^{Okp1/Ame1}$ binds microtubules but *not* centromeric nucleosomes [47], it appears that CENP-QU$^{Okp1/Ame1}$'s function may vary between organisms.

Taken together, there is now evidence for two conserved pathways of Ndc80c recruitment and hints of a possible third pathway in fungi. Multiple microtubule binding elements have been key to our conception of the kinetochore since the publication of Hill's seminal

"sleeve" model over 30 years ago [75]; only now are we beginning to understand how they are recruited.

Conclusion

Recent work has reshaped the field of kinetochore biology. For the first time we are able to visualize the protein complexes that make up this marvelous molecular machine. There is a growing sense that the organization of that machine may change throughout the cell cycle. And our understanding of the kinetochore as a multivalent microtubule coupler has been complicated by mounting evidence that there are distinct pathways whereby the microtubule-binding elements of the kinetochore are recruited.

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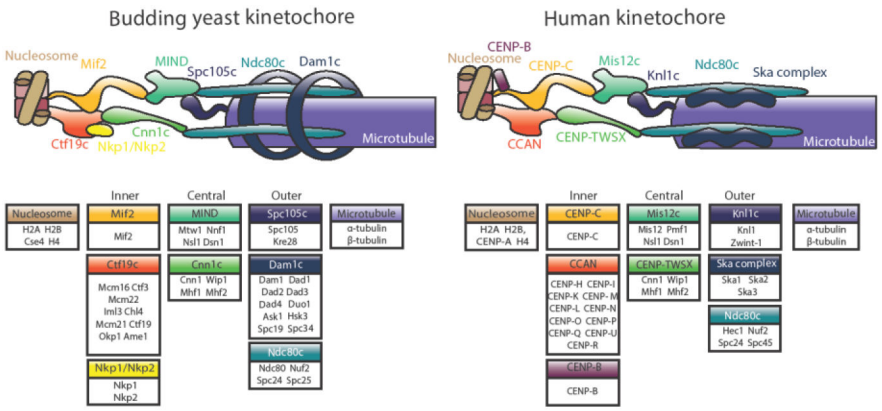

Figure 1.

Model for the budding yeast *(S.cerevisiae)* and human *(H. sapiens)* kinetochores. The individual proteins of each subcomplex are shown in boxes. Inner kinetochore proteins, called the constitutively centromere-associated network (CCAN), contact the centromeric nucleosome and recruit central kinetochore proteins. These contact the microtubule-binding elements that make up the outer kinetochore. Homologous subcomplexes are colored identically, with the exception of the Dam1 and Ska complexes, which are non-homologous functional counterparts.

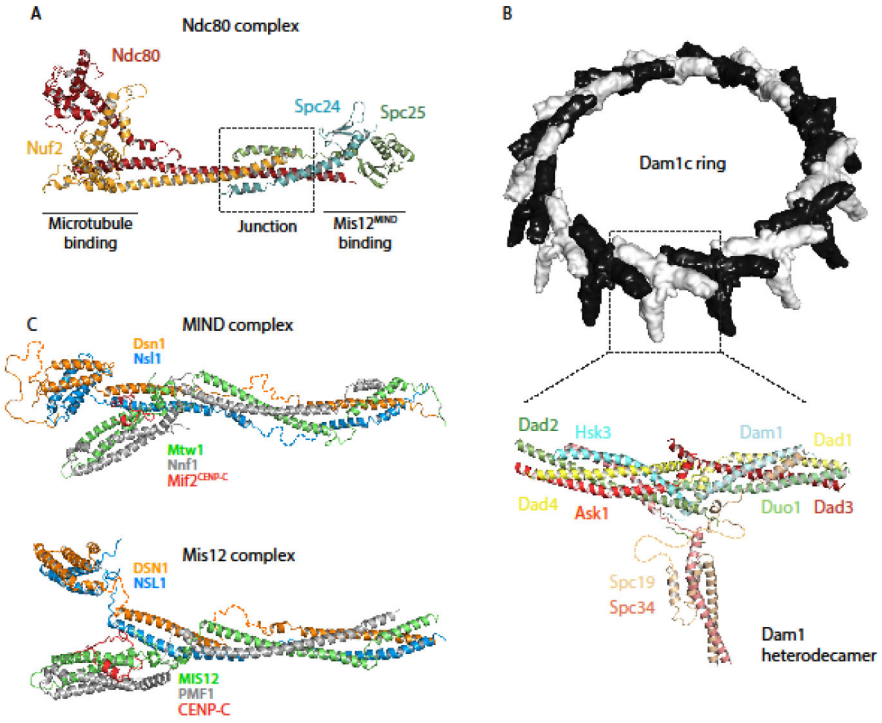

Figure 2.

Recently published structures of outer kinetochore protein complexes. (A) Ndc80c^{Dwarf} is an elongated heterotetramer comprised of a microtubule-binding Hec1^{Ndc80}-Nuf2 dimer and Mis12^{MIND}-binding Spc24-Spc25 dimer. The structure shows the complete junction region of the heterotetramer (PDB ID: 5TCS) [7,8]. (B) (top) *Chaetomium thermophilum* Dam1c heterodecamers oligomerize into 17-membered rings with an outer diameter of about 560 Å [9]. (bottom) Ribbon diagram of a heterodecamer subunit, containing amino acids 13-78 of Ask1, 18-76 of Dad1, 25-95 and 109-116 of Dad2, 18-82 of Dad3, 3-70 of Dad4, 53-107 of Dam1, 49-121 of Duo1, 22-77 of Hsk3, 7-112 of Spc19, 3-48 and 112-199 of Spc34 (PDB ID: 6CFZ) [9]. (C) (top) *K.lactis* MIND is a Y-shaped heterotetramer, the stem of which binds Spc24-Spc25 of Ndc80c. One globular head binds inner kinetochore proteins CENP-C^{Mif2} and CENP-U^{Ame1}, while a N-terminal extension of Dsn1 from the other head inhibits these interactions except when phosphorylated by Aurora B kinase (PDB ID: 5T58 and 5T51) [19]. (bottom) *H.sapiens* Mis12c with CENP-C shares a conserved structure with its budding yeast homolog; it also forms Y-shaped heterotetramer about 200 Å long, with conserved sites of interaction with CENP-C^{Mif2} and Ndc80(PDB ID:5LSK) [20].

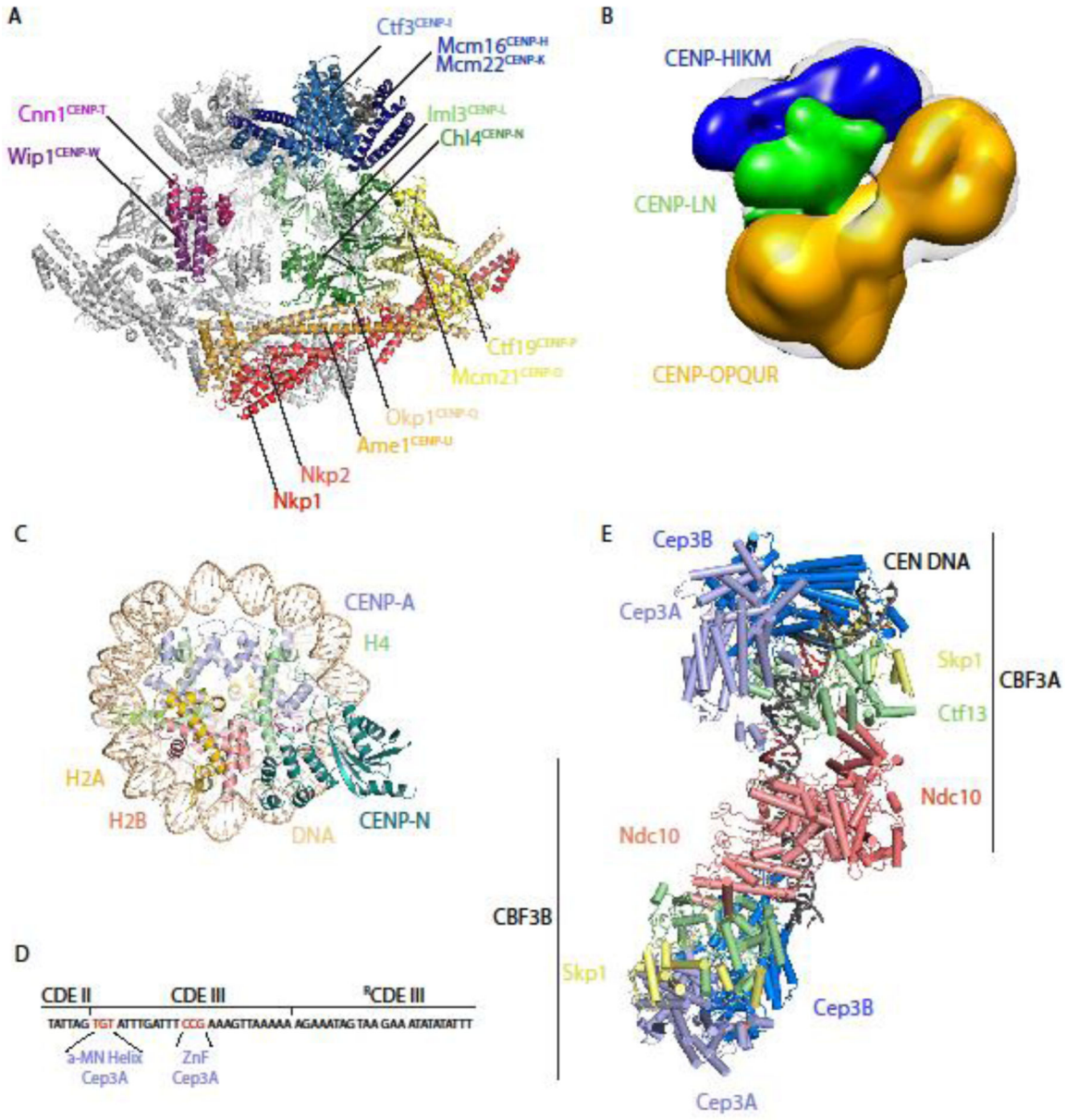

Figure 3. Recently published structures of inner kinetochore protein complexes.

(A) Dimers of the 13-protein *S. cerevisiae* Ctf19c flank a central cavity. CENP-NL$^{Ch14/Im13}$ occupies the middle of each protomer, and its CENP-A^{Cse4}-binding domain extends into the central cavity (PDB ID: 6NUW) [29]. (B) An ~22 Å negative stain 3D reconstruction of a reconstituted 11-protein human CCAN, in which CENP-HIKM (density of subcomplex in blue) and CENP-OPQUR (density of sub-complex in yellow) sandwich CENP-NL (density of subcomplex in green) [32]. (C) The N-terminus of human CENP-N specifically recognizes centromeric, CENP-A-containing nucleosomes by binding to the unique L1 loop (indicated in red) of CENP-A [27, 28]. (Model shown from PDB ID: 6C0W, related structures: 6BUZ and 6EQT) (D) Schematic of the 56 bp nuclease-resistant sequence bound to CBF3. Conserved TGT and CCG motifs in Centromere Determining Element III (CDEIII) are specifically bound by the α-MN helix and zinc finger (ZnF) motifs,

respectively, of the same Cep3A subunit [32]. (E) Budding yeast CBF3 binds centromeric DNA as a dimer, recognizing specific DNA sequences (colored red) prior to Cse4 deposition [32]. Orange spheres indicate zinc atoms. (Model shown from PDB ID: 6GYS, similar structures: 6GSA and 6F07).